

Doktorego Tesia

**Lipid regulation of Atg8 family protein
recruitment and membrane modulation of
mitophagy and autophagic cell death**

Zuriñe Antón Helas

2017

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Doctoral Thesis in English

Laburduren eta Sinboloen Zerrenda

2-AG	2-arachidonoylglycerol
2D/3D	two- or three-dimensional / bi edo hiru dimentsiokoa
4H	four-helix bundle
a.u.	arbitrary units
A_{260/280}	absorbance at 260 or 280 nm / absorbantzia 260 edo 280 nm-tan
A488	Alexa Fluor 488
AC	alternating current
AGO2	argonaute 2 / 2-argonauta
AIM	Atg8-family interacting motif / Atg8 familiarekin elkarreragiteko motiboa
AKT	RAC-alpha serine/threonine-protein kinase
ALPS	amphipathic lipid packing sensor / lipido anfiptikoen paketamenduaren sentsoarea
Ambra1	autophagy and beclin 1 regulator 1 / autofagia eta beclin 1 proteinaren 1-erregulatzailea
Amp	ampicillin / anpizilina
AMPK	AMP-dependent protein kinase / AMP-menpeko proteina kinasa
Ams1p	α -mannosidase / α -manosidasa
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
AP	autophagosome / autofagosoma
Ape1p	aminopeptidase 1 / 1-aminopeptidasa
A-SMase	acid sphingomyelinase / esfingomielinasa azidoa
ATG	autophagy-related / autofagiarekin erlazionatua
ATP	adenosine triphosphate
ATPB	ATP synthase subunit beta
AVd	degradative autophagic vacuole
AVi	initial autophagic vacuole
BafA1	bafilomycin A1

BAK	BCL-2 associated killer protein
BAR	bin–amphiphysin–rvs domain
BAX	BCL-2 associated X protein
BCA	bis-cinchoninic acid
BCL-2	B-cell CLL/lymphoma 2
BCL-x	B-cell lymphoma-extra
BDMA	N-benzyl dimethylamine
Beclin 1	coiled-coil myosin-like BCL-2-interacting protein / <i>coiled-coil</i> miosina-moduko BCL-2-rekin elkarregiten duen proteina
BH	BCL-2 homology domain
BID	BH3 interacting domain death agonist
BNIP3	BCL-2 interacting protein 3 / BCL-2-rekin elkarregiten duen 3-proteina
BSA	bovine serum albumin / behi-serumaren albumina
CALCOCO2/NDP52	calcium-binding and coiled-coil domain-containing protein 2 / kaltzioa-lotzeko eta <i>coiled-coil</i> domeinuak dauzkan 2-proteina
CAPK	ceramide-activated protein kinase / zeramidak aktibatutako proteina kinasa
CCCP	carbonyl cyanide m-chlorophenyl hydrazine / karbonil zianida m-klorofenil hidrazina
CD	circular dichroism / dikroismo zirkularra
CD-95	cluster of differentiation 95
CDase	ceramidase / zeramidasa
cDNA	complementary DNA / DNA konplementarioa
CDP-DAG	cytidine diphosphate diacylglycerol / zitidina difosfato diazilglicerola
Cer	ceramide / zeramida
CERS	ceramide synthase
CFP	cyan fluorescent protein
Chol/Ch	cholesterol
CL	cardiolipin / kardiolipina
CL*	<i>E.coli</i> cardiolipin / <i>E.coli</i> -ren kardiolipina
CLEM	correlative light and electron microscopy / argizko mikroskopia eta mikroskopia elektronikoko korrelatiboa

CMA	chaperone-mediated autophagy / txaperona bidezko autofagia
CMV	cytomegalovirus
CNR/CB	cannabinoid receptor
COL4A3BP/CERT	collagen type IV α -3-binding protein
c_p	heat capacity / bero-ahalmena
CTSB/L	cathepsin B or L
Cvt	cytoplasm-to-vacuole targeting
CYCS	cytochrome c somatic
Cyt c	cytochrome c / c-zitokromoa
DABCO	1,4-diazabicyclo[2.2.2]octane
DAG	diacylglycerol / diazilgizerola
DAPI	4',6-diamidino-2-phenylindole / 4',6-diamidino-2-fenilindola
ddH₂O	double distilled water / bi aldiz destilatutako ura
DDSA	dodecanyl succinic anhydride
DEGS1/2	delta(4)-desaturase, sphingolipid 1 or 2
Deptor	DEP-domain-containing mTOR-interacting protein / DEP domeinua daukan eta mTOR-ekin elkarreragiten duen proteina
DFCP-1	double FYVE domain-containing protein 1 / FYVE domainu bikoitza daukan 1-proteina
dhCer	dihydroceramide
dhSM	dihydrosphingomielin
DLCL	dilysocardiolipin / dilisokardiolipina
DLS	dynamic light scattering / argiaren dispertsio dinamikoa
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide / dimetilsulfoxidoa
DNase	deoxyribonuclease
DNM1L/DRP1	dynamamin-1-like protein / 1-dinamina-moduko proteina
Dodec/EtOH	dodecane/etanol
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphatidylethanolamine
DPX	<i>p</i> -xylene-bis-pyridinium bromide
DSC	differential scanning calorimetry / ekorketa kalorimetria diferentziala

dsDNA	double-stranded DNA / helize bikoitzeko DNA
DsRed	discosoma red fluorescent protein
DTT	DL-dithiothreitol / DL-ditiotreitola
E2F1	E2F transcription factor 1 / E2F 1-transkripzio faktorea
EBSS	Earle's balanced salt solution
EC50	half maximal effective concentration
ECD	evolutionarily conserved domain / eboluzioan zehar kontserbaturiko domeinua
eCer	egg ceramide
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1 / endosoma goiztiarraren 1-antigenoa
EM	electron microscopy / mikroskopia elektronikoa
EPR	electron paramagnetic resonance / elektroien erresonantzia paramagnetikoa
ER	endoplasmic reticulum / erretikulu endoplasmatikoa
ERGIC	ER-Golgi intermediate compartment / ER-Golgi bitarteko konpartimentua
ESCRT	endosomal sorting complex required for transport / garraiorako beharrezkoa den banaketa-konplexu endosomikoa
FA	fatty acid / gantz azidoa
FBS	fetal bovine serum / fetuko behi-seruma
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FIP200	focal adhesion kinase family interacting protein 200 kDa / 200 kDa-ekoa den atxikidura-fokal kinasa familiarekin elkarreragiten duen proteina
Fis1	mitochondrial fission 1 protein / fisio mitokondrialaren 1-proteina
FLM	fluorescence light microscopy / fluoreszentiako mikroskopia optikoa
FM	fluorescence microscopy / fluoreszentiako mikroskopia
FoxO1/3	forkhead box O1 or 3
FT	flow-through
FT-IR	Fourier-transform infrared spectroscopy / Fourier-en transformatuaren bidezko espektroskopia infragorria
FUNDC1	FUN14 domain containing 1 / FUN14 domeinua daukan 1

FYCO1	FYVE and coiled-coil domain-containing protein 1 / FYVE eta <i>coiled-coil</i> domeinuak dauzkan 1-proteina
GABA(A)	GABA type A / A-motako GABA
GABARAP	GABA type A receptor-associated protein / A-motako GABA hartzaileei lotutako proteina
GABARAPL1/2/3	GABA type A receptor-associated protein like 1, 2 or 3 / A-motako GABA hartzaileei lotutako proteina-moduko 1, 2 edo 3
GATE-16	Golgi-associated ATPase enhancer of 16 kDa
GCS	glucosylceramide synthase / glukosilzeramida sintasa
GFP	green fluorescent protein
GlcCer	glucosylceramide / glukosilzeramida
GOS28	Golgi SNAP receptor complex member 1 / Golgiko SNAP hartzaile konplexuaren 1-kidea
gp78	glycoprotein 78 / 78-glikoproteina
GPI	glycosylphosphatidylinositol / glikosilfosfatidilinositola
GSLs	glycosphingolipids / glikoesfingolipidoak
GSSG	glutathione disulfide / glutation disulfuroa
GST	glutathione S-transferase / glutation S-transferasa
GT11	N-[(1R,2S)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide
GTP	guanosine triphosphate / guanosina trifosfatoa
GUV	giant unilamellar vesicle / lamela bakarreko besikula erraldoia
GβL	G-protein β-subunit-like protein / G-proteina β-azpiunitate-moduko proteina
HEK293T	human embryonic kidney 293T
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
H_{II}	inverted-hexagonal lipid phase / alderantzizko lipido-fase hexagonala
hIPS	human induced-pluripotent stem cells
HIV/GIB	human immunodeficiency virus / giza immuno-eskasiaren birusa
HOPS	homotypic fusion and protein sorting / fusio homotipiko eta proteina banaketa
HPF	high-pressure freezing
HRP	horseradish peroxidase / errefau peroxidasa

HSP60	heat shock protein 60 kDa
IANBD	<i>N,N</i> -dimethyl- <i>N</i> -(iodoacetyl)- <i>N</i> -(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine
IMM	inner mitochondrial membrane / mitokondrioaren barne-mintza
iPSC	induced pluripotent stem-cell
IPTG	isopropyl- β -D-1-thiogalactopiranoside / isopropil- β -D-1-tiogalaktopiranosidoa
IRGM	immunity related GTPaseM / inmunitatearekin lotutako M-GTPasa
ISP-1	myriocin
ITC	isothermal titration calorimetry / kalorimetria bidezko balorazio isotermikoa
KAP1	KRAB associated protein 1 / KRAB-ari lotutako 1-proteina
k_B	Boltzmann constant / Boltzmann-en konstantea
KDSR	3-ketodihydrosphingosine reductase
LAMP2	lysosomal-associated membrane protein 2 / lisosomari lotutako mintz proteina 2
LB	Luria-Bertani
LC3A/B/B2/C	MAP1LC3; microtubule-associated protein 1 light chain 3 A, B, B2 or C / mikrotubuluei lotutako 3 A, B, B2 edo C kate arindun 1-proteina
LDL	low-density lipoprotein
LIR	LC3-interacting region / LC3-rekin elkarreagiteko eskualdea
LM	light microscopy / argizko mikroskopia
LMP	lysosomal membrane permeabilization
L_o	liquid-ordered lamellar lipid phase / fase lipidiko lamelar likido ordenatua
LPC	lysophosphatidylcholine / lisofosfatidilkolina
LUV	large unilamellar vesicle / lamela bakarreko besikula handia
L_{α}	fluid, liquid-disordered or liquid-crystalline lamellar lipid phase / fase lipidiko lamelar jariakorra, likido desordenatua edo likido-kristalinoa
L_{β}	gel or solid-ordered lamellar lipid phase / fase lipidiko lamelar gel edo solido ordenatua
L_{β'}	oblique lamellar lipid phase / fase lipidiko lamelar zeharra
L_{βi}	interdigitated lamellar lipid phase / fase lipidiko lamelar interdigitatua

MAPK	mitogen-activated protein kinase / mitogenoen bidez aktibatzen den proteina kinasa
mAU	milli absorbance units
MEF	mouse embryonic fibroblast / sagu enbrioien fibroblastoa
Mff	mitochondrial fission factor / fisio mitokondrialaren faktorea
Mfn	mitofusin / mitofusina
Miro	mitochondrial Rho GTPase
MLCL	monolysocardiolipin / monolisokardiolipina
MLV	multilamellar vesicle / lamela anitzeko besikula
MNA	methyl nadic anhydride
MtCK	mitochondrial creatine kinase / mitokondrioaren kreatina kinasa
mtDNA	mitochondrial DNA / DNA mitokondriala
mTORC1	mammalian target of rapamycin complex 1 / ugaztunen rapamizina 1-komplexuaren itua
MTS	mitochondrial targeting sequence
Mu1	mitochondrial ubiquitin ligase activator of NFKB 1 / NFKB-ren aktibatzailea den 1-ubikitina ligasa mitokondriala
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
NBR1	neighbor of BRCA1 (breast cancer early-onset 1) gene 1
NIX	NIP3-like protein X / NIP3-moduko proteina X
NMR	nuclear magnetic resonance / erresonantzia magnetiko nuklearra
Norm.	normalized
Nox	NADPH oxidase
NSF	N-ethylmaleimide sensitive factor / N-etilmaleimida faktore sentikorra
nSMase	neutral sphingomyelinase / esfingomielinasa neutroa
NUPR1/p8	nuclear protein 1
OATL1	ornithine aminotransferase-like 1
OD₆₀₀	optical density at 600 nm / 600 nm-tara neurtzen den dentsitate optikoa
OMM	outer mitochondrial membrane / mitokondrioaren kanpo-mintza
OPA1	optic atrophy 1 protein / atrofia optikoaren 1-proteina
OPTN	optineurin / optineurina

PA	phosphatidic acid / azido fosfatidikoa
PARK2/Parkin	E3 ubiquitin-protein ligase parkin
PARL	presenilin-associated rhomboid-like protein / prenisilinari lotutako erronboide-moduko proteina
PAS	phagophore assembly site / fagoforoaren mihiztadura gunea
PBS	phosphate buffered saline / fosfato gatz indargetzailea
PC/PtdCho	phosphatidylcholine / fosfatidilkolina
PCR	polymerase chain reaction / polimerasaren kate-erreakzioa
PD	Parkinson disease / Parkinson-en gaixotasuna
PDI	polydispersity index / polidispersitatearen indizea
PDI (protein)	protein disulfide isomerase
PE/PtdEtn	phosphatidylethanolamine / fosfatidiletanolamina
PFA	paraformaldehyde / paraformaldehidoa
PG	phosphatidylglycerol / fosfatidilglizerola
PH	pleckstrin homology / pleckstrin-en homologia
PI3KC1/3	class I or III phosphatidylinositol-3-kinase / I. edo III. motako fosfatidilinositol-3-kinasa
PINK1	PTEN-induced putative kinase 1 / PTEN-eragindako ustezko 1-kinasa
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PIs	phosphoinositides / fosfoinositidoak
PKA	cAMP-dependent protein kinase / cAMP-menpeko proteina kinasa
PKCα	protein kinase C alpha / C-proteina kinasa alfa
PLA2/C/D	phospholipase A2, C or D / A2-, C- edo D-fosfolipasa
PLO	protein-lipid overlay / proteina-lipido gainjartzea
PLS3	phospholipid scramblase-3 / fosfolipidoen 3-eskranblasa
PP1/2A	protein phosphatase 1 or 2A / 1- edo 2A-proteina fosfatasa
PRAS40	proline-rich AKT substrate 40 kDa / prolinan aberatsa den 40 kDa-eko AKTren substratua
PROPPINs	β -propellers that bind polyphosphoinositides / polifosfolipidoetara lotzen diren β -helizeak
PS	phosphatidylserine / fosfatidilserina
pSM	palmitoylsphingomyelin / palmitoilesfingomielina

Pt	platinum
PtdIns	phosphatidylinositol / fosfatidilinositola
PtdIns[3,4,5]P₃	phosphatidylinositol-3,4,5-trisphosphate
PtdIns[3,4]P₂	phosphatidylinositol-3,4-bisphosphate / fosfatidilinositol-3,4-bisfosfato
PtdIns[4,5]P₂	phosphatidylinositol-4,5-bisphosphate
PtdIns3P/PI3P	phosphatidylinositol-3-phosphate / fosfatidilinositol-3-fosfato
PtdIns4P	phosphatidylinositol-4-phosphate
PTFE	polytetrafluoroethylene / politetrafluoroetilenoa
P_β	rippled lamellar gel phase / fase gel lamelar uhindua
QELS	quasi-elastic light scattering / argiaren dispertsio kuasi-elastikoa
Rab	Ras-related GTP-binding protein / Ras-ekin erlazionatutako GTP-lotzen duen proteina
Raptor	regulatory associated protein of mTOR / mTORen proteina erregulatzailerik gertatzen
R_h	hydrodynamic radius
Rho-PE	Rhodamine-PE / Rodamina-PE
RING	really interesting new gene
ROI	region of interest / intereseko eskualdea
ROS	reactive oxygen species / oxigeno espezie erreaktiboak
Rot	rotenone
RPE1	hTERT-immortalized human retinal pigment epithelial 1
rRNA	ribosomal RNA / RNA erribosomikoa
S1P	sphingosine 1-phosphate / esfingosina-1-fosfatoa
SASA	solvent-accessible surface area
SDM	site-directed mutagenesis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis / sodio dodezil sulfatozko poliakrilamidazko elektroforesi gela
SEC	size exclusion chromatography
SH2	src homology 2
shRNA	small hairpin RNA
SIN	self-inactivating

siRNA	small interfering RNA / interferentziako RNA laburra
SLRs	sequestosome 1-like receptors / 1-sekuestosoma-moduko hartzaileak
SM	sphingomyelin / esfingomielina
SMase	sphingomyelinase / esfingomielinasa
SMPD1	sphingomyelinase phosphodiesterase 1
SMS	sphingomyelin synthase / esfingomielina sintasa
SNARE	SNAP soluble NSF attachment protein receptor
SphK	sphingosine kinase / esfingosina kinasa
SPR	surface plasmon resonance / gainzaleko plasmioen erresonantzia
SPT	serine palmitoyltransferase
SQSTM1/p62	sequestosome-1 / 1-sekuestosoma
SR	serine-arginine / serina-arginina
Stbd1	starch-binding-domain-containing protein 1 /almidoia lotzeko domeinua duen 1-proteina
SUV	small unilamellar vesicle / lamela bakarreko besikula txikia
TAX1BP1	Tax1 binding protein 1 / Tax1 lotzen duen 1-proteina
TBC1D2B	TBC1 domain family member 2B / TBC1 domeinudun 2B familia kidea
tBID	truncated-BH3 interacting domain death agonist
TCEP	tris(2-carboxyethyl) phosphine
TEM	transmission electron microscopy / transmiziozko mikroskopia elektronikoa
THC	Δ 9-tetrahydrocannabinol
TIM	translocase of the inner membrane / barne-mintzeko translokasa
TLCL	tetralinoleoylcardiolipin / tetralinoleilkardiolipina
T_m	lipid phase transition temperature / lipido-fase trantsizio tenperatura
TMCL	tetramyristoylcardiolipin
TNF	tumor necrosis factor
TOCL	tetraoleoylcardiolipin
TOM	translocase of the outer membrane / kanpo-mintzeko translokasa
TOMM20	mitochondrial import receptor subunit TOM20 homolog
TR-FRET	time-resolved fluorescence resonance energy transfer

TRIB3	tribbles pseudokinase 3
tRNA	transfer RNA / RNA transferentea
Ub	ubiquitin / ubikitina
UBL	ubiquitin-like / ubikitina-moduko
ULK1/2	uncoordinated-51 (unc-51)-like kinase 1 or 2 / koordinatu gabeko-51 (unc-51)-moduko 1- edo 2-kinasa
UPR	unfolded protein response
Uth1p	probable secreted beta-glucosidase UTH1
UVRAG	UV-radiation resistance associated gene / erradiazio ultramorearekiko erresistentziarekin lotutako genea
V-ATPase	vacuolar H ⁺ -ATPase
VCP/p97	type II AAA+ ATPase valosin-containing protein / II. motako AAA+ ATPasa den balosinadun proteina
VDAC	voltage dependent anion channel / boltai menpeko kanal ionikoa
Veh	vehicle
VHL	von Hippel-Lindau
VPS15/34	vacuolar protein sorting mutant 15 or 34
WIPI1/2	WD repeat domain phosphoinositide-interacting protein 1 or 2 / WD domeinu errepikatua daukan eta fosfoinositidoekin elkarreragiten duen 1- edo 2-proteina
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
WT	wild-type
ΔH	lipid phase transition enthalpy / lipido-fase trantsizio entalpia
$\Delta T_{1/2}$	width at half-height lipid phase transition / zabalera lipido-fase trantsizioaren altueraren erdian
$\Delta\pi$	surface pressure increase / gainazaleko presioaren igoera
$\Delta\psi$	membrane potential / mintz potentziala
ϵ	molar extinction coefficient / iraungipen molarraren koefizientea
θ	ellipticity / eliptikotasuna
$\lambda_{em(max)}$	wavelength at maximum fluorescence emission / fluoreszentszia igorpen maximoaren uhin luzera
$\lambda_{ex/em}$	fluorescence excitation or emission wavelength / fluoreszentszia kitzikapen edo igorpenaren uhin luzera
π	surface pressure / gainazaleko presioa

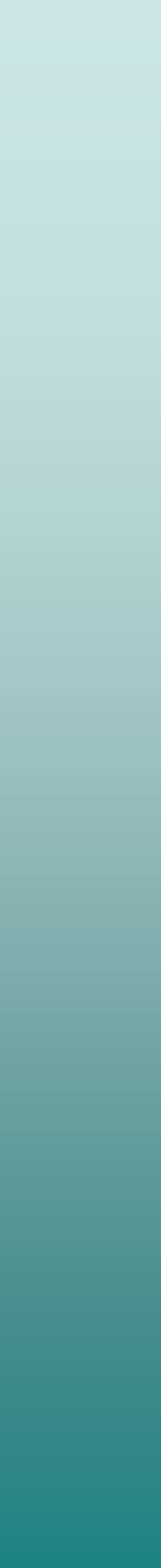
π_0/π_i	initial surface pressure / hasierako gainzaleko presioa
π_c	critical surface pressure / gainazeko presio kritikoa

Protokolo Esperimentalen Aurkibidea

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1. Kapitulua

Sarrera eta Helburuak



1. Kapituluua:

Sarrera eta Helburuak

1.1 Mintz Zelularrak

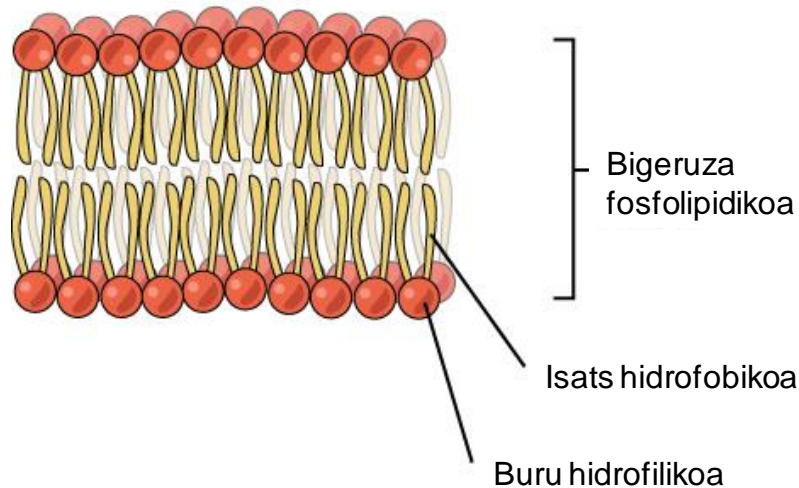
1.1.1 Ezaugarri Orokorak eta Funtzioak

Zelula bizidunak elkarrekin hartu-emanean eta era berean kanpo ingurutik babestuta egon behar dute. Mintzek inguruan egon daitezkeen baldintza ezberdinen aurrean biziraupenerako babesak eskaintzen dute. Mintz plasmaticoak zelula inguratzen eta mugatzen du molekula disolbagarrientzako iragazkortasun hesi hautakor bat sortuz. Prokariotoek ez bezala, zelula eukariotoek mintz plasmaticoa izateaz gain, organulu ezberdinak inguratzen dituzten zelula barneko mintzak dauzkate. Mintz guzti hauek barne-mintz sistema zabal baten parte dira eta zelulako konpartimentu ezberdinen artean molekulen hartu-emanaren erregulatzen duen hesi hautakorra osatzen dute.

Mintz plasmaticoa lipido geruza fin batez eta bertan barneratuta dauden proteina askoz osatua dago (Singer eta Nicolson, 1972; Goñi, 2014). Bai lipidoek zein proteinek, mintza zitoeskeletona ainguratzen parte hartzen dute, horrela, zelulari forma emateaz gain, kanpo matrizearekin elkarrekin, ehunen antolaketa egokia mantentzea lortzen dute. Gainera, mintzean aurkitzen diren osagaiak, zelulen seinalizazioa, zelulen arteko atxikidura, ioien eroankortasuna, elikagaien harrera edota behar ez diren materialen kanporaketa bezalako beste prozesu asko erregulatzen dituzte, eta prozesu hauek mintzean edota bere gertutasunean ematen dira.

Mintz zelularra era hautakorrean iragazkorra da eta biziraupenerako beharrezkoak diren osagaien garraioa ahalbidetzen du. Garraio hautakor honi esker, mintzek zelularen potentziala mantentzen dezakete. Askotan mintzaren alde batetik besterako solutuen mugimendua proteinen parte hartzea beharrezko duen mekanismo

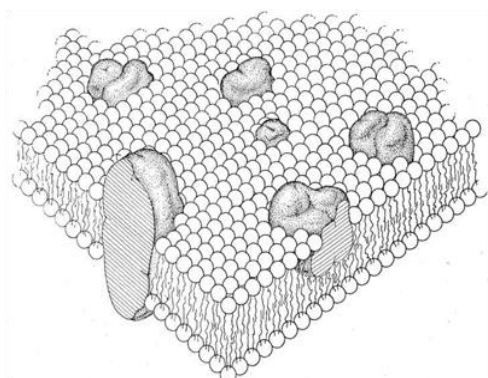
batzuen bidez kontrolatua dago. Karbono dioxidoa edota oxigenoa bezalako molekula txikiek, difusio sinple bidez zeharka dezakete mintza. Bestalde, karga elektrikoa duten partikulek (ioiak) edo mantenugai handiagoek (aminoazidoak, azukreak) energia gastua suposatzen duten eta proteinen parte hartzea behar duten garraio mekanismo konplexuagoak behar dute.



1.1 Irudia. Bigeruza lipidiko baten diagrama eskematikoa.

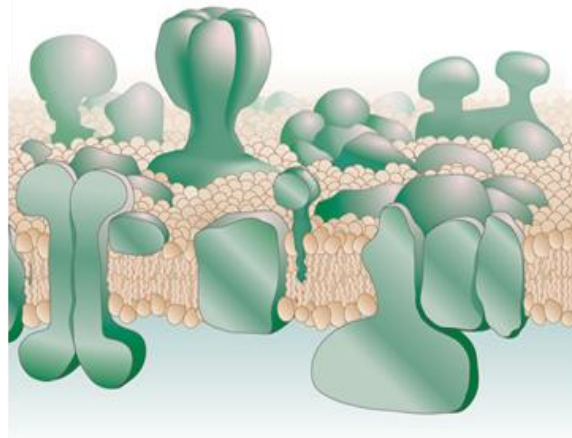
(<https://www.boundless.com/biology/textbooks/boundless-biology-textbook/biological-macromolecules-3/lipids-55/phospholipids-300-11433/>-etik moldatua).

Mintzetako lipidoak, gehienetan bigeruza lipidikoak eratuz mihizta daitezkeen isats hidrofobikodun eta buru polardun molekula anfipatikoak dira (**1.1 Irudia**). Lipido hauek, ura bezalako disolbatzaile baten aurrean, berez antolatu daitezke beraien buru hidrofilikokoak ur molekulekin elkarreaginez eta isats hidrofobikoak ur ingurutik isolatuta utziz (Israelachvili *eta* *lank.*, 1980). Beraz, mintz zelularretako lipidoek bigeruza egitura hartzen dute, non isats hidrofobikoak elkarri begira eta buru polarrak zitoplasmarekin edota zelularen kanpo inguruarekin kontaktuan geratzen diren. Proteinak, beste osagai nagusia, mintzan zehar egokitzen dira gehienetan mintz plasmaticoaren masaren %50etik gora hartuz.



1.2 Irudia. Singer eta Nicolson-en lipido eta proteinen "mosaiko fluido" izeneko biomintzen egituraren eredu. (Singer eta Nicolson, 1972-etik hartua).

Testuinguru honetan, Singer eta Nicholson-ek mosaiko fluidoa izenez ezagutzen den zelula mintzen eredua proposatu zuten. Eredu honetan lipido eta proteinak, modu errazean, mintzaren planoan biratu eta barreiatu daitezkeela defendatzen da (**1.2 Irudia**). Beraz, mintza asimetrikoa izango litzateke bi geruzen arteko mugimendu mugatua dela eta. Mintz proteinak gehienetan proteina periferiko edota integral bezala sailkatzen dira. Proteina integralek (intrintsekoak ere deituak) gutxienez beraien segmentu bat bigeruz fosfolipidikoan barneratuta daukate. Proteina integral gehienek fosfolipidoen kate azilikoekin elkarreragiten duten hondar hidrofobikoak dauzkate, horrela proteina mintzera ainguratzea lortuz. Hala ere, lotura kobalenteen bidez gantz azidoetara ainguratuta dauden proteinen kasuan gertatzen den moduan, kate polipeptidikoa ez da beti bigeruz fosfolipidikoan barneratzen. Bestalde, proteina periferikoek (estrinsekoak ere deituak) gehienetan lipidoen buru polarrekin edota proteina intrintsekoen atal polarrekin elkarreragiten dute.



1.3 Irudia. Singer-Nicolson ereduaren bertsio zuzendua eta eguneratua. (Engelman, 2005-etik moldatua).

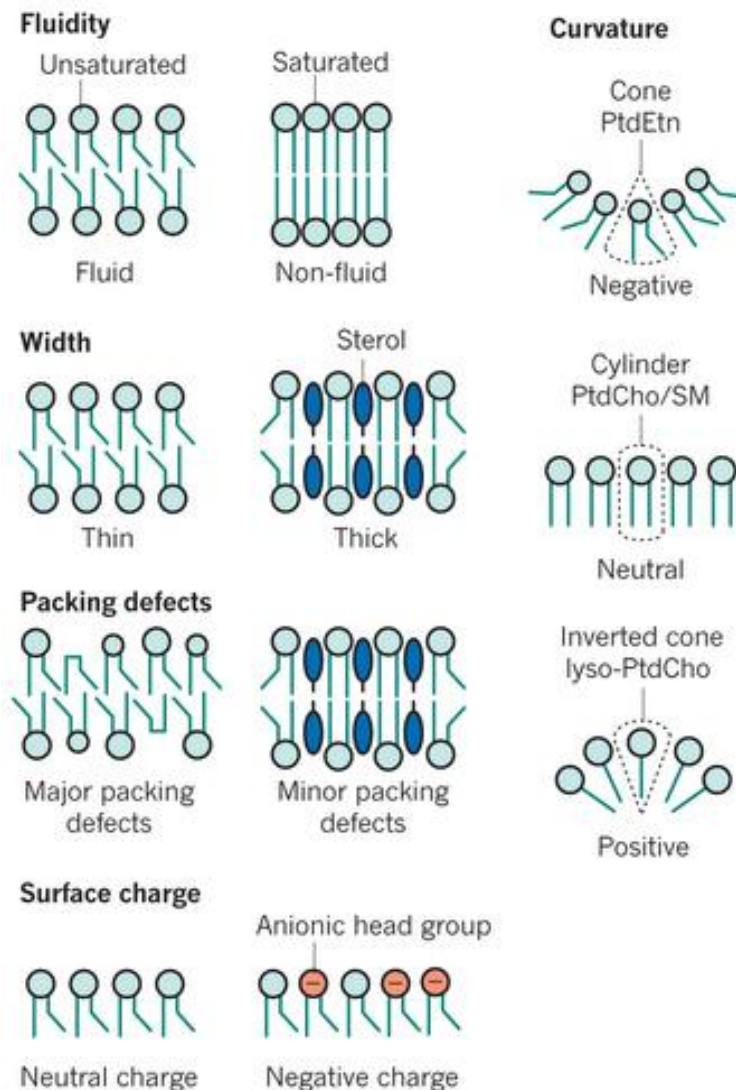
Ondorengo hamarkadetan eredu honen geroko doitze eta eguneratzeak plazaratu dira (Jacobson *eta* *lank.*, 1995; Engelman, 2005; Goñi, 2014). Gaur egun aintzat hartzen da proteina intrintsekoak oso ugariak izateaz gain, mintzean zehar modu homogeneo batean sakabanatuak daudela (**1.3 Irudia**). Zenbait proteina ez daude beti mintzarekin kontaktuan, hau da, denbora-tarte batzuetan zitosolean eta beste tarte batzuetan mintzera itsatsita aurkitzen dira. Gainera, mintz biologikoek albo heterogeneotasuna aurkezten dute. Proteina eta lipido espezifiko osatutako domeinuak izan ditzakete, horrela mintzekin lotutako zenbait prozesu biologikoen banaketa espaziala ahalbidetuz, adibidez ioi eta metabolitoen garraioa, seinalizazioa, zelulen atxikimendua eta migrazioa bezalako prozesuena. Gainera, errealitatean, bigeruz ez da laua. Lipidoen asimetria eta antolaketa ez-lamelarraren ondorioz mintzaren kurbadura lokala eragin daiteke. Funtzio biologiko garrantzitsuak dauzkaten zenbait lipido-egitura ez-lamelarrak modu iragankorrean eratu daitezke, adibidez,

mintzen arteko fusioa edota zatiketa zelularra bezalako prozesuetan. Mintzean, lipidoen asimetria, flipasa edo eskranblasa proteinen bidez kontrolatuta dago. Lipidoen flip-flop mugimendua oso garrantzitsua da zenbait prozesutan, adibidez, zelularen erantzun autofagiko batean. Erantzun honetan, gehien bat mitokondrioaren barne-mintzean aurkitzen den fosfolipido baten, kardiolipinaren (CL, ingelesetik *cardiolipin*), kanporaketa eragiten da mitokondrioaren ezagutzea eta mitofagia sustatzeko (Chu *eta lank.*, 2013; Maguire *eta lank.*, 2016). Tesi lan honetan sakonki ikertua izan den gertaera hau, aurrerago zehaztasun gehiagoz azaldua izango da.

1.1.2 Mintzetako Lipidoak

Animalien mintz zelularren %50 inguru, lipidoz (batez ere fosfolipidoak, glikolipidoak eta esterolak) osatua dago. Lipidoek funtzio biologiko garrantzitsuak dauzkaten molekula talde heterogeneoa osatzen dute. Zelulan, mintz zelularren eraketa prozesuan parte hartzen dute. Dena den, lipidoen gehiengoa energia erreserba gisa erabiltzen da eta, batez ere triglizerido eta esterol ester moduan, tanta lipidikoetan aurkitzen dira. Gainera, lipidoak seinaleen transdukzio eta molekulen ezagutze-prozesuetan lehen eta bigarren mezulari gisa joka dezakete ere (Bartke *eta Hannun*, 2009; van Meer, 2005; van Meer *eta lank.*, 2008).

Mintz zelularrak atal hidrofobiko eta hidrofilikoa daukaten lipido polarrez osatuta daude. Mintzen berezko eraketaren oinarri fisikoak, atal hidrofobikoek elkarrekin lotzeko joera eta atal hidrofilikoen ingurune urtsuekin elkarrekintzak izateko joera dira. Buruaren karga kontuan edukiz, lipido hauek neutralak (zwitterionikoak) edo elektrikoki kargatuak (gehienetan negatiboki) izan daitezke. Gehienetan buru honi 2 karbonotik 28ra eduki ditzaketen isats hidrofobiko aziliko 1 edo 2 lotzen zaizkio. Gainera hauek aseak edota *cis* edo *trans* lotura bikoitzez asegabeak izan daitezke (Berg *eta lank.*, 2002) **(1.4 Irudia)**.



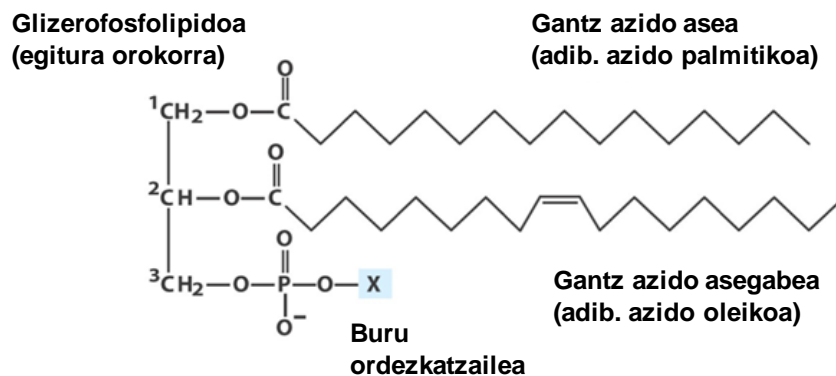
1.4 Irudia. Lipido konposaketak mintzaren ezaugarri fisikoak baldintzatzen ditu. Gantz azido labur eta asegabeek jariakortasuna sustatzen dute. Lotura bikoitzek kate azilikoek paketatze dentsitatea jaistea eragiten duten kurbadurak eratzen dituzte eta honek mintza fase jariakor batetik solido-gel fase batera aldatzea eragozten du. Lodiera kate azilikoek luzeerak eta esterolek baldintzatzen dute eta, azken hauek, kate azilikoak antolatu eta luzatzeaz arduratzen dira. Kate aziliko asegabeak eta buru txikiak dauzkaten lipidoetan paketatze akatsak aurkitu ohi dira. Gainazaleko karga fosfatidilserina (PS, ingelesetik *phosphatidylserine*) eta fosfoinositidoak (PIs, ingelesetik *phosphoinositides*) bezalako lipido anionikoek baldintzatzen dute. Lipidoen itxurak kurbadura baldintzatzen du. Beraien buru polarrek, kate azilikoarekiko azalera ratio txikia daukaten lipidoek (kono itxura eratu) kurbadura negatiboa eragiten dute, buruek katearekiko azalera ratio berdina daukaten lipidoak (zilindro itxura eratu) neutralak dira, eta burua kate azilikoaren azalera baino handiagoa daukatenean (kono alderantzikatuaren itxura eratu) kurbadura positiboa eragiten dute. (Holthuis eta Menon, 2014-etik moldatua).

1.1.2.1 Mintzetako Lipidoen Sailkapena

Zelula mintzetan aurkitzen diren lipidoak, batez ere, glizerolipidoak, esfingolipidoak eta esterolak dira.

A) Glizerolipidoak

Mintz eukariotikoetan aurkitzen diren eta egitura funtzioa duten lipido gehienak glizerolipidoak dira. Bi motatakoak bereiz daitezke, glizerofosfolipidoak (adib. kardiolipina) eta glizeroglikolipidoak. Glizerofosfolipidoen egitura glizerol molekula batean oinarritzen da. Glizerolari, gehienetan ester loturen bidez, *sn-1* eta *sn-2* posizioetako karbonoetara, gantz azido bana lotzen zaizkio. Glizerolaren *sn-3* posizioako karbonoari fosfato talde bat lotzen zaio eta fosfato honi talde polar bat esterifikatzen zaio (**1.5 Irudia**). Glizerolari lotutako kate azilikoaren luzera eta asetasunaren arabera, eta fosfatora lotutako talde polarraren arabera glizerofosfolipido espezie desberdin asko sortzen dira.



1.5 Irudia. Glizerofosfolipidoen egitura orokorra.

(<https://www.studyblue.com/notes/note/n/lipids/deck/1546284>-etik moldatua).

Beste motako glizerolipidoak diren glizeroglikolipidoek, animali zeluletako mintzetan askoz urriagoak diren arren, landare eta zenbait bakterio mintzetan kantitate handietan ageri dira. Fosfato talde bat falta dute, baina horren ordez, glizerolaren *sn-3* karbonora, lotura glikosiliko baten bitartez, mono- edo oligo-sakarido talde bat atxikia daukate.

B) Esfingolipidoak

Esfingolipidoak fosfoesfingolipido eta glikoesfingolipidoetan sailka daitezke. Beraien oinarri hidrofobikoa esfingosina izeneko kate asegabe luzeko amino oktadezil

alkohol bat da. N-hexadekanoil-D-*eritro*-esfingosina, edo zeramida, seinalizazio zelularrean garrantzitsua den esfingolipido bat, amida lotura baten bitartez esfingosina bati kate aziliko bat lotuz sortzen da (**1.6 Irudia**). Zeramida bati fosforilkolina molekula polarra lotuz sortzen den esfingomielina, zelularen mintz plasmaticoaren kanpoaldeko geruzan ugaria den fosfoesfingolipido bat da.

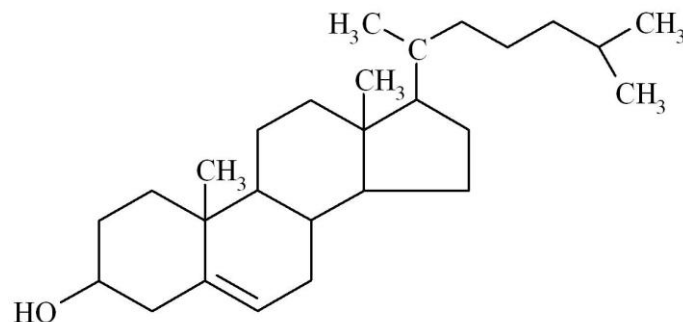


1.6 Irudia. Esfingolipidoen egitura orokorra. Esfingosina laukizuzen gorri batean, gantz azidoa beltzez, eta R buru aldakorrari dagokio (Fuller, 2010-etik moldatua).

Azukre egitura konplexuen loturak glikoesfingolipido izeneko esfingolipido mota baten eraketa dakar, hauek azukre talde kopuruaren eta azido sialikoen presentziaren arabera zerebrosido eta gangliosido gisa sailka daitezke.

C) Esterolak

Zelula mintzetan ageri diren hirugarren lipido taldea osatzen dute. Lipido hauek zelula mintzean ageri diren lipido apolar ugariak dira. Esterolen egitura zurruna, 4 eraztun hidrofobiko eta buru polar txiki batek osatzen dute. Gehienetan, esterolak isoprenoideen metabolismoko mebalonatoaren bidezidorrean sintetizatzen dira. Ugaztunetan kolesterola da esterol ugariena (**1.7 Irudia**). Bien bitartean, legamietan ergosterola da nagusi.

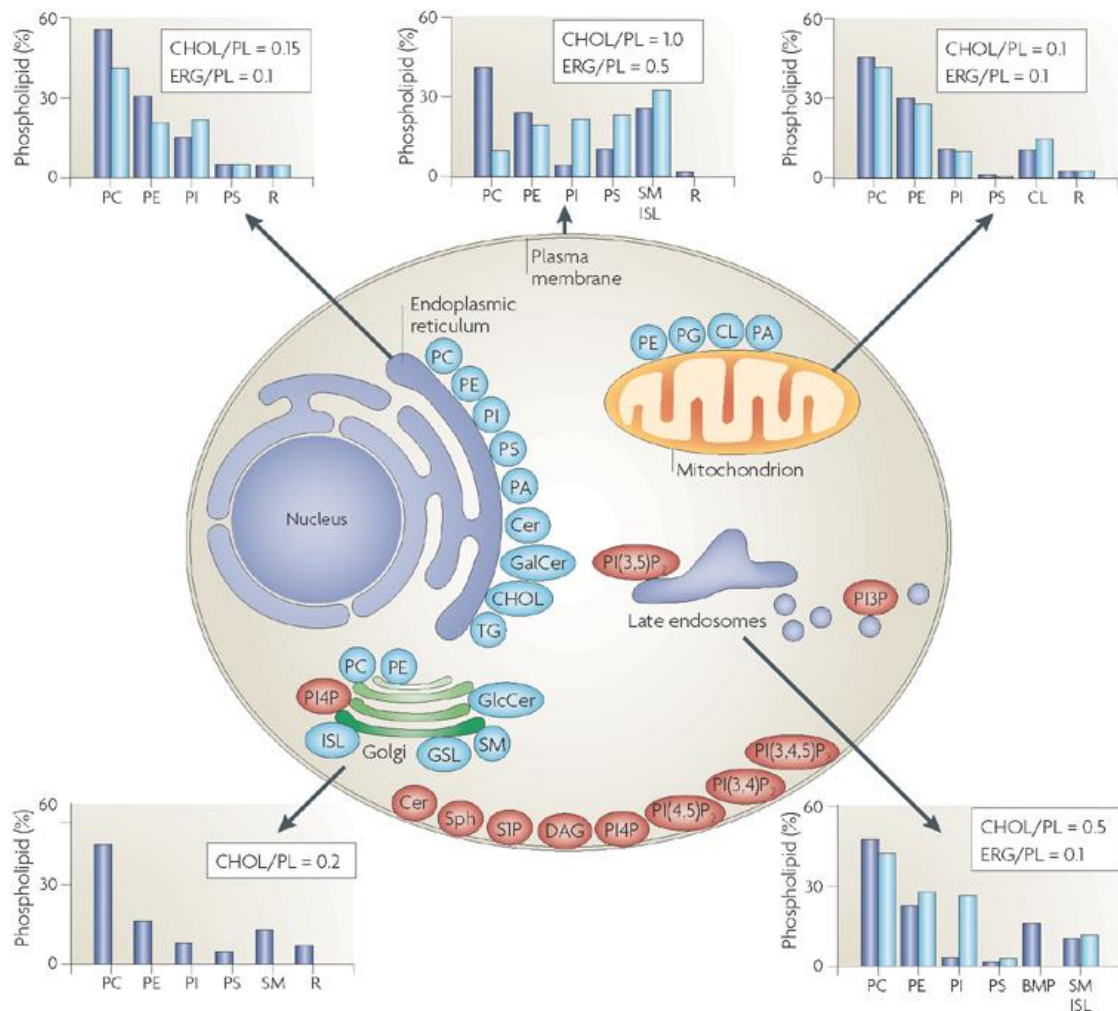


1.7 Irudia. Kolesterolaren egitura.

(<http://chemed.chem.purdue.edu/genchem/topicreview/bp/1organic/hydro.html>-etik hartua).

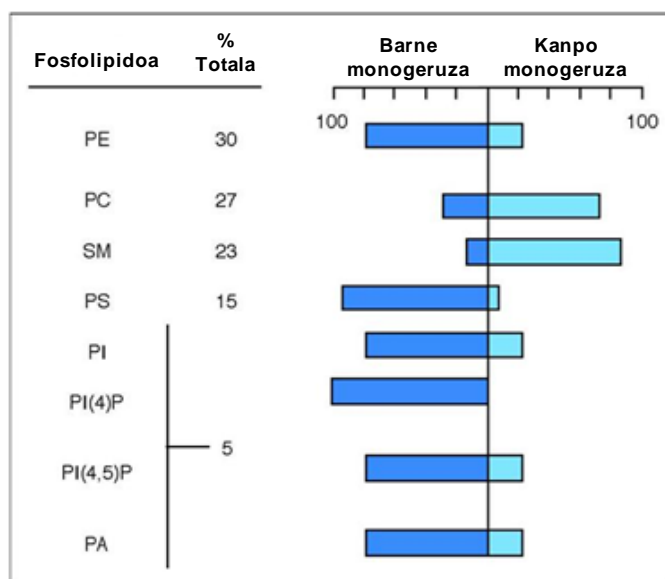
1.1.2.2 Lipidoen Banaketa eta Asimetria

Zelula eukariotoek 1000 lipido espezie ezberdinetik gora dauzkate beraien mintzetan. Aniztasun lipidiko handi hau, lipido mota bakoitzaren buru eta kate alifatiko desberdinen ondorioa da. Interesgarria suerta daiteke ugaztunen organulu nagusietan fosfolipidoak, esfingolipidoak eta esterolak era homogeen batean banatuak ez egotea. Izan ere, banaketa heterogeneo honek ezaugarri bereziak ematen dizkie (**1.8 Irudia**). Banaketa honek agerian uzten ditu lipido bakoitzaren eginkizun fisiologiko ezberdinak, lipido bakoitzak zelulan daukan metabolismo lokala eta lipido konposaketa ezberdinek sortzen dituzten mintzaren ezaugarri fisiko-kimiko espezifikoak (**1.4 Irudia**). Adibidez, esfingomielina eta kolesterola batez ere mintz plasmatikoa aurkitzen diren bitartean, kardiolipina ia soilik mitokondrioen mintzetan aurki daiteke.



1.8 Irudia. Zelulan aurki daitezkeen mintz ezberdinen lipido konposaketa. (van Meer *eta* lank., 2008-etik hartua).

Gainera, lipidoen heterogenitatea, bai zelulan aurkitzen diren mintz ezberdinen artean zein mintz berean ematen da. Lipidoak bigeruz lipidikoaren geruza bakoitzean era asimetriko batean sakabanatuak egon daitezke. Ezaugarri honi lipidoen zeharkako asimetria deritzo eta mintz zelular guztiek funtzionalak izateko beharrezko duten ezaugarria da. Giza eritrozitoetan, adibidez, beraien buru polarrean kolina daukaten ia lipido guztiak [fosfatidilkolina (PC, ingelesetik *phosphatidylcholine*) eta esfingomielina (SM, ingelesetik *sphingomyelin*)] batez ere kanpo monogeruzan kokatuak daude. Bestalde, amino taldea daukaten lipidoak [fosfatidiletanolamina (PE, ingelesetik *phosphatidylethanolamine*), PS] gehien bat barne monogeruzan aurkitzen dira (**1.9 Irudia**). Beraz, konposaketaz gain bi hemimintzen karga ere oso ezberdina da, barnekoak karga negatiboa daukan bitartean, kanpokoa neutroa da. Bi monogeruzen arteko lipido konposaketa ezberdinaren arrazoi nagusia, zenbait lipidoen sintesi asimetrikoa edo lipidoen noranzko bakarreko translokazioa ahalbidetzen duten garraiatzaileen presentzia da.



1.9 Irudia. Fosfolipidoen sakabanaketa asimetrikoa eritrozitoen mintz plasmaticoan.

Mintz ereduetan, bigeruzetako geruza bakoitzaren artean gertatzen den lipido polarren berezko mugimenduaren ratioa geldoa da (orduetatik egunetara bitartekoa PC lipidoarentzako) eta buruaren tamaina, karga eta polaritateak baldintzatua dago. Glikoesfingolipido (GSL, ingelesetik *glycosphingolipid*) konplexuen translokaziorako bataz-besteko denbora egunetakoa den bitartean, zeramidarentzat (Cer, ingelesetik *ceramide*), diazilglizerolarentzat (DAG, ingelesetik *diacylglycerol*) eta esterolentzat segundoetakoa da. Hori dela eta, mintz zelularretan zenbait proteinek (flipasek, flopasek, eskranblasek) mugimendu hau errazten dute, kasu batzuetan, energia gastua suposatzen duelarik (Contreras *eta* *lank.*, 2010).

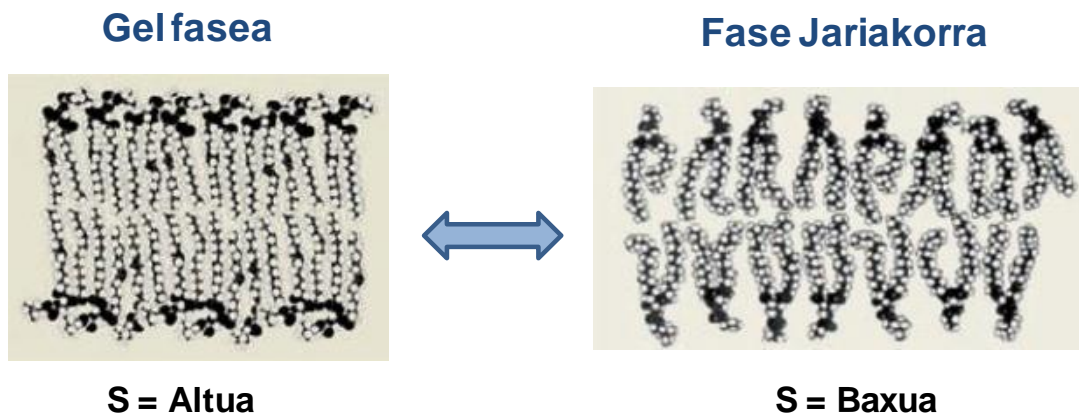
Gainera, bigeruzako monogeruza bakoitzean zehar ere, lipidoak ez daude modu homogeneo batean sakabanatuak. Lipidoek albo asimetria ageri dute. Mintzaren jariakortasuna lipidoen mugimenduekin lotuta dago eta albo mugimendu librearen ahalmena erregulatzen du. Testuinguru honetan, lipido domeinuak albo heterogeneotasun honekin lotuta daude.

1.1.2.3 Lipidoen Polimorfismoa

Mintz biologikoen plastikotasun morfologikoa funtsezkoa da zelulen biziraupenerako. Izan ere, zelulek, jasotako estimulu baten aurrean erantzun egoki bat eman ahal izateko, mintzak erraz berrantolatzeke ahalmena eduki behar dute. Mintz zelularrak dimentsio bakarreko egitura lamelar gisa antolatzen dira. Hala ere, gertaera espezifikoko batzuetan lamelarrak ez diren egitura konplexuagoak ageri da, horren adibide dira alderantzikako fase hexagonalak edo fase kubikoak. Makromolekulen mintz zeharreko mugimendua edota zatiketa zelularra bezalako hainbat prozesuetan, egitura ezegonkor hauen eraketa beharrezkoa da (Epan, 1998). Mintzen fusio eta fisio prozesuak, adibidez, birusen infekzioa, erretikulu endoplasmatikotik (ER, ingelesetik *endoplasmic reticulum*) Golgirako besikulen garraioa, edota garraio endozitiko eta exozitikoak, energetikoki aldekoak ez diren eta lamelarrak ez diren egitura iragankorren eraketa beharrezkoa duten gertaeren adibide onak dira (Gilbert, 2016; Kozlov eta Chernomordik, 2015). Hala eta guztiz ere, lipidoen polimorfismoa ez dago soilik bi dimentsioko egitura aldaketetara lotuta. Adibidez, mintz batean alboz banatutako egituren eraketa, non proteina konplexuek konformazio egonkorragoa har dezaketen, dimentsio bakarreko aldaketen arabera da, hain zuzen, lipidoen antolaketa eta jariakortasunaren arabera. Lipido konposaketa (**1.4 Irudia**) eta tenperaturak, mintzaren jariakortasuna baldintzatzen dute eta hau ongi erregulatua egon behar da, adibidez, garraioa edo entzimen aktibitatea egokia izateko.

Fase lipidikoa kontzeptu termodinamiko bat da. Kontzeptu honek, lipido batek edo lipido nahastura batek soluzio urtsu batean hartzen duen egitura patroia ezberdinei egiten dio erreferentzia. Oreka egoeran dauden mintz biologikoak konfigurazio lamelarrean aurkitzen diren bigeruzko lipidiko osatzen dira, non fase adierazgarriak: fase jariakorra, likido desordenatua edo likido-kristalinoa (L_{α}), fase gel edo solido ordenatua (L_{β}), eta fase jariakor eta solidoaren tarteko ezaugarriak dituen fase likido ordenatua (L_o) diren. L_{α} fasean lipidoak alboz barreiatzeko eta biratzeko libre dira eta beraien kate azilikoak elastikotasun handiko egoera desordenatu batean dauzkate. Normalean, gutxienez kate azilikoetako bat, asegabea izaten da. L_{β} faseko lipidoek

ordena altuko kate azilikoak eta proportzio handian C-C *trans* konformeroak dauzkate, honek mugimendua asko mugatzen duenez ezinezkoa dute albo mugimendua edo biraketa (Marsh, 1980). L_{β} fasetik L_{α} faserako trantsizioa, trantsizio-fase nagusi gisa ezagutzen da (**1.10 Irudia**).



1.10 Irudia. Gel fase lamelarretik fase lamelar jariakorrerako trantsizioa eta honi lotutako altuera eta kateen antolamenduaren aldaketa. S-k kate azilikoaren ordenamendu parametroa adierazten du. (van Meer *eta* *lank.*, 2008-etik moldatua).

Beste fase lamelar batzuk, kate azilikoak mintzaren planoarekiko okertuta ageri diren L_{β} fasea, bi geruzetako kate azilikoak interdigitatuak dauzkan $L_{\beta I}$ fase interdigitatua (kate aziliko labur eta luze bana dauzkaten lipidoetan ohikoa dena), eta fosfatidilkolina eta fosfatidilglicerol asetu gutxi batzuen bidez lor daitekeen P_{β} fase uhindua dira. Azken honetan, mikroskopia elektronikoa erabiliz izozketaren bidez apurtutako lipido laginen gainazalean uhinak beha daitezke.

Lipidoaren geometria molekularrak, lamelarrak ez diren zenbait egituren eraketa asko baldintzatzen du. Mintzetako lipidoen %30 inguruk sistema ereduatan lamelarrak ez diren egiturak hartu edota eragiten dituzte. Buru polar eta isats hidrofobikoaren arteko zeharkako ebaketaren azaleran ageri diren ezberdintasunek, lipido pilaketen egitura orokorra zehaztu dezakete (Israelachvili *eta* *lank.*, 1980). Ikuspuntu honetatik, lipidoak 3 talde nagusitan sailkatzen dira: kono, zilindro edo alderantzikatutako kono itxurako lipidoak (**1.11 Irudia**). Itxura hauek S parametro morfologikoaren bidez laburbil daitezke:

$$S = V/A_0L_c$$

1 Ekuazioa. S parametro morfologikoa.

non:

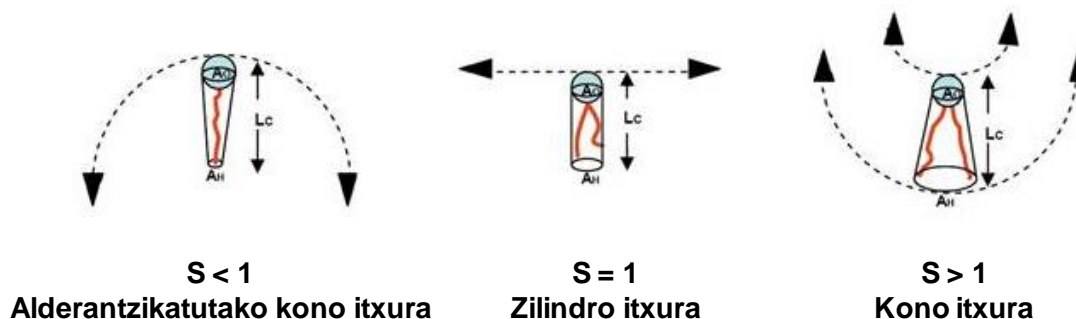
V = lipido molekularen bolumena den,

A_0 = lipido-ur interfasean dagoen molekularen azalera den eta

L_c = kate aziliko luzatuaren luzera.

Parametro hauek eta lipidoaren isats hidrofobikoaren zeharkako ebaketaren azalera, " A_H ", kontuan izanda, zilindro itxura daukan lipido baten kasuan $V = A_H L_c$ bolumena adieraziko luketena, geometria morfologiko ezberdinak honela adierazi ditzakegu:

- $A_0 = A_H$ ($S = 1$): Molekulak zilindro itxura dauka.
- $A_0 < A_H$ ($S > 1$): Molekulak kono itxura dauka.
- $A_0 > A_H$ ($S < 1$): Molekulak alderantzikatutako kono itxura dauka.



1.11 Irudia. Lipido fisiologikoen itxura molekularra.

Dena den, zaila da lipido faseen itxura eta kurbadura kuantitatiboki kalkulatzeko. Izan ere, molekularen azalera eta bolumena, ur kantitatearen, indar ionikoaren, pHaren, tenperaturaren, katioi dibalenteen eta proteinen menpe ere egoten dira. Lipido puruak soluzio urtsuetan berez elkartuko dira beraien geometriaren arabera egiturak eratuz. Zilindro itxura daukaten PC edo SM bezalako lipidoak bigeruz lamelar gisa antolatuko dira. Kono itxurako PE, DAG edo esterolek egitura "hexagonalak" (tubularrak) sortzeko joera edukiko dute, bestalde, lisofosfolipidoen (lisofosfatidilkolina, LPC, ingelesetik *lysophosphatidylcholine*) kasuan bezala, alderantzikatutako kono itxurako lipidoak,

mizeletan antolatuko dira (**1.4 Irudia**). Hala ere, zilindrikoak ez diren lipido hauek, PC, SM edo PS bezalako lipidoak aurkitzen diren bigeruzako lipidikoen parte ere izan daitezke. Adibidez, egitura lamelar bat eratzeko, beharrezkoa da lipido konikoekin batera zilindro itxurako lipidoen %20-%50 mol bitartean nahastuak egotea (Cullis *eta lank.*, 1986).

Laburbilduz, lipidoen polimorfismoak lipidoek baldintza fisiko espezifikoetan daukaten jokaera islatzen du, non fase konkretu batean dauden lipidoek egitura ordenatu ezberdinak eratzen dituzten. Lipidoen polimorfismoa zelularen funtzio askorekin zuzenean lotuta egongo litzateke, adibidez, mintzen fusioa, zatiketa zelularrean zeharreko besikulen eraketa, proteinen garraioa, endozitosia, mintzean proteina konplexuen antolaketa edo mintzean bertan eta mintzean zeharreko molekulen mugimendua. Prozesu guzti hauek, mintzeko zonalde batzuetan egitura iragankor ez lamelarren eraketa behar dute.

1.1.3 Proteina-Lipido Elkarrekintzak

Mintz proteinak mintzarekin era ezberdinetan elkarreragiteak funtzio ezberdinak aurrera eramatea ahalbidetzen du. Bigeruzako bakoitzak, ezaugarri konkretu batzuk ematen dizkion proteina talde espezifiko bat edukiko du.

1.1.1 atalean azaldu den moduan, mintz proteinak gehienetan proteina periferiko edo integral gisa sailkatzen dira. Proteina integralak (intrintsekoak ere deituak) gutxienez atal bat bigeruzako fosfolipidikoan barneratuta daukate. Proteina integral gehienek, proteina mintzera ainguratzeko, fosfolipidoen kate azilikoekin elkarreragiten duten hondar hidrofobikoak dituzte. Dena den, kate polipeptidikoa ez da beti bigeruzako fosfolipidikoan barneratzen, horren adibide da gantz azidoetara lotura kobalenteen bidez ainguratzen diren proteinen kasua. Beste muturrean proteina periferikoak (estrinsekoak ere deituak) daude, hauek gehienetan lipidoen buru polarrekin edo proteina intrintsekoekin elkarrekintzen dute.

Gainera, mintz proteina askok ez dute beraien biziraupen guztia mintzei ainguratuta igarotzen. Zenbaitak, soilik egoera konkretu batzuetan elkarreragiten dute bigeruzako lipidikoarekin, horrela, mintzari lotuta geratu (bai proteina integral zein periferiko gisa) edo medio urtsura azkar itzuli (mintz proteina ez-iraunkorrak) daitezke. Honi dagokionez, proteina ez-iraunkor hauek, mintzarekiko elkarrekintzaren itzulgarritasunaren arabera edo elkarrekintza motaren arabera sailka daitezke. Lehen

irizpideak, zenbait proteinek mintzarekin era itzulgarri batean eta beste batzuek era iraunkorrean (ez-itzulgarrian) elkarrekiten dutela onartzen du. Bigarrenak, proteinak elkarrekintzaren indarraren arabera sailkatzen ditu, mintzarekiko elkarrekintzak, bai itzulgarriak zein iraunkorrak, ahulak edo sendoak diren kontuan hartuz (Goñi, 2002).

Proteina-mintz elkarrekintzek, mintzaren berezitasun zehatz batzuk ezagutzen dituzten proteinen domeinu espezifikoaren menpe ere egon daitezke (Hurley, 2006; Lemmon, 2008; Moravcevic *eta* *lank.*, 2010). Gutxienez 10 proteina domeinu mota desberdin lotzen dira mintz gainazaleko fosfolipidoetara. Elkarrekintza hauek 2 motakoak izan daitezke: oso espezifikoak, zeintzuek mintzeko fosfolipido zehatz bat ezagutzea suposatzen duten, edo ez-espezifikoak, zeintzuek mintzaren gainazaleko ezaugarri fisiko bat dakarten (Lemmon, 2008). Adibidez, C1, C2, pleckstrin-en homologia (PH, ingelesez *pleckstrin homology*), PX, FYVE, edo polifosfoinositidoetara lotzen diren β -helizeak (PROPPINs, ingelesez *β -propellers that bind polyphosphoinositides*) domeinuak lehen motari dagokie. Bestalde, BAR domeinuak, lipido anfipatikoen paketamenduaren sentsorea (ALPS, ingelesez *amphipathic lipid packing sensor*) motiboak edo N-muturreko α -helize anfipatikoak, mintzaren kurbadura bezalako ezaugarri fisikoak antzematen dituzte.

Tesi lan honen ikerkuntza gai nagusia den sare autofagikoan, mintz zelular ezberdinekin lotzen diren zenbait proteina daude. Proteina horiek motibo lipidiko, gantz azido edo prenil taldea, glikosilfosfatidilinositol (GPI, ingelesez *glycosylphosphatidylinositol*) aingura, alfa helize anfipatikoa, FYVE [PtdIns3P-ra (ingelesez *phosphatidylinositol-3-phosphate*) lotzen dena] edo PH [PtdIns[3,4]P₂-ra (ingelesez *phosphatidylinositol-3,4-bisphosphate*) lotzen dena] bezalako domeinu baten bidez lotu daitezke. Gehienetan, C-muturreko glizina hondarrari kobalenteki lotuta dagoen PEn bitartez, Atg8 eta ugaztunen Atg8 proteina familia mintz autofagosomikoetara lotzen dira. Aldaketa mota hau oraingoz soilik Atg8 familian aurkitu da. Zenbait mintzeko proteina periferikoak autofagosomaren eraketan parte hartzen dute. Adibidez, koordinatu gabeko-51 (*unc-51*)-moduko 1- eta 2-kinasak (ULK1 eta ULK2, ingelesez *uncoordinated-51 (unc-51)-like kinase 1 and 2*), gatz eta detergenteekiko erresistentea den mintz frakzio batean ageri direnak, eta FYVE domeinua daukan DFCP1 proteina (ingelesez *double FYVE domain-containing protein 1*) (Tooze, 2010). Gainera, elikagairik gabeko egoeretan, seinalizazio autofagikoaren hasiera mintz plasmatikoa ematen da, non fosfoinositidoak proteinen bitartez ezagutuak diren (fosfatidilinositol-3-kinasa edo fosfoinositido fosfatasak). Honetaz gain, autofagosomaren eraketa prozesu guztian zehar mintz-material kantitate izugarriak uneoro mugimenduan dihardu zelularen zonalde desberdinetan zehar non

hainbat proteina modu iragankorrean lotuta dauden, adibidez, ATG9, ATG5-12-16 konplexua, ATG3, ATG14 edo WIPI (ingelesetik *WD repeat domain phosphoinositide interacting protein*) proteinak (Ktistakis eta Tooze, 2016 artikuluan berrikusia). Lan honen testuinguruan, duela gutxi proposatu da kardiolipinak eta zeramidak Atg8aren ortologoa den LC3 (microtubuluei lotutako 3 kate arindun 1-proteina, ingelesetik *MAP1LC3, microtubule-associated protein 1 light chain 3*) proteinaren hartzaile gisa joka dezakeela. Hauek mitokondrioaren kanpo-mintzean elkarreragingo lukete, mitokondrioaren ezabapen hautakorra gauzatuz (Sentelle *eta lank.*, 2012; Chu *eta lank.*, 2013; Maguire *eta lank.*, 2016).

1.1.4 Mintz Domeinuak

Mintzak, eta batez ere mintz plasmatikoa, ez dira homogeneoak eta, askotan, domeinu izenez ezagutzen diren albo heterogeneotasunak dauzkate. Hauek ezaugarri funtzional bereizgarriak ematen dizkien lipido eta proteina konkretu batzuez aberastuta daude eta ehundaka nanometrotik mikra bateko tamaina artekoak izan daitezke (Engelman, 2005; Goñi, 2014). Gaur egun ez dago oso argi domeinu ezberdinak mintz jarrai baten gutxieneko zonalde diren, edota mintz plasmatikoa batez ere elkarren alboan jarritako eskualde heterogeneoz osatua dagoen.

Lipido batzuk bigeruzan daukaten nahaskortasun baxua, mintz zelularreko domeinuen jatorriaren faktore garrantzitsu bat izango litzateke. Teknika ezberdinak erabiliz sortutako fosfolipido eta kolesterol nahasturen arteko fase diagrama trianguluarrek (de Almeida *eta lank.*, 2003; Veatch *eta Keller*, 2005; Goñi *eta lank.*, 2008; Heberle *eta Feigenson*, 2011) aldi bereko fase ugariren presentzia adierazten dute, adibidez, $L_d + L_o$, $L_d + L_o + L_\beta$, $L_o + L_\beta$, tenperatura konkretu batean. Kolesterolaren, fosfatidilkolina ase baten eta bi asegabeen nahasturen ondorioz sortutako $L_\alpha + L_\beta$ domeinuetan, domeinu likidoaren tamaina handitu egiten da eta L_o eta L_d bigeruzen arteko lodiera desorekatzen da (Heberle *eta lank.*, 2013). Lipidoen nahaskortasun baxuaz gain, proteinen mugikortasuna (difusio translazionala) zailtzen duten beste mintz ezaugarri batzuk ere garrantzitsuak dira (Tomishige *eta lank.*, 1998). Testuinguru honetan garrantzia berezia du mintzeko proteina integralak zitoeskeletoko proteinetara ainguratzeak, honek mintzeko proteinen difusio translazionala (baina ez errotaziozkoa) galarazten baitu. Ainguratutako proteina hauetako batek, beste hainbatekin elkarrekiteko gai dela kontuan hartuz, mintza proteinaz beteta egotearen ondorioz sortzen den difusio eragozpena eta lipido konkretu batek proteina zehatz bati

lotzeko daukan lehentasuna aurretik azaldu den lipidoen nahaskortasun baxuarekin batera, logikoa izango litzateke mintz domeinuen eraketa maiztasun handiz gertatzea.

Mikretako tamaina daukaten domeinu handienak, normalean “plataforma” gisa ezagutzen dira. Honen adibide dira, esfingomielinasa azidoaren bidezko esfingomielinaren degradazioaren ondorioz eratutako zeramidadun domeinu handiak. Hauek estres seinale baten aurrean eratzten dira zeina segidako seinalizazioen aktibazioaren bidez zelula apoptosira daraman (Stancevic eta Kolesnick, 2010; Zhang *eta lank.*, 2009). Hala ere, zelula, ehun edota organuluaren mintz motaren arabera egoera alda daiteke.

Simons eta Ikonen-ek (1997) “mintz *raft* hipotesia” proposatu zuten (Simons eta Ikonen, 1997). *Raft*-ak, txikiak (10–200 nm), heterogeneoak, oso dinamikoak, esterol eta esfingolipidoz aberastuak eta zelularen prozesuak konpartimentuetan banatuko lituzketen domeinuak izango lirateke (Pike, 2006; Shaikh eta Edidin, 2006). *Raft*-ak, zelula barneko esfingolipido garraioarekin lotuta egongo lirateke eta zenbait seinalizazio funtzio gauzatuko lituzkete. Dena den, beraien tamaina txiki eta biziraupen laburra dela eta ez dago existitzen direla frogatzen duen ebidentzia argirik.

Laburbilduz, albo heterogenotasuna, lipidoen nahaskortasun baxuaren, lipido-paketatze parametro ezberdinen, proteina-proteina kontaktuen (proteina integral eta periferikoen elkarrekintzak barne), proteina-lipido elkarrekintzen eta proteina integral ugari egotearen ondorioz ematen diren ektodomeinu proteikoen arteko talken ondorioa da (Goñi, 2014). Mintz domeinuen proteinen antolamenduan laguntzen dute, besikula txikiagoetan garraiatuak izateko edo baita beraien funtzioa konplexuetan gauzatzeko ere. Domeinu hauek oso garrantzitsuak dira seinalizazio prozesu, zelula barneko lipidoen garraio, seinaleen transdukzio, zelularen morfogenesi, mintz zelularren zeharreko garraio eta toxina eta birusen sarrerarako (Carquin *eta lank.*, 2016).

1.2 Autofagia

Autofagia (grekotik auto-, “norbera” eta phagein, “jan”) eukariotoetan kontserbatua dagoen prozesu biologiko orokor bat da, zeinak beharrezkoak edo funtzionalak ez diren material zelularren degradazio erregulatua ahalbidetzen duen. Gertaera hau garapen zelularrean, diferentziazio zelularrean edo immunitatean baldintza fisiologikoetan ematen da. Dena den, gaixotasunen testuinguruan ere aktibatu daiteke, biziraupena sustatzeko asmoz edo zenbait kasutan ere zelularen

heriotza eraginez. Gainera, elikagai gabeko baldintzetan mantenugaiak hornitzen ditu, horrela zelularen ezinbesteko funtzioak ahalbidetuz. Hori dela eta, autofagia ezegokia egoera patologiko askoren oinarria da.

1.2.1 Jatorria eta Autofagia Motak

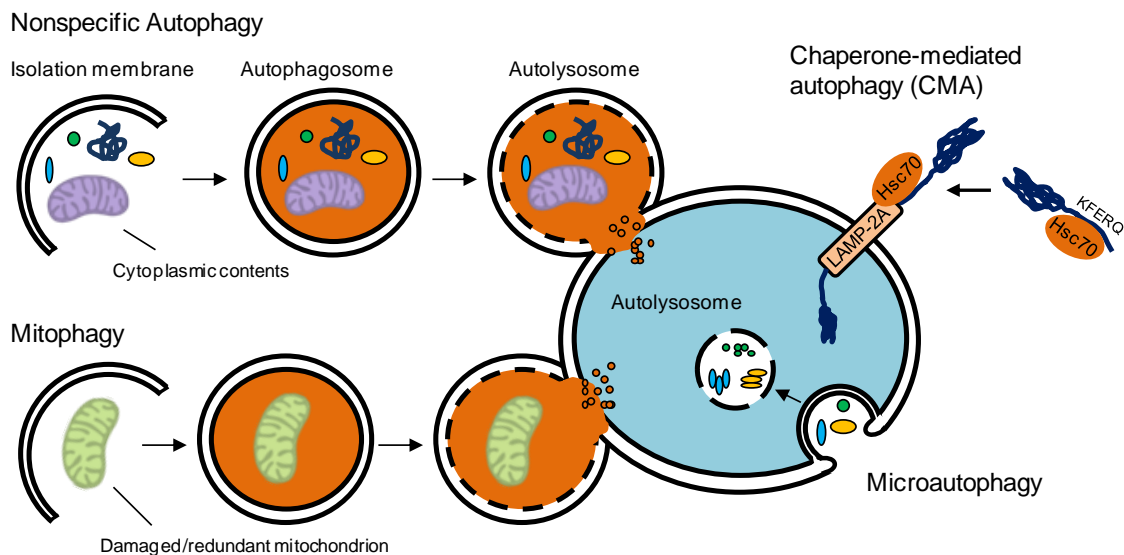
Autofagia prozesua, Keith R. Porter eta bere doktoretza-ondoko ikaslea zen Thomas Ashford-ek aurkitu zuten Rockefeller Institutuan, New York-en. 1962ko urtarrilean arratoi gibel zeluletan glukagoia gehitu ondoren, lisosoma kopurua asko handitzen zela ohartu ziren. Gainera zelularen erdialdera desplazatutako zenbait lisosomak beraien barnean mitokondrioak bezalako beste organulu batzuk zeuzkatela konturatu ziren. Hau izan zen ingelesez deskribaturiko zelula barneko organuluaren digestioaren lehenengo ebidentzia zientifikoa. 1963. urtearen hasieran, *American Journal of Pathology* izeneko aldizkari amerikarrak, degradazio zitoplasmatikoko fokalaren deskribapen ultraestruktural zehaztua argitaratu zuen, non kalteak eragindako bahiketa aipatzen zuen 1955eko ikerketa alemaniar bati erreferentzia egiten zitzaion. Z. Hruban eta lankideek, bahitutako zitoplasmaren lisosomara bideko garapenean 3 fase jarrai deskribatu zituzten. Gainera, prozesua estres egoeretan gertatzeaz gain, material zelularren berrerabilpenerako, baldintza fisiologikoetan ere aktibatzen zela ikusi zuten (Hruban *eta* lank., 1963). Aurkikuntza honetatik abiatuta, lisosomen eta peroxisomen aurkitzailea den Christian de Duve (de Duve, 1963) Nobel sarituak, "autofagia" terminoa plazaratu zuen. Porter eta Ashford-ek ez bezala, de Duve-ek, autofagia terminoa lisosomaren funtzio gisa erabili zuen, glukagoia gibel zeluletako degradazio-eragile nagusia zela deskribatzerakoan (Klionsky, 2008). Bere doktoretza-ondoko ikaslea zen Russell L. Peter-ekin batera, lisosomak glukagoiak eragindako autofagiaren arduradunak zirela defendatu zuen. Momentu horretan, lisosomak zelula barneko autofagiaren lekuak zirela ezarri zen.

Ugaztun zeluletan 3 autofagia mota desberdin deskribatu dira (**1.12 Irudia**) (Okamoto, 2014):

- a) Makroautofagia, autofagiaren bidezidor nagusia da eta batez ere kaltetutako zelula organulu edo erabiltzen ez diren proteinak ezabatzeko gertatzen da. Honek beharrezko du material zitoplasmatikokoaren inguruan mintz bikoitz bat eratzea, autofagosoma izenez ezagutzen den organulua emango duena. Autofagosoma, zitoplasman zehar lisosoma batera mugitzen da eta bi organuluak fusionatzen dira; prozesu honetan bidezidor endosomalekiko elkargurutzamenduak ere gertatzen dira. Behin autofagosoma lisosomaren

barruan dagoela, bere edukia lisosomako hidrolasa azidoen bidez degradatzen da. Bidezidor autofagiko hau legamietatik gizakietara kontserbatua dago.

- b) Mikroautofagiak berriz, inbaginazio bidez, lisosomak material zitoplasmatikoa zuzenean irenstean datza. Beraz, beharrezkoa da lisosomaren mintza barrurantz tolestea edota protruzio zelularra. Mota hau ere legamietatik gizakietara kontserbatua dago.
- c) Txaperona bidezko autofagia (CMA, ingelesetik *chaperone-mediated autophagy*) zitosolean disolbatuta dauden proteinen txaperona-menpeko aukeraketan datza. Hauek, lisosometara eramanak dira eta zuzenean translokatuak izango dira bertan degradazioa emateko. Autofagia mota hau soilik ugaztunetan aurkitu da eta zelularen metabolismoaren erregulazioan laguntzen du (Bejarano eta Cuervo, 2010; Tasset eta Cuervo, 2016).



1.12 Irudia. Ugaztunen bidezidor autofagikoak. Makroautofagia ez espezifikoa, makroautofagia espezifikoa (adib. mitofagia), CMA eta mikroautofagia.

Zelulako osagai espezifiko bat degradatzeko seinaleak egon edota ausazko prozesu bat izatearen arabera, autofagia hautakor edota autofagia ez-hautakorrak bereizten dira. Hau oso garrantzitsua da zelularentzat organulu espezifiko batzuk soilik kaltetuak dauden kasuetan (Sica *eta* *lank.*, 2015). Testuinguru honetan, organulufagia hitza sortu zen. Organulu ezberdinak espezifikoki hautatuak izango litezke makroautofagia edo mikroautofagia bidezidorren bidez degradatuak izateko. Azken urteotan ia zelularen organulu guztiak autofagia hautakor honetarako hautagai izendatuak izan dira. Adibidez, mitokondrioa (mitofagia), peroxisoma (pexofagia), tanta

lipidikoak (lipofagia), nukleoa (nukleofagia), lisosoma (lisofagia), ER (erretikulofagia) edo erribosomak (errifofagia) (Okamoto, 2014; Choi *eta lank.*, 2016; Khaminets *eta lank.*, 2015; Mochida *eta lank.*, 2015).

Tesi lan honetan, mitofagia (mitokondrioen ezabapena espezifikoa) eta autofagia ez-hautakorra ikertuak izan dira, beraz, lan honen testuinguruan, autofagia makroautofagia gisa ulertua izan behar da, autofagia mota ahaltzuen eta gehien ikertua izan dena.

1.2.2 Autofagiaren Eginkizunak

1.2.2.1 Autofagiak Zelularen Biziraupena Sustatzen du

Autofagia bidezko zelularen osagaien birziklapena ezinbestekoa da aminoazido edo nitrogenoaren gabezia (Kuma *eta lank.*, 2004; Takeshige *eta lank.*, 1992), hipoxia (Decker *eta Wildenthal*, 1980) edo banaketa zelularra (Debnath, 2009) bezalako hainbat ingurunekei erronkei aurre egiteko. Autofagiak zelulen biziraupenean ere laguntzen du, estres oxidatiboa edota ER estresa bezalako zelula barneko erronkei erantzuten eta beraien maila jaisten. Adibidez, oxigeno espezie erreaktiboak (ROS, ingelesetik *reactive oxygen species*) ematen dituzten mitokondrio kaltetuen mitofagia hautakorraren bidez (Twig *eta lank.*, 2008; Narendra *eta lank.*, 2008; Nowikovsky *eta lank.*, 2007; Scherz-Shouval *eta Elazar*, 2007) edo tolestu gabeko proteinen aurreko erantzuna bermatuz (Ding *eta lank.*, 2007; Ogata *eta lank.*, 2006). Autofagiak, zelularen mantenuan ere parte hartzen du proteinen agregatu toxikoak ezabatuz [adib. Huntington-en gaixotasunean ematen diren proteinen konformazio akatsekin lotuta dauden eta pilatzeko joera daukaten proteinak (Bjørkøy *eta lank.*, 2005; Ravikumar *eta lank.*, 2002)] eta patogeno inbasore intrazelularrak ezabatuz (xenofagia) (Gutierrez *eta lank.*, 2004; Miao *eta lank.*, 2015). Azkenik, autofagiak, zelulen hazkuntza (Mathew *eta White*, 2011) eta garapena (Cecconi *eta Levine*, 2008) bezalako prozesu garrantzitsuen zeharreko zelulen birmoldaketan parte hartzen duela aipatu behar da. Beraz, autofagia ezinbestekoa da zelulen mantenu eta biziraupenerako, eta hau legamietan eta *Caenorhabditis elegans*-en autofagia biziraupenaren luzapenarekin lotuta dagoela dioten ikerketek egiaztatzen dute (Bergamini *eta lank.*, 2003; Meléndez *eta lank.*, 2003; Tang *eta lank.*, 2008). Aspektu negatibo gisa, tumoreen zelula transformatuek ere beraien biziraupena bermatzeko autofagia erabiltzen dutela dirudi (Mathew *eta White*, 2011).

Bestalde, gutxiegiako edo akasun autofagia, burmuinean tolestu gabeko proteinen agregatu toxikoen pilaketarekin zerikusia duten Huntington, Alzheimer edo Parkinson-en gaixotasunak bezalako gaixotasun neurodegeneratiboen hasiera berantiarrekin lotzen da (Vidal *eta lank.*, 2014; Rubinsztein, 2006). Transformazio kaltegarrian zehar ere autofagia askotan motelduta edo inhibitua dagoela ikusi da, eta honek onkogenesian eta minbiziaren garapenean lagundu lezake (Galluzzi *eta lank.*, 2015; Mizushima *eta lank.*, 2008; Mowers *eta lank.*, 2016). Adibidez, autofagia bidezidorraren aktibatzaile garrantzitsua den Beclin 1 proteina, tumore supresorea den gene bezala identifikatua izan da (Liang *eta lank.*, 1999). Era berean, autofagiarekin lotuta dauden zenbait proteinen delezioa [adib. ATG5, ATG4C, UVRAG] ehun espezifikoetan minbiziaren sentikortasunarekin lotu da (Levine eta Kroemer, 2008). Azkenik, patogeno ugari (bakterioak, birusak eta parasitoak) xenofagia bidezko degradazioa burutzeko lisosometara garraiatuak izatea eta autofagia berezko sistema immune eta sistema immune egokitzailen parte hartzeak, autofagia ere hainbat gaixotasunen aurkako *bona fide* prozesu immunologiko garrantzitsua dela pentsatzera garamatza (Deretic, 2011; Levine eta Kroemer, 2008; Münz, 2015).

1.2.2.2 Autofagia eta Heriotza Zelularra

Autofagia gehienetan prozesu zitobabegarri gisa ulertzen den bitartean, hiltzen ari diren zeluletan autofagosoma kopurua handitzeak, heriotza zelular autofagikoaren kontzeptua ekarri du. Autofagiaren lotura apoptosiaren menpekotasunik gabeko heriotza zelular espezifiko programatu batekin (heriotza zelular autofagikoa, aurrerago bigarren motako heriotza zelular programatua ere deitua) (Baehrecke, 2003; Pyo *eta lank.*, 2005; Shimizu *eta lank.*, 2004), hasiera batean morfologikoki deskribatu zen. Dena den, termino honen erabileraren hedapena autofagia “rekin” gertatutako heriotza deskribatzeko eztabaida iturri da gaur egun. Berriki zehaztu den heriotza zelular autofagikoaren definizioak, autofagia “bidez” gertatutako heriotzera mugatu du (hau da, fluxu autofagiko handitua, autofagiaren inhibizioaren bidez zelulen heriotza saihestu daitekeenean), eta beraz, zenbait kasuetan ez dago oinarri sendorik autofagia deskribatzen den heriotza zelular autofagikoaren arduraduna dela baieztatzeke (Shen eta Codogno, 2011; Shen *eta lank.*, 2012). Horren ordean, autofagiaren aktibazioa heriotza zelularren aurreko biziraupen mekanismo gisa jokatzek azalduko luke ikerketa hauetako askotan deskribatu den autofagosoma kopuru handia hiltzen ari diren zeluletan (González-Polo *eta lank.*, 2005; Ravikumar *eta lank.*, 2006). Adibidez, U2OS osteosarkoma zelulak erabiliz, luzaroan minbizi-zeluletan heriotza zelular

autofagikoaren inguruan egon den eztabaida bat argitzea lortu da. Horretarako, minbizi-zelula hauek minbiziaren aurkako terapietan erabilitako agente zitotoxiko desberdin askorekin tratatuak izan ziren (Shen *eta lank.*, 2011). Ikerketak, erabilitako errektibo hauetatik 80k autofagiaren aktibazioa eragiten dutela baieztatu zuen – fluxu autofagikoa handitzea edota autofagosomen degradazioa urritzearen ondoriozko GFP-LC3 punteaketaren (ingelesez *puncta*) pilaketa – baina, konposatu hauek eragindako heriotza zelularra ATG5 edo ATG7 shRNA bidezko autofagiaren inhibizioarekin saihestu ezin zela ikusi zen (Shen *eta lank.*, 2011; Shen *eta lank.*, 2012). Horrez gain, Velasco eta lankideen zenbait aurkikuntzek (Salazar *et lank.*, 2009), kanabinoideek minbizi-zelula mota ezberdinetan, adibidez, glioma/astrozitoma eta pankreako minbizi-zeluletan, autofagikoa bidezko heriotza zelularra eragiten dutela erakutsi zuten. Aurrerago zehaztasun gehiagorekin azalduko den moduan, kasu honetan, autofagiaren aktibazioa mekanismoaren ezinbesteko pausua izango litzateke.

Beste hainbat ikerketek heriotza zelularren erregulazioan autofagiak daukan ekarpena nabarmentzen dute. Hau, luzaroko autofagiaren aktibazioa eta heriotza zelularren artean dagoen loturan oinarritzen da [adib. hipoxian (Azad *eta lank.*, 2008) edo estres oxidatiboan (Chen *eta lank.*, 2008; Chen *eta lank.*, 2007)]. Gainera, apoptosiarekiko erresistenteak diren Bax/Bak *knockout* bikoitz sagu enbrioien fibroblastoak (MEFs, ingelesez *mouse embryonic fibroblasts*), apoptosisia aktibatzen duten errektiboekin (adib. etoposida, estauroesporina) tratatu ostean, Beclin 1 eta ATG5 proteinen bidez gauzatutako heriotza zelular ez-apoptotiko bat deskribatu da (Shimizu *eta lank.*, 2004). Hala ere, aurretik aipatu bezala, beste kasu batzuetan autofagia eta apoptosiaren arteko elkargurutzamendua ezinbestekoa da (Salazar *eta lank.*, 2009; Shen *eta lank.*, 2012; Yu *eta lank.*, 2004). Hortaz, autofagiak heriotza zelularrean modu askotan lagunduko luke. Hautatutako adibide hauek, autofagia eta heriotza zelular apoptotiko eta ez-apoptotikoen arteko lotura nahasi eta guztiz ez ulertua islatzen dute, zeina ziur aski estimuluaren jatorriaren eta ehunaren arabera desberdina den.

1.2.2.3 Autofagia Garapen eta Diferentziazioan

Hainbat dira garapen zelular eta diferentziazioan zehar, zelulen birmoldaketarako zelulen osagaien degradazioa beharrezkoa denean, autofagiak funtzio ezberdinak dauzkala dioten ebidentziak. Saguetan autofagiaren erregulatuzailea den Beclin 1 proteinaren delezioak heriotza zelular orokorra eta enbrioien hilkortasun goiztiarra eragiten ditu (Qu *et lank.*, 2003; Yue *eta lank.*, 2003). Hala eta guztiz ere,

autofagosoma goiztiarraren mihizadura makinariaren funtsezko osagaien delezioak (ATG5 edo ATG7), edoskitzean akats bat sortzen du, zeinak ez duen gainontzeko garapen arazorik eragiten, animaliak aldi neonataleko elikagaien gabezia zeharreko estres metabolikoaren ondorioz hiltzen den arte (Komatsu *eta lank.*, 2005; Kuma *eta lank.*, 2004). Hala ere, animalia hauetan duela gutxi deskribatutako ATG5/ATG7ren menpekoea ez den autofagia alternatiboak, delezio hauek garapenean duten efektu arina azalduko luke (Nishida *eta lank.*, 2009). Estrategia esperimental desberdinak erabiliz lortutako ehunarekiko espezifikoak diren ATG5 eta ATG7 proteinen delezioek, autofagiak zelulen biziraupenean eta garunaren garapenean (adib. motore funtzioak, neurodegenerazioa) eginkizun garrantzitsuak dauzkala erakutsi dute (Aufschnaiter *eta lank.*, 2016; Cecconi eta Levine, 2008; Hara *eta lank.*, 2006; Read *eta lank.*, 2011; Komatsu *eta lank.*, 2007). *Knockout* saguak erabiliz, autofagia moteldua edo inhibitua garapen defektu espezifikoekin - adib. barne belarriarena ATG4B proteinarene kasuan (Mariño *eta lank.*, 2010) edo sistema immunearena ATG5aren kasuan - eta tumoreak garatzeko joerarekin (Cecconi eta Levine, 2008; Levine eta Kroemer, 2008; Mariño *eta lank.*, 2007) ere erlazionatu da.

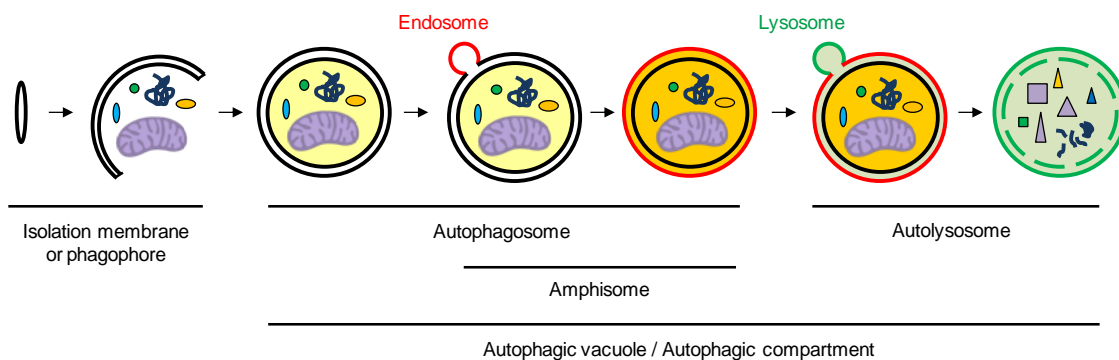
Autofagia zenbait zelula moten diferentziazio prozesuan zeharreko birmoldatzearekin ere lotu da. Adibidez, podozitoen *in vitro* diferentziazioan zehar, autofagosomen eraketa modu espezifiko batean positiboki erregulatuta dago (Asanuma *eta lank.*, 2003). Antzera, C2C12 zelulen miohodietarako *in vitro* diferentziazioan, autofagia aktibatzen da eta era espezifikoan erregulatuta dago (Tanida *eta lank.*, 2006). Gainera, 1960ko hamarkadatik aurrera autofagosomak zelula eritroideen diferentziazio prozesuan deskribatuak izan dira (Kent *eta lank.*, 1966). Dena den, zelula eritroideen azken diferentziazio faseetan hau nola erregulatzen den eta autofagiaren parte hartzea erretikulozitoen heltzean oraindik ez dago oso argi (Griffiths *eta lank.*, 2012).

1.2.3 Makroautofagiaren Makinariaren Muina

Autofagian ematen den gertaera nagusia autofagosoma (AP, ingelesetik *autophagosome*) izeneko mintz bikoitzeko egituraren eraketa da (Noda eta Inagaki, 2015). Prozesu honen lehenengo pausua mintz isolatu baten sorrera da. Oraindik ez da ezagutzen hasierako mintz honen jatorri zehatza, dena den hainbat izan dira jatorriaren inguruko proposamenak: ER, ER-Golgi bitarteko konpartimentua (ERGIC, ingelesetik *ER-Golgi intermediate compartment*), endosomak, mintz plasmatikoa edota

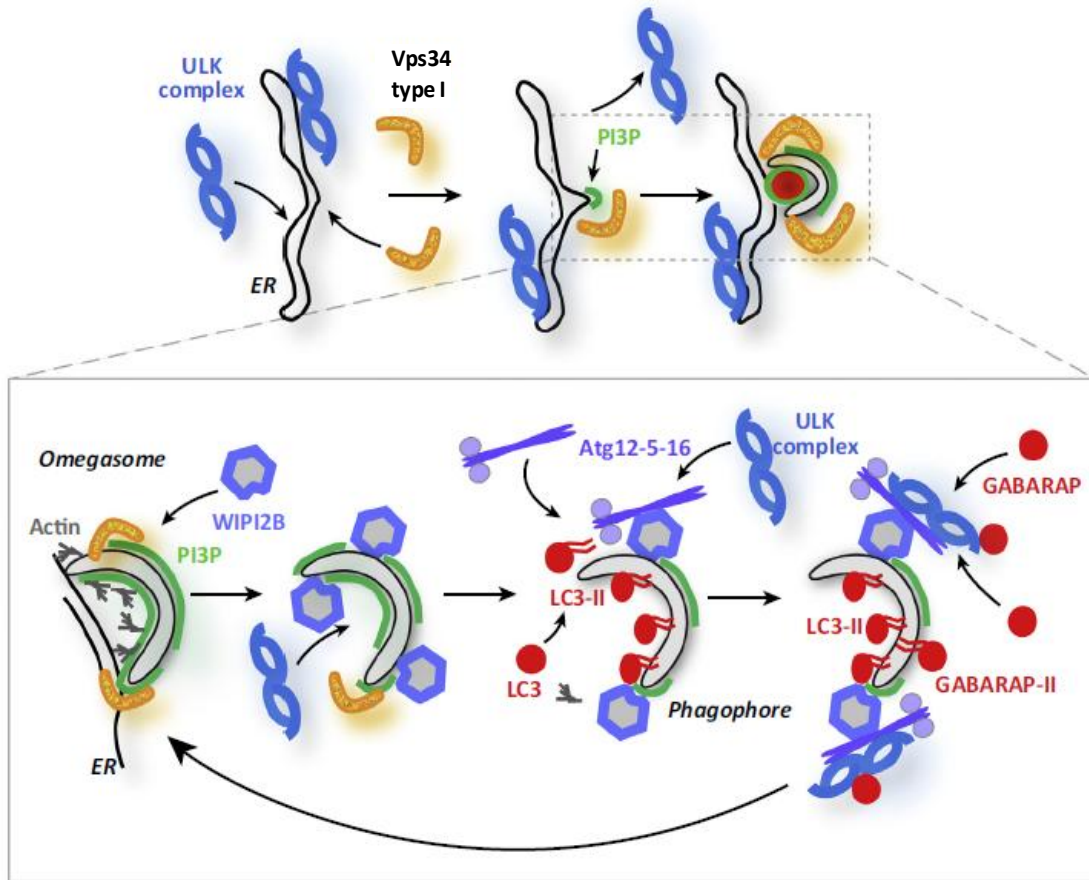
baita mitokondrioa ere (Ge *eta lank.*, 2013; Hamasaki *eta lank.*, 2013; Mari *eta lank.*, 2011; Mizushima *eta lank.*, 2011; eta duela gutxi Ktistakis eta Tooze, 2016-en berrikusapena).

Hemendik aurrera, autofagosomaren elongaziorako mintz egituren arteko atxikidura eta fusioa beharrezkoak dira. Mintz materiala besikula gisa aurre-autofagosomara iristen doan bitartean, hautatutako edo ez-hautatutako eduki zelularrak egitura honetara sartzen doaz. Azken urrats batean, autofagosoma itxi eta lisosoma batekin fusionatzen da barruko edukiak hidrolasa lisosomalaren bidez degradatuak izateko (**1.13 Irudia**) (Tooze eta Yoshimori, 2010; Hasegawa *eta lank.*, 2016).



1.13 Irudia. Ugaztunen makroautofagiaren prozesu orokorraren eskema. Autofagosomak isolatze-mintz (edo fagoforo) baten luzapenez, ausazko edo hautatutako material zitoplasmakoaren inguruan eratzen diren mintz bikoitzeko bakuoloak dira. Autofagosomak hainbat endosoma goiztiar eta berantiarrekin fusionatzen doazen bitartean heltzen doaz. Era berean heltzeko beharrezko dituzten zenbait mintz proteina endosomal (gorriz) eta beraien edukiaren azidifikazioa gauzatzeko (laranjaz) beharrezko duten protoi ponpa hartzen dute. Autofagosomak besikula anitzeko endosoma berantiarrekin fusionatu ostean anfisoma gisa izenda daitezke. Azkenik, beraien kanpo-mintzek lisosomekin (berdez) fusionatzeak kanpo- eta barne-mintzen artean entzima lisosomalaren askapena eragiten du, horrela barne-mintzaren eta bere edukiaren degradazioa gauzaturik. Lisosomekin fusionatu ostean autofagosomak autolisosoma bilakatzen dira. (Eskelinen, 2005 eta Mizushima, 2007-etik moldatua).

Gaur egun arte, gutxienez autofagiarekin erlazionatutako (ATG) 35 gene aurkitu dira (**1.1 Taula**), hauek, AP biogenesian parte hartzen duten momentuaren arabera, hainbat taldetan bereizten dira (Lamb *eta lank.*, 2013). Beraien artean, tesi lan honetan ikertu diren ubikitina-moduko giza Atg8 proteinak aurkitzen dira, zeinak APra lotzen diren autofagosoma berantiarren proteina espezifikoki diren eta fagoforoaren espantsioan laguntzen duten (**1.14 Irudia**) (Berrikuspenetarako ik. Mizushima *eta lank.*, 2011; Yang eta Klionsky, 2010; eta Ktistakis eta Tooze, 2016).



1.14 Irudia. Makroautofagiaren bidezidorraren urrats molekularrak. ULK konplexuaren [ULK1/2, FIP200, ATG13, ATG101] eta Vps34-ren (ingelesetik, *vacuolar protein sorting mutant 34*) I.motako konplexuaren (BECLIN 1, VPS34, p150, ATG14) errekrutatzea, ER mintzetan gertatzen da PI3Pn aberastuak diren domeinuak eratuz. Era berean, domeinu hauek FYVE domeinu bikoitzeko proteina 1-n (DFCP-1, ingelesetik *double FYVE domain-containing protein 1*) aberastutako omegasomak eratzen dituzte (DFCP-1 ez da erakusten). Aktina fagoforoaren barruan sare bat mihizatzen erakusten da eta bitartean aktinak kanpoaldea aktina kometa-isatsak eratzen ditu. Ondoren, LC3 familia kideen lipidazioa gauzatzeko asmoz, WIPI2B PI3P-ra lotu eta ATG12–5-16L1 konplexua errekrutatzen du. ATG16L1 proteina, ULK konplexuaren FIP200ra ere lotzen da. ATG12–5-16L1ren bidez errekrutatutako ULK konplexuek, aktiboak diren ULK1n kopurua handitu, Vps34 konplexuaren aktibitatea indartu eta PI3P eraketa mantenduko lukete. ULK konplexuko kideetako bakoitzak LC3 familiako kideak lotzen dituen “LC3-rekin elkarreragiteko eskualdea” (LIR, ingelesetik *LC3-interacting region*) domeinuak dauzka. LIRen bidezko LC3/GABARAP familiako kideen lotura ULK–ATG12–5-16L1 mihiztadurara ere ULK1 aktiboaren kopurua handitu eta Vps34 aktibitatea mantentzeko (Kistakis eta Tooze, 2016-tik moldatua).

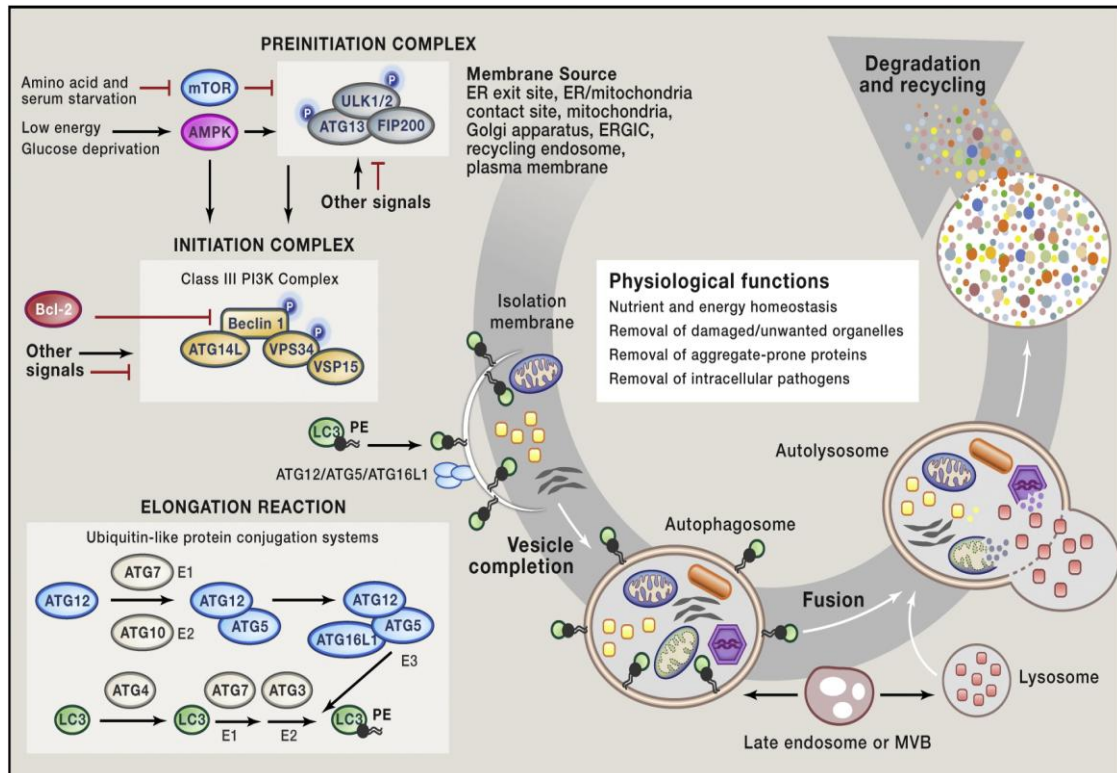
Proteina	Kokapena autofagiaren bidezidorean	Izen alternatiboa eta funtzioa
ULK1 eta ULK2	ULK konplexua	Atg1en ortologoak; mTOR seinalizazioa eta ATG9ren zikloa gauzaten duten Ser/Thr kinasak
ATG13	ULK konplexua	ULK1 eta ULK2ren substratua, ULK konplexuaren aktibitatea ere modulatzeko duena
FIP200	ULK konplexua	Atg17ren ortologoa; ULK1 eta ULK2ren substratua, ULK konplexuaren aktibitatea ere modulatzeko duena
ATG101	ULK konplexua	ULK1 eta ATG13rekin elkarrekin
Beclin 1	PI3K konplexua	Atg6ren ortologoa; PI3K konplexuaren parte eta autofagian ere hasiera, eraketa eta heltzean zehar eginkizunen bat dauka
VPS34	PI3K konplexua	PI3K konplexuaren katalitikoki aktiboa den azpiunitatea
p150	PI3K konplexua	Vps15aren ortologoa; PI3K konplexua mintzetara erakartzeko du
ATG14L	PI3K konplexua	Atg14ren ortologoa; PI3K konplexua omegasomara zuzentzen du; Barkor gisa ere ezagutua
WIPI1 eta WIPI2	PtdIns3Pra lotzen den proteina	Atg18ren ortologoak; autofagosoman PtdIns3Pra lotzen dira
ATG3	LC3-PE elkarketa	E2 ubikitina entzima elkartzailearen antzekoa, LC3 PEra elkartzen du
ATG4	LC3-PE elkarketa	LC3 paralogoen C-muturreko Gly hondarrak moztu dituen Cys proteasa. LC3 autofagosomaren kanpo-mintzetik birziklatzeko ere beharrezkoa da
ATG7	LC3-PE eta ATG12 elkarketa	E1 ubikitina entzima aktibatzailearen antzekoa; ATG12 eta LC3 paralogoak aktibatzen ditu
LC3A, LC3B eta LC3C GABARAP, GABARAPL1 eta GABARAPL2	LC3-PE elkarketa	Atg8aren ortologoak; autofagosometara degradaziorako-materiala errekrutatzen duten eta mintzen fusioan lagunduko luketen ubikitina-moduko proteinak
ATG5	ATG5-ATG12 elkarketa	ATG12ri lotuta
ATG10	ATG5-ATG12 elkarketa	E2 ubikitina entzima elkartzailearen antzekoa; ATG12 ATG5aren barneko Lys hondar bati elkartzen dio
ATG12	ATG5-ATG12 elkarketa	ATG5ari lotutako ubikitina-moduko proteina, zeinak ATG3ren aktibazioan parte hartzen duen
ATG16L1	ATG5-ATG12 konplexua	ATG5-ATG12 konplexuari lotzen zaio eta isolatze-mintzan LC3 proteinaren elkarketa zuzentzen du
ATG9A eta ATG9B	Mintz proteina integralak	Atg9ren ortologoak; autofagosomaren eraketarako beharrezkoak
ATG2A eta ATG2B	Omeegasoman kokatuak	Atg2ren ortologoak; autofagosomak eratzeko behar diren isolatze-mintzen itxierarako beharrezkoak

1.1 Taula. Autofagosomaren eraketarako ezinbestekoak diren ATG proteinak. (Lamb eta lank., 2013-tik moldatua).

1.2.4 Autofagiaren Erregulazioa eta Aktibazioa

Autofagiaren aktibazioa elikagai, hazkuntza-faktore, hormona, ATP maila eta beste hainbat seinale ezberdin kontutan hartzen dituen erregulazio mekanismo konplexu batek kontrolatzen du. Seinale askok, ugaztunen rapamizina 1-konplexuaren ituan (mTORC1, ingelesetik *mammalian target of rapamycin complex 1*) bateratzen dira. mTORC1 osatzen duten osagaiak, mTOR, mTORen proteina erregulatzailerkarta (Raptor, ingelesetik *regulatory associated protein of mTOR*), DEP domeinua daukan eta mTORekin elkarreagiten duen proteina (Deptor, ingelesetik *DEP-domain-containing mTOR-interacting protein*), prolinan aberatsa den 40kDa-eko AKTren substratua (PRAS40, ingelesetik *proline-rich AKT substrate 40 kDa*) eta G-proteina β -azpiunitate-moduko proteina (G β L, ingelesetik *G-protein β -subunit-like protein*) dira (Wirawan *eta* *lank.*, 2012). Aminoazido eta hazkuntza-faktoreak daudenean, I. motako fosfatidilinositol-3-kinasak (PIK3C1, ingelesetik *class I phosphatidylinositol-3-kinase*) mTORC1 aktibatzen du, zeinak seinalizazio autofagikoa inhibitzen duen. mTORC1 aktiboak autofagia inhibitzen du ULK konplexuaren ULK1/ULK2 eta ATG13 lotuz eta fosforilatuz (Joachim *eta* *lank.*, 2015). Konplexu hau ULK1/ULK2k, ATG13k, 200kDa-ekoa den atxikidura-fokal kinasa familiarekin elkarreagiten duen proteinak (FIP200, ingelesetik *focal adhesion kinase family interacting protein 200 kDa*) eta ATG101ek osatzen dute (Noda *eta* Inagaki, 2015). Autofagia aktibatzekeo asmoz, elikagaien gabezi edo rapamizina tratamenduaren bidezko mTORC1aren errepresioa askotan erabiltzen da. Beraz, mTORC1 inaktibatzen denean, ULK konplexutik disoziatzen da ULK aktibo utziz (**1.15 Irudia**) (Noda *eta* Fujioka, 2015). Legamietan, Atg17, Atg29 eta Atg31 ere Atg1 konplexuaren parte dira eta, Atg1 proteina menpekoa den Atg17 eta Atg9ren elkarrekintza baten bidez, Atg9 besikulak fagoforoaren mihizadura gunera (PAS-era, ingelesetik *phagophore assembly site*) errekrutatzen parte hartzen dute (Ragusa *eta* *lank.*, 2012; Stanley *eta* *lank.*, 2014; Rao *eta* *lank.*, 2016). Beraz, honi dagokionez, legamia eta ugaztunen bidezidoren artean ezberdintasun handia dago.

Elikagai falta dagoenean, ULK1ek eginkizun garrantzitsua dauka ATG9ren garraioa erregulatzen. ATG9 egoera basaletan trans-Golgi sarearen eta konpartimentu endosomalaren artean ziklatzen den transmintz proteina bakarra da. Autofagiaren aktibazioaren ostean, ATG9 hazten ari den autofagosomara erakarria da, non mintzaren elongaziorako beharrezkoak diren lipidoak horni ditzazkeen (Orsi *eta* *lank.*, 2012; Webber *eta* *lank.*, 2007; Joachim *eta* *lank.*, 2015; Karanasios *eta* *lank.*, 2016).



1.15 Irudia. Makroautofagiaren seinalizazio bidezidorra. Goitik behera, seinale ezberdinak mTORen aurreko inhibitzaile gisa jarduten dute. ULK eta PI3KC3 konplexuak mintz nukleazioan parte hartu eta proteinen errekrutamenduan laguntzen dute. Bai Atg8, baita Atg5 ubikitina-moduko (UBL, ingelesetik *ubiquitin-like*) sistemak ere, autofagosomaren elongazio eta ixtean elkarrekin lan egiten dute (Green eta Levine, 2014-tik hartua).

Duela gutxiako emaitzak, mitokondrioek zelula barneko Ca^{2+} ren bidezko autofagiaren erregulazioan eginkizun garrantzitsua dutela frogatu dute (Cárdenas *eta* *lank.*, 2010). Inositol trifosfatoaren hartzaileen aktibazioaren ostean, mitokondrioek ERak kanporatutako Ca^{2+} hartzen dute. Gainera, mitokondrioen Ca^{2+} harrerak fosforilazio oxidatibo eraginkorra, ATP eraketa eta AMPK aktibitatearen errepresioaren bidezko autofagiaren inhibizioa dakartza. Interesgarria suerta daiteke mitokondrioek, autofagiaren aktibazioa, ATParen bidez erregulatzeaz gain, mitokondrioen menpeko glutaminolisian eratutako amoniakoaren bidez ere erregulatu dutela (Eng *eta* *lank.*, 2010). Amoniakoak, autofagia positiboki erregulatu du ohikoa ez den era batean, izan ere, ez du ez ULK1 ezta ULK2ren beharizanik (Cheong *eta* *lank.*, 2011). Amoniakoak autofagia positiboki erregulatzeko daukan ahalmena garrantzitsua izan daiteke minbizian, izan ere, minbizi-zelulek glutaminolisi maila altuak izaten dituzte, eta autofagiak tumoregenesian ezinbestekoa dela dirudi, baldintza batzuetan behintzat (Galluzzi *eta* *lank.*, 2015; Guo *eta* *lank.*, 2011; Yang *eta* *lank.*, 2011). Berriki ikerketa batek aurkitu duenez, legamietan ere mitokondrioak cAMP-menpeko proteina

kinasaren (PKA, ingelesetik *cAMP-dependent protein kinase*) aktibitatearen erregulazioaren bidez autofagia aktibatzeke ezinbestekoak dira (Graef eta Nunnari, 2011). Kasu honetan, mitokondrioen arnasketaren defizientziak PKA aktibitatearen erregulazio positibo batera daramala ikusi zen, autofagia bi modu ezberdinetan inhibitzen zuena: Atg1-Atg13 konplexuaren aktibitatea (ULK1-ATG13-FIP200 konplexuaren parekoa) blokeatuz eta Atg8aren adierazpena inhibituz.

Autofagiaren aktibazioaren ostean, autofagosoma heldua eratzeko asmoz, zenbait proteina konplexuk isolatze-mintzaren elongazioan parte hartzen dute. Prozesu hau 3 urratsetan banatu daiteke: besikularen nukleazioa, elongazioa eta heltzea. Ekintza hauek gauzatzeko asmoz urrats bakoitzean proteina espezifikoak jarduten dute (Ktistakis eta Tooze, 2016-an berrikusia). Autofagosomaren nukleazioa izeneko lehenengo pausuan, autofagosomaren eraketa gunera proteina ezberdinen kopuru handia errekrutatzen da. PI3KC3, p150, autofagia eta Beclin 1 proteinen 1-erregulatzaila (Ambra1, ingelesetik *autophagy and beclin 1 regulator 1*) eta BCL-2rekin elkarreragiten duen proteinak (Beclin 1, ingelesetik *BCL-2-interacting protein*), PI3KC3 konplexuaren muina dira, zeina erradiazio ultramorearekiko erresistentziarekin lotutako genearen (UVRAG, ingelesetik *UV-radiation resistance associated gene*) produktuari edo ATG14Lri lotu daitekeen. Konplexuan, PtdIns3Pren eraketa modulatzeko, PI3KC3ren zenbait proteina erregulatzaila Beclin 1 proteinara lotzen dira. Fosfolipido honek, nukleazio gunera ATG proteina gehiago erakartzeko eginkizuna dauka (Horenkamp eta *lank.*, 2015; Juris eta *lank.*, 2015). PtdIns3Prekin elkarreragiten duten proteinen artean, WD domeinu errepikatua daukan eta fosfoinositidoekin elkarreragiten duen 1- eta 2-proteinak (WIPI1 eta WIPI2, ingelesetik *WD repeat domain phosphoinositide-interacting protein 1 and 2*) (Dooley eta *lank.*, 2014; Polson eta *lank.*, 2010), ATG2 eta DFCP-1 proteinak dira ezagunenak, nahiz eta beraien eginkizun zehatza sakonago ikertua izan behar den.

Ondoren bi UBL konjugazio sistemek, autofagosomaren mintzaren elongazioan, forma ematean eta ixtean parte hartzen dute. Bi sistema hauek elkarlotuta daude eta *in vivo* autofagosomaren hazkuntza egoki baterako batera jardun behar dute. Lehenengo UBL sistema ATG5, ATG12, ATG10 eta ATG16L1-ek osatzen dute. Hasteko, ATG12, ATG7ren (E1-moduko entzima) bidez aktibatzen da, ATG10era (E2-moduko entzima) mugitzen da eta azkenik ATG5ari kobalenteke lotzen zaio. ATG12-ATG5-ek, ATG16L1ekin ere elkarreragiten du homodimerizatzen den konplexu bat eratzeko konplexu multimeriko bat eratuz (**1.15 Irudia**). Konplexu honen azken ekintza, autofagosomaren kanpo-mintzarekin elkarreragin eta bigarren UBL konjugazio sistemaren E3-ligasa entzima bezala jardutea da (Romanov eta *lank.*, 2012; Walczak

eta Martens, 2013). Bigarren UBL sistema hau, ATG4, ATG7, ATG3 eta LC3-k osatzen dute (**1.15 Irudia**). Hasteko LC3ren C-muturra ATG4 bidez mozten da, glizina bat agerian utziz. Gero, LC3 ATG7rekin lotu eta ATP-menpeko trans-tioesterifikazio prozesu baten bidez ATG3ra eramana da. Behin ATG3ri lotuta, azken honek, LC3 mintz autofagosomikora darama, eta E3-ligasa konplexuaren laguntzarekin LC3 PErekin elkartzen du, horrela LC3-PE emanez (Nakatogawa *eta lank.*, 2007; Noda *eta lank.*, 2011; Tanida *eta lank.*, 2004b; Hsu eta Shi, 2016).

Azken produktua, LC3-PE, zeluletan erabiltzen den autofagiaren markatzaile espezifiko nagusia da eta autofagosomaren elongazioan parte hartzen duen ekintzaile ezagunena da ere (Hsu eta Shi, 2016). Gainera, LC3k, bere LIR motiboaren bidez, mintz aurre-autofagosomikoan autofagiaren erregulazioan eta autofagosomaren hazkuntzan laguntzen duten proteinak errekrutatzen ditu (Hurley eta Schulman, 2014; Noda *eta lank.*, 2010; Stolz *eta lank.*, 2014). Behin autofagosoma eratuta dagoela eta degradatu beharreko materiala bere barnean daukala, ATG4k berriz ere parte hartzen du, oraingo honetan LC3 proteinaren birziklapenean LC3-PE autofagosomaren kanpo-mintzetik askatuz. Hala eta guztiz ere, autofagosomaren barne-mintzari lotuta dagoen LC3, entzima lisosomalen bidez degradatua izango da. 1.2.5 Atalean Atg8aren UBL sistema sakonkiago deskribatuko da.

Autofagiaren azken pausuan, eta barneratutako materialaren degradazioari dagokionez, autofagosoma mikrotubuluaren bitartez zitoplasman zehar garraiatu eta lisosomekin fusionatzen da (Shen eta Mizushima, 2014). Autofagosomaren kanpo-mintza lisosomekin fusionatzen da eta, beraz, autofagosomaren barne-mintza eta bere edukia lisosomen hidrolasa eta lipasekin kontaktuan jartzen dira. Hainbat dira autofagosoma-lisosoma fusioan parte hartzen duten proteinak, adibidez, lisosomari lotutako mintz proteina 2 (LAMP2, ingelesetik *lysosomal-associated membrane protein 2*), Ras-ekin erlazionatutako GTP-lotzen duen proteina (Rab, ingelesetik *Ras-related GTP-binding protein*), fusio homotipiko eta proteina banaketa (HOPS, ingelesetik *homotypic fusion and protein sorting*), SNAREak (ingelesetik *SNAP soluble NSF attachment protein receptor*), LC3, garraiorako beharrezkoa den banaketa-konplexu endosomikoa (ESCRT, ingelesetik *endosomal sorting complex required for transport*), FYVE eta *coiled-coil* domeinuak dauzkan 1-proteina (FYCO1, ingelesetik *FYVE and coiled-coil domain-containing protein 1*) eta UVRAG-C-Vps lotura konplexua (Wirawan *eta lank.*, 2012). Autofagia prozesuaren emaitza erreakzio anaboliko berriak gauzatzeko zitosolera itzuliko diren birziklatutako aminoazido, gantz azido eta nukleosidoen sorrera da.

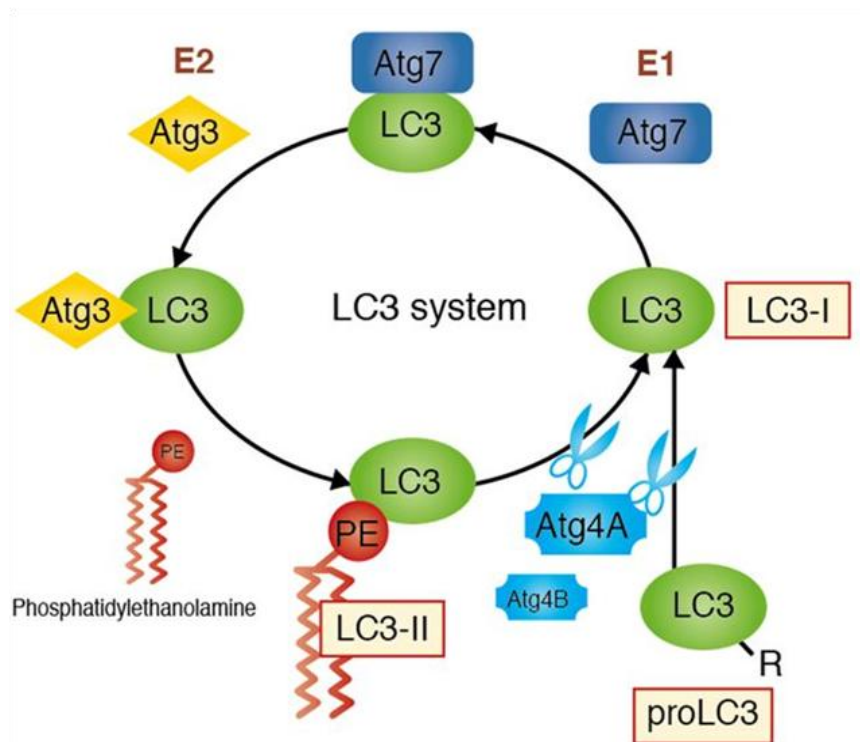
1.2.5 Atg8 Konjugazio Sistema

Aurretik aipatu bezala, autofagosomaren elongazio eta ixte prozesua 2 UBL sistemen, Atg8 eta Atg5, beharrezkoa da. *In vivo* biek batera jarduten dute, dena den, *in vitro* beraien eginkizun nagusirako, hau da, Atg8aren lipidazioa burutzeko, bigarren sistema ez da ezinbestekoa. Tesi lan honetan ikerketa batez ere Atg8aren giza ortologoei bideratuta dagoenez, aurrerago Atg8 UBL sistemaren deskribapen zehatza ematen da (berrikuspenetarako ik. Yang eta Klionsky, 2010 eta Mizushima *eta lank.*, 2011).

Atg8, legamien autofagosomaren elongazioan ezinbestekoa den proteina, UBL sistemaren funtsezko partaidea eta amaierako efektorea da. Ugaztunetan gutxienez Atg8aren 8 ortologo identifikatu dira, zeintzuek LC3 eta GABARAP izeneko bi azpifamilietan banatzen diren. LC3Ak, LC3Bk, LC3B2k eta LC3Ck, LC3 azpifamilia osatzen duten bitartean, GABARAPek (A-motako GABA hartzaileei lotutako proteina, ingelesez *GABA A type receptor-associated protein*), GABARAPL1/GEC1ek, GABARAPL2/GATE-16k eta GABARAPL3k, GABARAPena osatzen dute (Weidberg *eta lank.*, 2010). Aipatzekoa da GABARAPL3ren adierazpena soilik maila transkripzionaletan erakutsi dela (Xin *eta lank.*, 2001); beraz dagokion irakurtarau irekia (ingelesez *open reading frame*) pseudogene bat adieraz dezake. Tesi lan honen testuinguruan, LC3B, GABARAP, GABARAPL1 eta GABARAPL2 ikertuak izango dira.

Atg8 UBL sistemak, beste 3 proteina dauzka, ATG4, ATG7 (E1-moduko entzima) eta ATG3 (E2-moduko entzima). ATG4, Atg8aren giza ortologoaren C-muturreko azken aminoazidoak itzulpen ostean mozten dituen zisteina proteasa bat da. Mozketa horren ondorioz, kontserbatua dagoen C-muturreko glizina bat agerian geratzen da [Gly120, LC3Barentzako eta Gly116, GABARAP azpifamilia kide guztientzako (Kabeya *eta lank.*, 2004; Tanida *eta lank.*, 2003; Tanida *eta lank.*, 2004a; Tanida *eta lank.*, 2006)]. Glizina hau ezinbestekoa da azkenean PERekin elkartzeko eta beraien lipidazio egoeraren arabera autofagosomen mintzetan kokatzeko (Kabeya *eta lank.*, 2000, 2004; Tanida *eta lank.*, 2004a). Mozketa horren ostean, LC3k (eta beste Atg8aren ortologoek) sistemaren E1-moduko entzima den ATG7arekin elkarreragiten dute (**1.16 Irudia**). ATG7, bi zisteina katalitiko dauzkan homodimero simetrikoa da eta hondar hauekin bi UBL proteina desberdinen, LC3 eta ATG12ren, aktibazioa ahalbidetzen du (Hong *eta lank.*, 2011; Kaiser *eta lank.*, 2013). Sistema honen E2-moduko entzima den ATG3k, ATG7tik LC3 jasotzen du eta LC3ren azken lipidaziorako, LC3 mintzera ekartzearen arduraduna dela uste da. Azken erreakzio hau autofagian

parte hartzen duen beste UBL sistemak, ATG5-ATG12-ATG16 konplexuak, bizkortzen du. Konplexu honek erreakzio sofistikatu honen E3-ligasa entzima gisa jarduten du.



1.16 Irudia. Atg8 UBL sistemaren irudikapen eskematikoa.

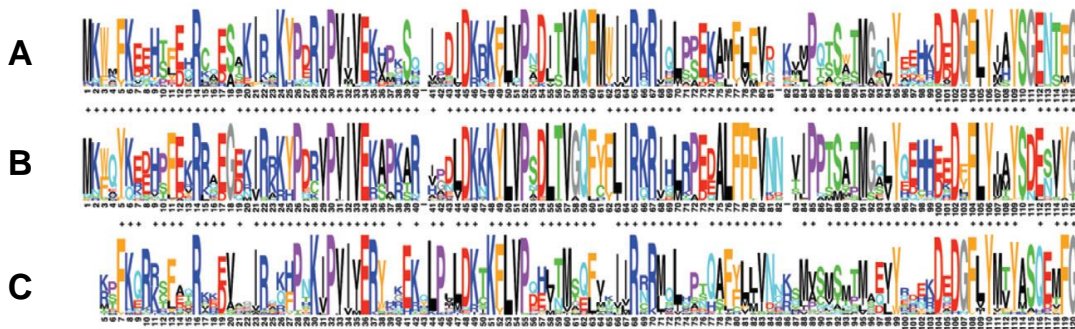
(<http://ruo.mbl.co.jp/g/product/protein/autophagy/autophagy.html>-tik hartua).

1.2.5.1 Atg8aren Giza Ortologoak

Aurretik aipatu den bezala, ugaztunetan Atg8aren ortologoak bi azpifamilietan banatzen dira: LC3 (LC3A, LC3B, LC3B2 eta LC3C) eta GABARAP (GABARAP, GABARAPL1, GABARAPL2 eta GABARAPL3) (Weidberg *eta* *lank.*, 2010). Ugaztunetan gehien ikertua izan den ortologoa LC3B da. Honek, Atg8ak legamietan egiten duen moduan, autofagosomaren eraketan parte hartzen du. GABARAP eta GABARAPL2 (A-motako GABA hartzaileri lotutako proteina-moduko 2, ingelesetik *GABA type A receptor-associated protein like 2*) Golgi barruko besikulen garraio faktore eta mikrotubuluekin batera GABA(A) (ingelesetik *GABA type A*) hartzaileren taldekatzean laguntzen duen proteina gisa identifikatu dira, hurrenez hurren (Tanida *eta* *lank.*, 2004b). Giza ortologoaren artean azken bi hauek dira, autofagosomaren eraketan daukaten eginkizunari dagokionez, gutxien ikertuak izan direnak.

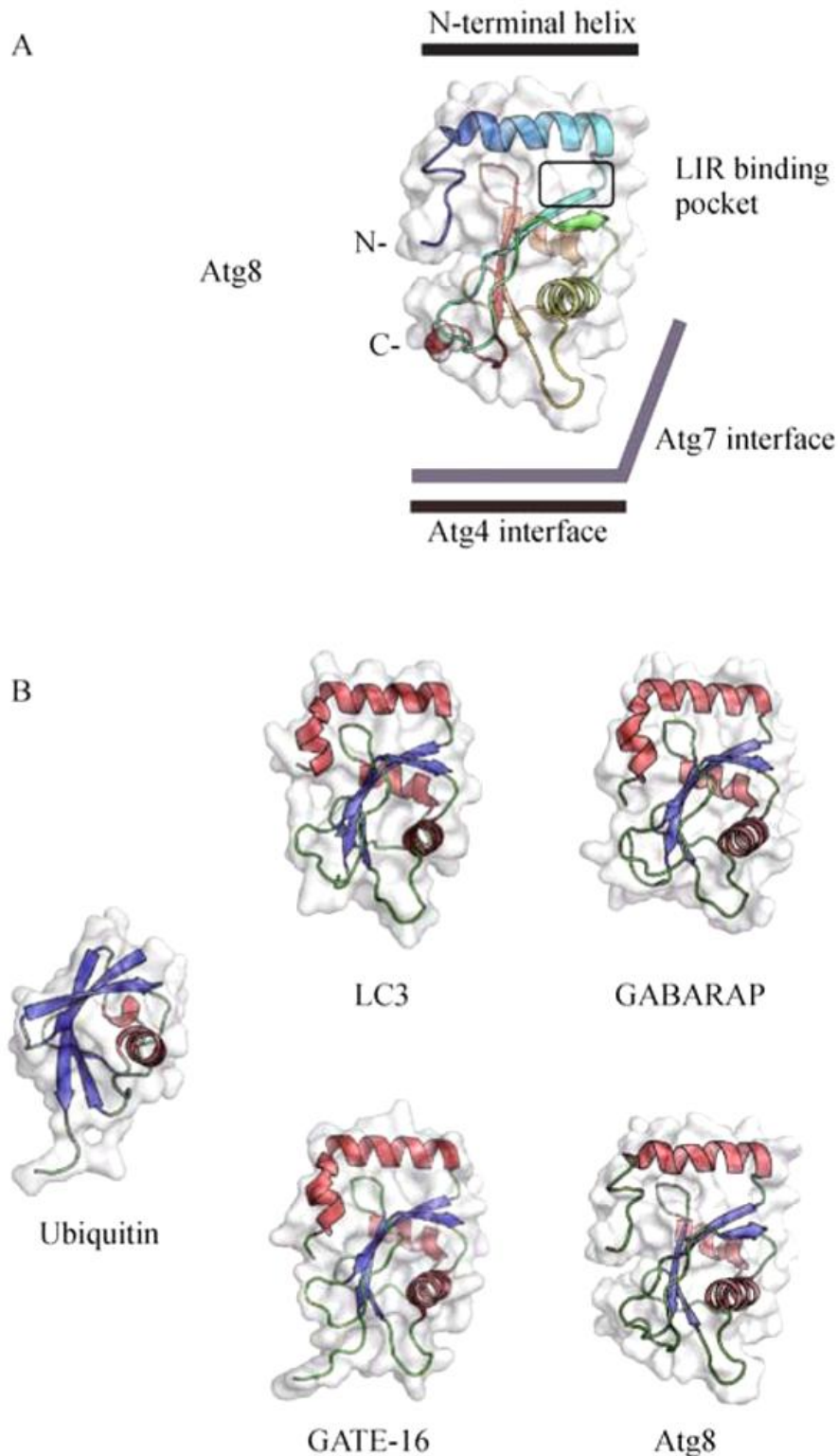
A) Sekuentzia eta Egiturazko Ezaugarri Bereizgarriak

Atg8 azpifamilia bakoitza, osotasunean oso ongi kontserbatua dago (LC3ren amino-muturreko hondar gutxi batzuk izan ezik) eta proteinek posizio gehienetan ere beraien artean antzekotasun handia erakusten dute (Schaaf *eta lank.*, 2016; Shpilka *eta lank.*, 2011). Familia bakoitzean soilik kontserbatuak diren eta beraz azpifamilien artean ezberdinak diren posizio gutxi batzuk, beraien arteko ezberdintasun funtzional batzuen arrazoieta bat izan daiteke (**1.17 Irudia**). Dena den, gaur egun argitu gabe dago zeintzuk diren sekuentziaren ezaugarri espezifiko bakoitzarekin lotuta dauden funtzioak, baten bat badago.



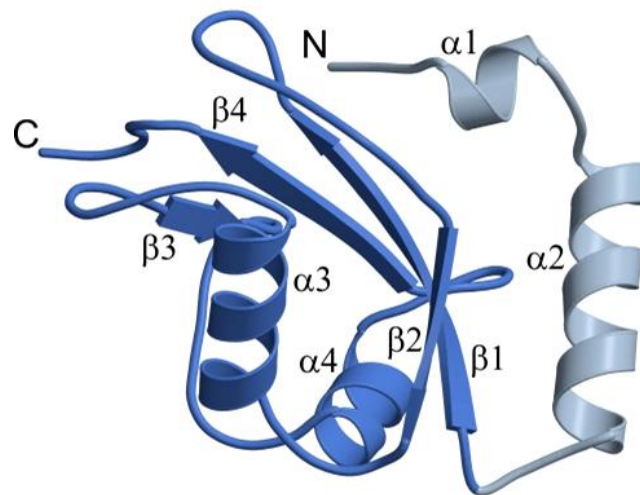
1.17 Irudia. Atg8aren giza ortologoaren sekuentziaren ezaugarriak. (A) GABARAPL2, (B) GABARAP eta (C) LC3. Sekuentzietako logoek kontserbazioa (altuera totala) eta lerrokatze ezberdinetan hondar bakoitzaren nagusitasuna adierazten dute (Schneider *eta* Stephens, 1990). Lerrokatzeek kontserbatutako sekuentzia zonalde nagusiak erakusten dute, soilik sekuentzia batzuen kontserbaturik gabeko zonalde distal eta laburrak baztertuz. Zeinu positiboek, GABARAPL2 eta GABARAPen arteko, eta GABARAP eta LC3 azpifamilien lerrokatzeen arteko antzeko posizioak erakusten dituzte. (Shpilka *eta lank.*, 2011-tik moldatua).

LC3Bk, GABARAPek eta GABARAPL2/GATE-16k (ingelesez *Golgi-associated ATPase enhancer of 16 kDa*), ugaztun, protozoo eta ondo zeluletako Atg8aren gainontzeko ortologoak bezala, ubikitinaren egiturarekiko antzekotasun handia daukate, nahiz eta aminoazidoen sekuentzian desberdinak diren (Shpilka *eta lank.*, 2011; Schaaf *eta lank.*, 2016). Ia guztiz kontserbatua dagoen C-muturreko ubikitinaren muina (β -heltze motiboa ere deitua, ingelesez *β -grasp motif*), normalean mota mistoko 4 β -orritz osatua dago (hau da, barneko bi harizpiak paraleloki kokatuta eta kanpoko segmentu antiparaleloekin alboratuak daude) eta helize batek edo bik orriaren aurpegi ahurra babesten dute (Coyle *eta lank.*, 2002; Kumeta *eta lank.*, 2010; Paz *eta lank.*, 2000; Schwarten *eta lank.*, 2010; Sugawara *eta lank.*, 2004) (**1.18 Irudia**). Domeinu honek, familia-kideen artean kontserbatzen den ezaugarri basiko bat dauka eta proteina-proteina elkarrekintzetan eginkizunen bat daukala proposatu da.



1.18 Irudia. Atg8aren, ugaztunen Atg8aren ortologoaren eta ubiquitinaren egitura kristalinoak. (A) Legamien Atg8aren NMR egitura. Zenbait elkarrekintza zonaldeak erakusten dira. Atg8k degradaziorako-material espezifiko eta autofagiaren makinariaren muina errekrutatzen ditu. Horretarako, motibo kontserbatu bat, LIR edo Atg8 familiarekin elkarrengaitzeko motiboa (AIM, ingelesetik *Atg8-family interacting motif*), Atg8aren poltsiko hidrofobiko espezifiko bati lotzen zaio. (B) Ubikitina-moduko tolestura erakusten duten Atg8 familia proteinen egituren alderaketa. PDB: 2KQ7 (Atg8), 1UGM (LC3), 1GNU (GABARAP), 1EO6 (GABARAPL2/GATE-16), 1UBI (ubikitina). (Chew eta Yip, 2014-etik hartua).

Atg8aren giza ortologoak, ubikitinaren muinaz gain, β -orriaren alde ganbilera lotutako bi helize gehigarri dauzkan N-mutur bat daukate. Eskualde hau Atg8-moduko proteina guztien bereizgarri bat da, zeinak ubikitinaren superfamiliarengandik bereizten dituen. Ezberdintasun handienak C-muturreko muina inguratzen duen zonalde honetan aurkitzen dira (**1.18 eta 1.19 Irudiak**). Adibidez, LC3ren N-muturreko sekuentzia erlatiboki basikoa den bitartean, GABARAP eta GABARAPL2rena azidoa da. GABARAPen egitura kristalinoak, Atg8 familiako proteinak bi konformazio ezberdin hartzeko ahalmena izango luketela erakutsi zuen, N-muturreko helizea beste Atg8 molekula batekin elkarrekintza intermolekular batean laguntzen: konformazio itxi bat, non α helizeak ubikitinaren muinetik beherantz begira kokatzen diren, eta konformazio ireki bat, non amino-muturreko 10 aminoazidoak ubikitina muinetik kanpo bideratzen diren (Coyle *eta lank.*, 2002). Azken konformazio hau proteinaren egoera oligomeroaren bidez egonkortzen da eta Atg8aren amino-muturreko eskualdeak daukan elastikotasuna adierazten du. Hipotesi honen alde, erresonantzia magnetiko nuklearrak (NMR, ingelesetik *nuclear magnetic resonance*), legamien Atg8aren N-muturreko egituraren elastikotasuna frogatu du (Schwarten *eta lank.*, 2010; Kumeta *eta lank.*, 2010). N-muturreko isats dinamikoaren bitartez Atg8k orden altuagoko homo-oligomeroak eratzeko daukan ahalmenak, mintzari atxikitutako Atg8-PEk daukan mintzak ainguratzeko funtzioaren oinarritzko mekanismoa izan daiteke.



1.19 Irudia. Atg8 familiako proteinen tolestura bereizgarria, GABARAPL2aren egitura kristalinoaren adibidez adierazia. β -heltze motiboa (urdin iluna) ubikitina superfamiliaren ezaugarria da. Atg8aren ortologo guztiek N-muturreko hedapen bereizgarri bat duten bitartean (urdin argia), beste ubikitina-moduko proteinetan antolaketa ezberdinak aurkitu izan dira. PDB: 1EO6. (Weiergräber *eta lank.*, 2013-etik moldatua).

GABARAPL2z eta GABARAPez gain, Atg8 familiako beste kide batzuen egitura tridimentsionalak ere zehaztuak izan dira, horien artean aurkitzen dira GABARAP proteinarekin estuki erlazionatuta dagoen GABARAPL1, LC3A, LC3B (Kouno *eta lank.*, 2005), eta legamien Atg8 bera (Schwarten *eta lank.*, 2010; Kumeta *eta lank.*, 2010). Zerrenda honetara azken gehitutakoa LC3C da, zeinaren egitura kristalinoa, autofagiako hartzaile batekin konplexu bat eratzen egonda zehaztu den (von Muhlinen *eta lank.*, 2012). Egitura guzti hauek esperotako tolestura orokorra erakutsi arren, N-muturreko azpidomeinuek daukaten elastikotasunaren inguruko eztabaidara batu ziren baita ere. Adibidez, LC3Baren NMR egiturak ez zuen α 1- α 2 zonaldearen inguruan inongo fluktuaziorik erakutsi (Kouno *eta lank.*, 2005), zeina desadostasunean dagoen aipatutako aurkikuntzekin, zeintzuek GABARAPen N-muturrean bi konformazio edo gehiagoren arteko oreka bat dagoela erakusten duten (Stangler *eta lank.*, 2002). Geroago, polimorfismo konformazionala, gutxienez Atg8-motako proteina batzuetan, N-muturreko azpidomeinuaren ezaugarri intrintseko bat dela proposatu da. Hala ere, kontuan eduki behar da, behaketa hauen garrantzia funtzionala oraindik zehaztua izan behar dela.

Aurkikuntza guzti hauek kontuan hartuta, badirudi Atg8ren amino-muturreko eskualdeak proteina hauen funtzioetan funtsezko eginkizuna betetzen duela (Nakatogawa *eta lank.*, 2007; Shvets *eta lank.*, 2011; Weidberg *eta lank.*, 2011; Schaaf *eta lank.*, 2016). Beraz, Atg8 proteinen arteko ezberdintasunak zonalde honetan, funtzio ezberdinak betetzearen seinale izango litzateke.

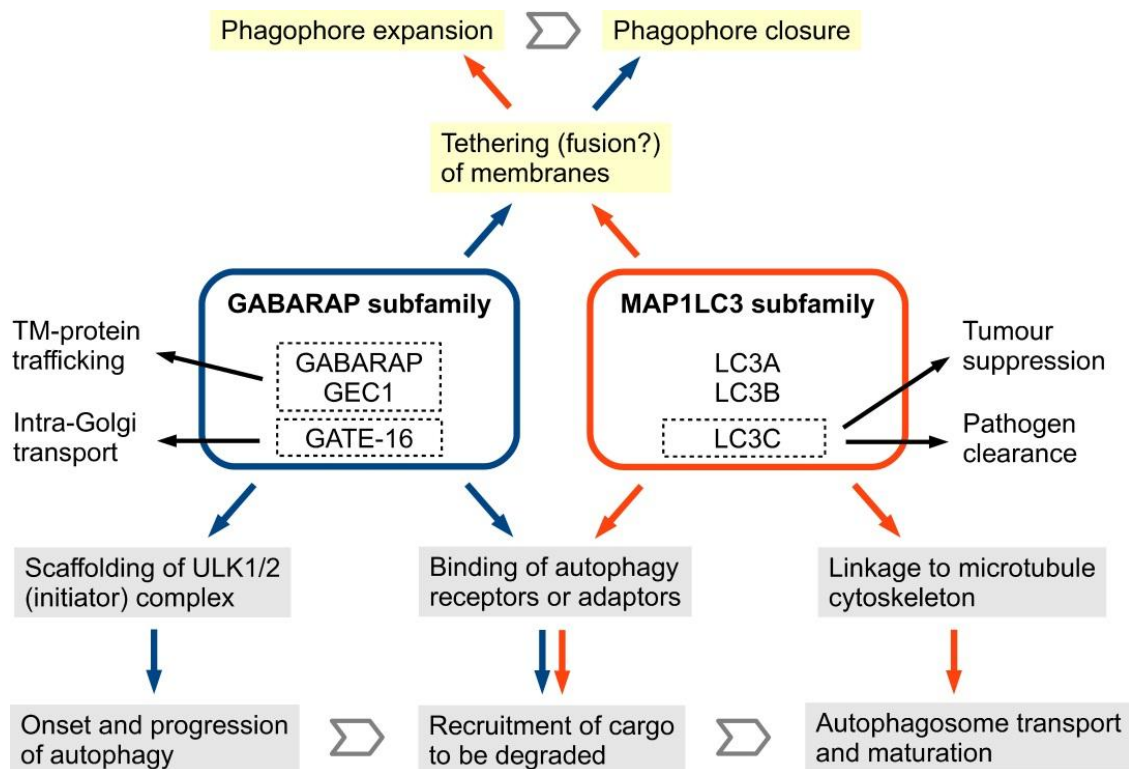
B) Atg8aren Ortologoaren Eginkizun Dibergenteak

15 urte inguru dira Atg8 legamietan autofagosomen eraketa egokirako ezinbestekoa dela jakin zenetik (Lang *eta lank.*, 1998). Parologo bat baino gehiago egotearen ondorioz, ugaztunak bezalako metazoo garatuagoetan egindako geneen delezio esperimentuek ez dute fenotipo zehatz bat erakutsi (O'Sullivan *eta lank.*, 2005). Honek Atg8 familiako kideen artean erredundantzia funtzional bat egon daitekeela iradokitzen du. Proteina hauek, ehunaren eta estres baldintzen arabera adierazpen maila desberdinak dauzkaten arren, ubikuoak dira (Shpilka *eta lank.*, 2011; Schaaf *eta lank.*, 2016). Adibide batzuk aipatzekotan, GABARAPL1ren adierazpena nerbio-sistema zentrolean altua da (Nemos *eta lank.*, 2003), GABARAPL2k garunean adierazpen maila altua du, eta LC3aren azpifamiliako gainontzeko kideak baino gutxiago transkribatzen den LC3C, batez ere biriketan adierazten da (He *eta lank.*, 2003; Xin *eta lank.*, 2001).

Zenbait estres baldintzetan Atg8 proteinen erregulazio positiboa behatua izan da (Egami *eta lank.*, 2005; Kirisako *eta lank.*, 1999; van Zutphen *eta lank.*, 2010; Zois *eta lank.*, 2011). Atg8 geneen adierazpena erregulatzen duten seinalizazio bidezidor eta transkripzio-faktoreak soilik partzialki karakterizatu izan diren arren, ugaztun zeluletan egindako zenbait ikerketek FoxO1ek, FoxO3k (ingelesetik *forkhead box O1 or 3*) eta E2F 1-transkripzio-faktoreak (E2F1, ingelesetik *E2F transcription factor 1*) prozesu honetan Atg8 proteinak erregulatzen dituztela erakutsi dute (Mammucari *eta lank.*, 2007; Polager *eta lank.*, 2008; Sengupta *eta lank.*, 2009; Zhao *eta lank.*, 2007). Atg8 gene espezifikoaren adierazpen patroiak transkripzio eta itzulpen mailan ezagutzeak, beraien eginkizun fisiologiko ezberdinen inguruko argibideak eman ditzake.

Zenbait Atg8aren ortologoak, autofagiarekin loturarik ez duten hainbat prozesu zelularretan deskribatu dira. LC3, lehen aldiz, mikrotubuluekin dauzkan elkarrekintzen bidez isolatua izan zen (Mann *eta Hammarback*, 1994). Gutxienez LC3B eta GABARAP, beraien N-muturreko azpidomeinuarekin bideratutako elkarrekintza elektrostatikoen bidez tubulina eta mikrotubuluak lotzeko gai dira (Coyle *eta lank.*, 2002; Kouno *eta lank.*, 2005; Wang *eta lank.*, 1999). LC3B proteinak mikrotubulu eta autofagosomen arteko proteina-egokitzaile gisa jotzen duela proposatu da, baina ez zaio autofagiarekin lotuta ez dagoen beste eginkizunik esleitu (Kouno *eta lank.*, 2005). Bestalde, GABARAP familiako kideek mintzen garraioan betetzen duten eginkizunen bidez identifikatuak izan dira (**1.20 Irudia**). GABARAPL2 daukan N-etilmaleimida faktore sentikorra (NSF, ingelesetik *N-ethylmaleimide sensitive factor*) eta Golgiko SNAREa den GOS28 (Golgiko SNAP hartzaile konplexuaren 1-kidea)-ren arteko lotura aktibitatearen bidez, Golgi barruko garraioaren modulatzailerik bat da (Sagiv *eta lank.*, 2000). Gainera, GABARAP eta GABARAPL1 ere NSFrekin elkarrengaitzeko gai dira (Chen *eta lank.*, 2006; Kittler *eta lank.*, 2001). GABARAPek, bere N-muturreko tubulina-lotzeko azpidomeinuaren bidez, tubulinaren polimerizazioa eragiten duela eta bere C-muturreko Ubl domeinuaren bidez GABA(A) hartzaileekin elkarrengaitzeko duela ikusi da, ziurrenik bere β -orrietako batekin emandako elkarrekintzen bidez (Coyle *eta lank.*, 2002). Bai GABARAP zein GABARAPL1, ER, Golgi sarearekin eta GABA(A) hartzaileen mintz plasmatikora bideratutako garraioarekin lotuta daude, honek, baliteke garun ehunetan daukaten adierazpen maila altua azaltzea (Chen *eta lank.*, 2006; Leil *eta lank.*, 2004). GABARAPL1ek G-ri lotutako κ -opioide hartzaileen garraioan ere parte hartzen du (Chen *eta lank.*, 2006). GABARAPek, berriz, transferrina hartzailearekin (Green *eta lank.*, 2002) eta ziur aski baita I. motako angiotensina II hartzailearekin ere elkarrengaitzeko du (Cook *eta lank.*, 2008). Beraz, ortologo hauek mintz plasmatikoko

beste proteina batzuen garraioan lagunduko lukete. GABA(A) hartzaileen baita opioide hartzaileen garraiorako ere, GABARAP eta GABARAPL1en kontserbatutako Gly116 hondarreen C-muturreko prozesamendua beharrezkoa da. Hala ere, garraioaren testuinguruan, lipidoekiko ubikitina-moduko konjugazioaren beharrezkotasuna ez da ikertua izan (Chen *eta lank.*, 2011; Chen *eta lank.*, 2007).



1.20 Irudia. Ugaztun zeluletan GABARAP eta LC3 azpifamiliako proteinen funtzioen laburpen eskematikoa. Autofagiaren bidezidorrerako ezinbestekoak diren funtzioak grisez eta horiz koloreztatuta daude, proteina-proteina eta proteina-lipido elkarrekintzen parte hartzea adieraziz, hurrenez hurren. Autofagiaren beste alderdi batzuetarako garrantzitsuak diren funtzioak (LC3Caren kasuan) edo autofagiarekin erlazionatuta ez dauden funtzio gehigarriak (GABARAP azpifamiliako kideen kasuan) ere adierazten dira (Weiergräber *eta lank.*, 2013-tik hartua).

Autofagiaren testuinguruan, molekula hauek autofagiaren funtsezko osagaien lotura bikote eta proteina-egokitzaila gisa jokatzen dutela jakina da; hori dela eta, degradatu beharreko material espezifikoaren errekrutatzean parte hartzeaz gain (Stolz *eta lank.*, 2014), konplexu funtzional multi-proteikoen antolakuntzan ere parte hartzen dutela proposatu da. Elkarrekintza bikotearen arabera, bi azpifamiliako kideen arteko lotura espezifitateak oso desberdinak izan daitezke (Schaaf *eta lank.*, 2016-ean berrikusia). ULK1/2 konplexuko osagaiek, adibidez, nahiago dituzte GABARAP-moduko proteinak LC3-modukoak baino (Alemu *eta lank.*, 2012) (**1.20 Irudia**), eta

egungo ikerketek, Atg1/ULK-k aktibazio fasean jarduteaz gain, fagoforoaren mintzean funtzio garrantzitsuak betetzen dituela iradokitzen dute (Kraft *eta lank.*, 2012; Nakatogawa *eta lank.*, 2012). Bestalde, autofagosomak mikrotubuluaren zitoeskeletora lotzen dituen FYCO1 proteina egokitzaileak, LC3rekiko lehentasun handia erakusten du (Pankiv *eta lank.*, 2010). Gaur egun arte identifikatu diren Atg8/LC3rekin lotzen diren proteina guztien artean muga handienak NDP52k erakusten ditu. Ubikitinazuzendutako autofagia hartzaile gehienek [p62, NBR1 (ingelesetik *neighbor of BRCA1 gene 1*) eta optineurina], bai GABARAP zein LC3 azpifamiliako proteinekin elkarreragiten duten bitartean (Kirkin *eta lank.*, 2009; Pankiv *eta lank.*, 2007; Wild *eta lank.*, 2011), NDP52k soilik LC3C lotzen duela ikusi da. LC3Ck alderdi gehiagotan ezohikoa dela dirudi. Duela gutxiko ikerketek diotenaren arabera, gainontzeko LC3 proteinekin erredundanteak ez diren edota baita aurkakoak ere diren funtzioak betetzen ditu (Stadel *eta lank.*, 2015). Mikrobioen garbiketaren eginkizunaz gain, minbiziaren aurkako ekintza bereizgarri bat esleitu zaio. Gibeledako minbizi-zelulekin egindako saiakuntzek, von Hippel-Lindau (VHL) proteinak, LC3B eta LC3Cren adierazpenak kontrako moduan erregulatzen dituela erakutsi dute, LC3Bren kasuan tumorearen hazkuntza sustatuz eta LC3Cren kasuan inhibituz (Mikhaylova *eta lank.*, 2012). Behaketa deigarri honek autofagiaren itu garrantzitsuen errekrutamendu bereizgarriarekin lotuta dagoela pentsatzera eramanez litekeen arren, itu hauek oraindik ezin izan dira identifikatu. Gainera, LC3ren isoformek (LC3A, B eta C) zenbait giza minbizi-zelula lerroetan, eta baita MRC5 fibroblastoetan ere, adierazpen patroi ezberdinak dauzkatela erakutsi da (Koukourakis *eta lank.*, 2015). LC3Ak kokapen perinuklearra eta nuklearra du, LC3B zitoplasman zehar era homogeneo batean banatuta eta eskualde nukleolarretan aurkitzen da, eta LC3C zitoplasman eta batez ere nukleoan aurkitzen da, non LC3A eta Beclin 1 proteinekin batera dagoen (Koukourakis *eta lank.*, 2015). Beraz, molekula hauen banaketa desberdina da; hala ere, oraindik ez dago argi hiru LC3 proteina hauek autofagian eginkizun biologiko desberdin bat duten edo ez.

Autofagosomaren eraketari dagokionez, legamien Atg8k *in vitro* mintzen arteko fusioa gauzatu dezake (Nakatogawa *eta lank.*, 2007), eta isolatze-mintzaren elongazioa, eta beraz autofagosomen tamaina, erregulatzen duela ikusi da (Xie *eta lank.*, 2008). Ugaztunetan, LC3B, GABARAP eta GABARAPL2 *in vitro* mintzen arteko fusioa gauzatzeko gai dira, eta beraien N-muturreko sekuentzietatik zenbait hondar ezabatzeak autofagosomaren eraketan eragina du eta ATG16rekin markatutako fagoforoen pilaketa eragiten du (Landajuera *eta lank.*, 2016; Weidberg *eta lank.*, 2011). Autofagosometan, LC3B Atg8aren ortologo adierazgarriena da, eta aurretik aipatu den

bezala, urteetan zehar autofagiaren markatzaile nagusi gisa erabilia izan da (adib. autofagiaren aktibazioa edo fluxua neurtzeko) (Klionsky *eta* *lank.*, 2008). Interesgarria da bai LC3 zein GABARAP familiako kideak, autofagosoma berdinetan aurki daitezkeela (Kabeya *eta* *lank.*, 2004; Weidberg *eta* *lank.*, 2010), eta duela gutxiko ikerketa batek biek autofagosomaren eraketarako beharrezkoak direla, baina ziurrenik bata bestearen ondoren jokatzen dutela erakutsi du (Weidberg *eta* *lank.*, 2010). Atg8ren azpifamilia bat zein bestea isilarazteak, guztiz osatu gabeko fagoforoen pilaketa eragiten du, baina egitura hauen arteko tamaina eta kopuru desberdintasunak eta baita beste kide batzuen (LC3B edo GABARAPL2) gainadierazpenaren metatze-efektua edo efektu motelgarria iradokitzen dutenez, LC3 azpifamiliako proteinak fagoforoaren mintzaren elongazioan garrantzitsuak dira, non GABARAP azpifamiliako proteinek aurrerago diharduten, ziur aski mintzaren ixtean (Weidberg *eta* *lank.*, 2010).

Atg8aren giza ortologoak dibertsitateak degradaziorako-materialaren ezagutzean ere eginkizun garrantzitsua izan dezake (Stolz *eta* *lank.*, 2014). NBR1ek eta p62k Atg8aren sei giza ortologo nagusiekin elkarreagiten duten bitartean (Behrends *eta* *lank.*, 2010; Kirkin *eta* *lank.*, 2009a; Shvets *eta* *lank.*, 2008), Nixek GABARAP proteinei lotzeko lehentasuna dauka (Novak *eta* *lank.*, 2010). Interesgarria suertatzen da, autofagia sarearen inguruko ikerketa proteomiko batek, Atg8aren sei giza ortologoekin elkarreagiten duten 67 proteina erakusten dituela, non horietatik 31 proteinek ortologo espezifiko batekin soilik elkarreagiten duten (Behrends *eta* *lank.*, 2010). Elkarrekintza hauek funtzio gehiago aurkitzen eta Atg8aren giza ortologo bakoitzaren eginkizunen espezifizitatea ulertarazten lagun ditzakete. Adibidez, TBC1 domeinudun 2B familia kidea (TBC1D2B, ingelesezik *TBC1 domain family member 2B*), Atg8ri lotzen zaion proteina gisa identifikatu zen (Behrends *eta* *lank.*, 2010), eta duela gutxiko ikerketa batean, Atg8ri, OATL1 eta TBC1D2B bidezko seinale transdukziorako proteina-aldamio (ingelesez *scaffold protein*) gisa, autofagosomaren heltzea erregulatzeko funtzioa esleitu zaio; OATL1ek, aztertutako hiru Atg8 ortologoetatik, GABARAParekiko afinitate altuena erakutsi zuelarik (Itoh *eta* *lank.*, 2011).

Atg8aren zenbait ortologoak funtzioa ere modu ezberdinean erregulatua egongo litzateke (Schaaf *eta* *lank.*, 2016-en berrikusia). Adibidez, LC3Aren Ser 12 hondarraren fosforilazioak autofagosomaren eraketa inhibitzen zuela (Cherra *eta* *lank.*, 2010), eta hondar hau, Atg8aren ortologo nagusien artean, GABARAPL2n soilik kontserbatua dagoela ikusi zen.

Laburbilduz, Atg8 proteinek zelula barneko garraio eta autofagia prozesu desberdinetan parte hartzen dute. Atg8 autofagosomaren mihiztaduran ezinbestekoa

da lipido fusio gertaeretan – isolatze-mintzen elongazio eta itxierarako - eta baita degradaziorako-materialaren aukeraketan ere betetzen dituen funtzioen bidez. Ugaztunen Atg8 azpifamiliak autofagosomen eraketan ezberdin jarduten dutela badirudi ere, azpifamilia bakoitzeko lau kideetik gorako beharrezana oraindik ez da azaldua izan. Batez ere, ezinbestekoa da azpifamilia bateko kide ezberdinak autofagosoma berdinean dauden edo kide bakoitzak autofagosoma ezberdin batean jarduten duen zehaztea. Honek, estres baldintza ezberdinen aurrean aktibatutako autofagia prozesuan Atg8 kide ezberdinak dituzten autofagosoma “espezializatuak” eratzen diren argitzen lagundu lezake.

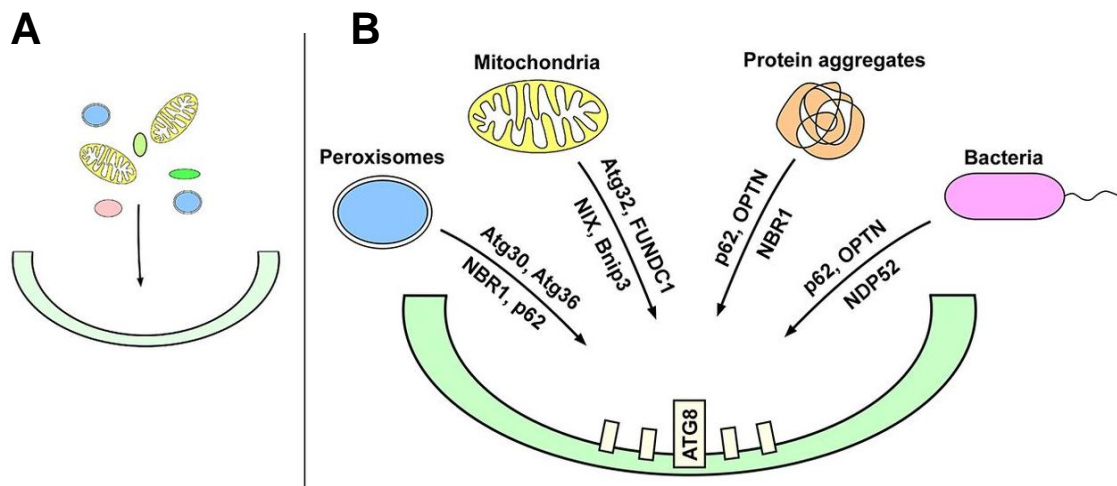
Beste galdera garrantzitsu bat da faktore hauek nola erregulatzen duten mintzen arteko fusioan eta degradaziorako-materialaren autofagosomarako errekrutatzean daukaten eginkizun bikoitza. Gero eta ikerketa gehiago daude erakusten dutenak ugaztunetan deskribatutako Atg8aren ortologo ezberdinek, funtzio eta elkarrekintza bikote amankomunak eta espezifikokoak dauzkatela. Beraz, ehun ezberdinetan daukaten adierazpenaren aldaketek helburu ezberdinetarako autofagiaren erregulazioan parte hartzen dutela pentsa liteke. Gainera, autofagia hautakor bat burutzeko Atg8 familiako kide ezberdinak ezinbestekoak izatea ulergarria den arren, Atg8 kide bakoitzaren degradaziorako-materialarekiko espezifikitatea zehazteko ikerketa proteomiko gehiago behar dira. Lan honen testuinguruan, Atg8aren giza ortologoen eginkizun espezifikoko posibleak aztertu dira, bai prozesu autofagiko hautakor zein ez-hautakorretan.

1.2.6 Autofagia Hautakorra vs Ez-Hautakorra

Hasiera batean, autofagia, estres baldintzetan elikagai eta energia birziklatzeko ausazko degradazio-mekanismo sinple gisa deskribatu zen arren, ikerketa askok argi utzi dute, autofagia proteolitikoaren itu espezifikokoak egoteak homeostasi zelularrean ezinbesteko garrantzia duela. Izan ere, gaur egun argi dago kaltetutako organuluaren birziklapena, esate baterako, mitokondrioak (mitofagia), peroxisomak (pexofagia), erribosomak (erribofagia) edo erretikulu endoplasmatikoa (erretikulofagia); proteina pilaketan ezabapena (agrefagia) eta zelula barneko patogenoen garbiketa (xenofagia), oso selektiboak diren eta estuki erregulatuak dauden prozesu autofagikoak direla (Khaminets *eta lank.*, 2015; Mochida *eta lank.*, 2015; Sica *eta lank.*, 2015).

Autofagia hautakorra, autofagosometara barneratzeko hautaketa positiboaren bidez [adib., ubiquitinazioa edo azetilazioa bezalako itzulpen osteko eraldaketan bidez

(Jeong *eta lank.*, 2009; Kirkin *eta lank.*, 2009b)] eta/edo hautaketa negatiboaren bidez (adib. autofagosoman degradatuko diren proteina edo organulu batzuetara iristea zaila denean) gerta daiteke. Espezifikotasuna proteina hartzaille (edo egokitzaille) autofagikoen bidez lortzen da, zeintzuek fagoforoaren gainazalean Atg8-moduko proteinekin lotzen diren eta autofagosoman degradatu beharreko material desberdinen aukeraketa hautakorra gauzatzen duten (**1.21 Irudia**). Autofagiaren erregulatzaille desberdinen inguruko ezagutza asko handitu da azkenaldian, honek oso hautakorra den eta estuki erregulatuta dagoen mintzen menpeko prozesu hau ulertzeko modua berritu duelarik.



1.21 Irudia. Autofagia hautakorra vs ez-hautakorra. (A) Ausazko autofagia ez-hautakorrean eratzen ari den autofagosomak, fagoforoak, edozein material zitoplasmatico bahitzen du. (B) Autofagia hautakorrak proteina hartzaille menpekoa den prozesu baten bidez fagoforoa degradaziorako-material espezifikoaz aberasten du. Hemen, identifikatu diren degradaziorako-material desberdinak eta hauen degradazio hautakorrarekin lotuta dauden zenbait hartzaille autofagiko adierazi dira. Azken horien artean aurkitzen dira OPTN (optineurina), NBR1, NDP52, NIX (NIP3-moduko proteina X) eta FUNDC1 (FUN14 domeinua daukan 1, ingelesetik *FUN14 domain containing 1*). (Svenning eta Johansen, 2013-tik moldatua).

1.2.6.1 Degradaziorako-Materiala Hautatzeko Mekanismo Orokorrak Ugaztunetan

Materialaren ezagutzeaz eta emateaz arduratzen diren hartzaille autofagiko berrien identifikazioa, degradaziorako-material horiei buruzko ezagutzaren handipenarekin batera gertatzen da (Stolz *eta lank.*, 2014). Beraien funtzioa lau irizpide amankomunen arabera deskriba daiteke:

(i) Hartzaile autofagikoak Atg8/LC3 proteinari lotzen dira kontserbatutako lotura guene baten bidez, LIR motiboa edo AIM – W/YxxL/I.

(ii) Hartzaile autofagikoak degradaziorako-materialarekin batera degradatzen dira.

(iii) Hartzaile autofagikoen ezabapenak ez luke autofagiaren oinarriko makinaria oztopatu behar.

(iv) Proteina-proteina lotura-domeinuek edo mintzari lotzeko domeinuek degradaziorako-materialarekiko elkarrekintzan parte hartzen dute.

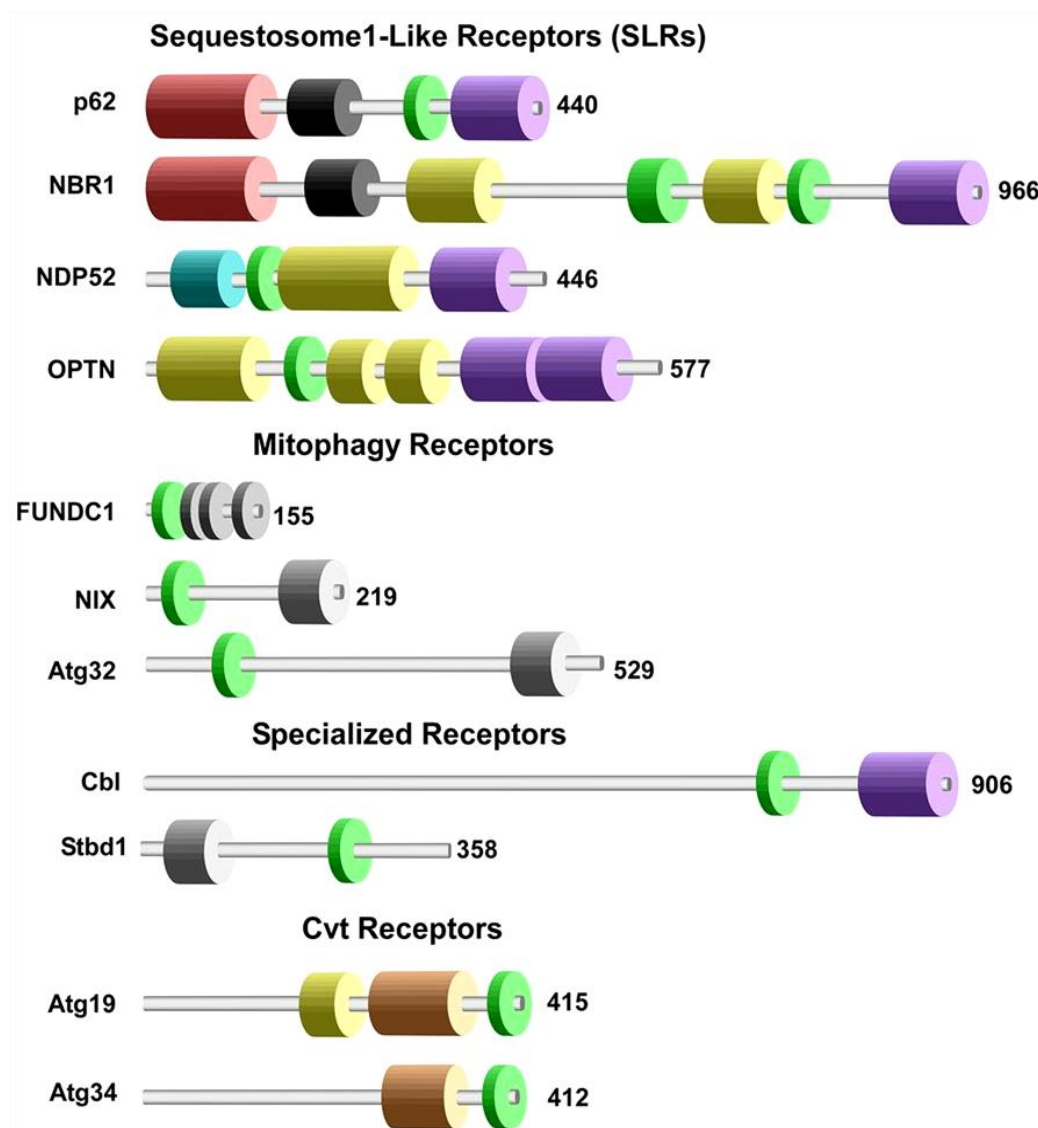
Beraien egiturazko ezaugarrien eta degradaziorako-material desberdinekin nola elkarreagiten dutenaren arabera, hartzaile autofagikoak ondorengo lau talde ezberdinetan bana daitezke (**1.22 Irudia**):

(i) 1-sekuestosoma-moduko hartzaileek (SLR-ak, ingelesetik *sequestosome 1-like receptors*), 1-sekuestosoma (SQSTM1/p62) proteinaren egitura eta funtzioarekiko zenbait antzekotasun dauzkate (Deretic, 2012). SLR guztiek dimerizazio edo polimerizazio domeinuak dauzkate, ubikitina lotzen dute eta giza Atg8 familiako proteinekin elkarreagiten dute. Hartzaile autofagiko hauek, ubikitina motibo ezberdinekiko afinitate zehaztuak dauzkaten C-muturreko ubikitina-lotzeko domeinuen bitartez degradaziorako-material ubikitinizatuak ezagutzen dituzte. Dena den, zenbait kasutan eraldatuta ez dagoen degradaziorako-materiala ere zuzenean lotu dezakete (Johansen eta Lamark, 2011; Shaid *eta lank.*, 2013).

(ii) Kaltetutako mitokondrioen autofagiaren bidezko ezabapena (mitofagia), mintzei lotutako hartzaileen bidez gauzatzen den autofagia hautakorraren adibiderik ikertuena da (Sica *eta lank.*, 2015). Ugaztunen NIX, BCL-2rekin elkarreagiten duen 3-proteina (BNIP3, ingelesetik *BCL-2 interacting protein 3*), FUNDC1 eta legamien Atg32, transmintz domeinuen bidez mitokondrioen kanpo-mintzari ainguratuak daude. Hauek ugaztunen Atg8 familiako proteinekin LIRen menpeko elkarrekintzen bidez mitokondrioen ezabapen hautakorra gauzatzen dute (Hanna *eta lank.*, 2012; Kanki *eta lank.*, 2009; Liu *eta lank.*, 2012; Novak *eta lank.*, 2010; Okamoto *eta lank.*, 2009; Sakakibara *eta lank.*, 2015).

(iii) Berriki, zenbait hartzaile autofagiko espezializatuak aurkitu dira. Horien adibide dira Stbd1 (almidoia lotzeko domeinua daukan 1-proteina) eta E3-ubikitina ligasa, Cbl. Stbd1ek glikofagia izeneko prozesu autofagiko batean, glukogenoaren hartzaile hautakor gisa dihardu (Jiang *eta lank.*, 2011).

(iv) Legamien Cvt (ingelesetik *cytoplasm-to-vacuole targeting*) bidezidorreran, 1-aminopeptidasa (Ape1p) eta α -manosidasa (Ams1p) hidrolasa bakuolarrak, Atg19 hartzaile autofagikoari zuzenean lotuz, era hautakorrean legamien bakuoloetara barneratuak dira. Lotura honek, Cvt konplexu multimeriko edo agregatu baten eraketa eragiten du. Ondoren, Atg19k Atg11 egokitzaillearekin elkarreragiten du, zeinak eratzen ari den autofagosomarako translokazioa gauzaten duen. Autofagosoman, hidrolasen ematea errazteko, Atg19 Atg8ri lotzen zaio (Liu *eta lank.*, 2016; Lynch-Day *eta Klionsky*, 2010). Atg19k elikagaien aberastasun baldintzetan jarduten duen arren, Atg34 proteina homologoak, elikagaien gabeziak aktibatutako autofagian jarduten du Ape1p eta Ams1p bakuoloetara eramanez (Suzuki *eta lank.*, 2010).



1.22 Irudia. Hartzaile autofagiko ezberdinen proteina domeinuen arkitektura irudi eskematikoa. Cbl proteinak irudia errazteko adierazi ez diren domeinu gehiago dauka (N-muturrean: 4H, EF, SH2 eta RING). (Svenning *eta Johansen*, 2013-tik moldatua).

Gaur egun arte, ubikitina lotzen duten 4 giza hartzaile autofagiko deskribatu dira; p62/SQSTM1, NBR1 (Kirkin *eta lank.*, 2009a), NDP52 (Thurston *eta lank.*, 2009) eta optineurina (Wild *eta lank.*, 2011). Ugaztunetan, gehien ezagutzen diren autofagiaren substratu hautakorrek p62/SQSTM1 eta NBR1 dira (**1.22 Irudia**). Biek, LIR motibo baten bidez zuzenean Atg8aren LC3B giza homologoarekin elkarrengaitan dute eta p62, gutxienez Atg8aren 5 giza homologo nagusiri lotu daiteke, elkarrekintza hau ugaztunen Atg8aren ortologo guztietan kontserbatuta dagoela iradokiz (Bjørkøy *eta lank.*, 2005; Noda *eta lank.*, 2010; Pankiv *eta lank.*, 2007). p62k eta NBR1ek elkarrengaitan eta oligomeroak sor ditzaketen arren, zeluletan modu independentean jarduten dute (Kirkin *eta lank.*, 2009a). Atg8/LC3Bren bai N-muturreko zein C-muturreko sekuentziak, p62 (Ichimura *eta lank.*, 2008; Shvets *eta lank.*, 2008) eta NBR1 (Kirkin *eta lank.*, 2009a) proteinen AIM motiboekin elkarrengaitan garrantzitsuak dira. Atg8/LC3B isolatze-mintzean egoteak, autofagosomaren eraketaren ostean barne-mintzan bilakatuko den azalera ere kontuan hartuz, elkarrekintza hau p62 eta NBR1 isolatze-mintzera bideratzeko eta autofagosometan irenstea gauzatzeko gidari nagusia dela iradokitzen du (Kirkin *eta lank.*, 2009a). Autofagiaren bidezko p62 eta NBR1en birziklapena gauzatzeko AIM motiboen beharizanak ideia hau indartzen du (Ichimura *eta lank.*, 2008; Kirkin *eta lank.*, 2009a). Dena den, p62 oligomerizatua autofagosomaren eraketa gunean ere aurki daiteke LC3Bren errekrutatzea gertatu baino lehen eta bere AIM motiboarekiko menpekotasunik gabe. Honek p62 degradaziora eramateko AIM motiboak LC3B/Atg8ekin elkarrengaitan nahikoa ez dela edo p62 autofagosomaren eraketa gunea zehazteko eta eraketa aktibatzeke gai izango litzatekeela iradokitzen du (Itakura *eta Mizushima*, 2011). Errekrutamendu gunea eta ordena argi ez dauden arren, AIMren menpeko proteina-egokitzailak autofagikoekin eta Atg8aren ortologoen arteko elkarrekintzak degradaziorako-materialaren hautaketan lagunduko luke, material hauek mintz autofagikoetara lotuz (Noda *eta lank.*, 2010-ean berrikusia).

p62 eta NBR1 degradaziorako-materialaren hartzaile egokitzailak bezala ere ezagutzen dira, proteina ubikitinizatuak lotu eta autofagiaren bidezko degradaziora bideratzen dituztelako (Kirkin *eta lank.*, 2009a; Pankiv *eta lank.*, 2007). Adibidez, p62k proteina pilaketan degradazioan parte hartzen du, baina baita peroxisomen, mitokondrioen eta zelula barneko bakterioen degradazioan ere ubikitinazio seinaleen bidez (Bjørkøy *eta lank.*, 2005; Geisler *eta lank.*, 2010; Kim *eta lank.*, 2008; Zheng *eta lank.*, 2009). Bai p62 zein NBR1, Parkinson edo Huntington-en gaixotasunekin eta orokorrean gaixotasun neurodegeneratiboekin lotuta dauden autofagiaren menpeko

proteinen pilaketa toxikoen garbiketan parte hartzen dute (Bjorkoy *eta lank.*, 2005; Pankiv *eta lank.*, 2007; Ravikumar *eta lank.*, 2002; Rubinsztein, 2006).

Beste bi SLRak, optineurina eta NDP52 (**1.22 Irudia**), biak bakterioen autofagia hautakorrekarekin (xenofagiarekin) erlazionatuak izan dira (Thurston *eta lank.*, 2009; Wild *eta lank.*, 2011). Honetaz gain, optineurinak zenbait proteinen pilaketen ubikitinaren menpeko autofagia hautakorrean laguntzen duela proposatu da (Korac *eta lank.*, 2013). Bi proteinen domeinuen arkitekturak, *coiled-coil* motako domeinuak, LIR motibo funtzionalak eta C-muturreko ubikitina lotzeko domeinuak barne hartzen ditu. Optineurina hainbat gaixotasun neurodegeneratiboen proteina pilaketetan identifikatua izan da, eta *coiled-coil* domeinuen bidez bere buruarekin elkarreragiteko eta oligomerizatzeko duen gaitasuna kontuan hartuz, ziur aski material pilatuen hartzaille gisa dihardu p62 bezala. Optineurinak, ubikitinaren menpekoa ez den huntingtina pilaketen agregagian ere parte hartzen du (Korac *eta lank.*, 2013). NDP52ren agregagiari buruz gutxiago ezagutzen da, baina duela gutxi microRNA prozesatzen duen DICERaren eta AGO2 (2-argonauta) efektorearen autofagia hautakorrean parte hartzen duela erakutsi da (Gibbins *eta lank.*, 2012).

1.3 Mitofagia: Bidezidor Makroautofagiko Hautakor bat

Behin oxigenoa erabiltzen zuten bakterio autonomo gisa existitu zirela proposatuak izan diren horiek, mitokondrioek, gaur egun zelula eukariotikoak energiaz hornitzen dituzte. Argizko mikroskopia bidez barra itxurako egiturak diruditen mitokondrioak, 2 mintzez osatzen dira. Barne-mintza, tolestuta dago eta arnasketa kateko proteina konplexuak eta fosforilazio oxidatiboaren bidezko ATParen sintesirako beharrezkoak diren entzimak biltzen ditu (Rich *eta Maréchal*, 2010). DNA mitokondrialetik (mtDNA) ostalariaren nukleorako material genetikoaren garraio jarraiak, zelulak aktibitate mitokondrialaren kontrol zehatza izatea ahalbidetzen du. 22 RNA transferente (tRNA) eta 2 RNA erribosomiko (rRNA)-rekin batera, soilik mitokondrioaren arnasketa kateko 13 polipeptidok mtDNA zirkularrean kodetuta daude (Taanman, 1999). Gaur egun, mitokondrioak ATParen eraketaz gain Ca^{2+} aren seinalizazioan eta apoptosian bezalako beste prozesu zelular batzuetan ere parte hartzen duela dakigu. Mitokondrioek burutzen dituzten eginkizun ezberdinak direla eta, zelulak mitokondrioen funtzioaren eta osasunaren inguruko erregulazio mekanismo hertsia izatera behartuta daude. Izan ere, mitokondrioen kalitate kontrolean ematen diren akatsek, mitokondrio kaltetuen eta ez funtzionalen populazioa handitzea eragiten

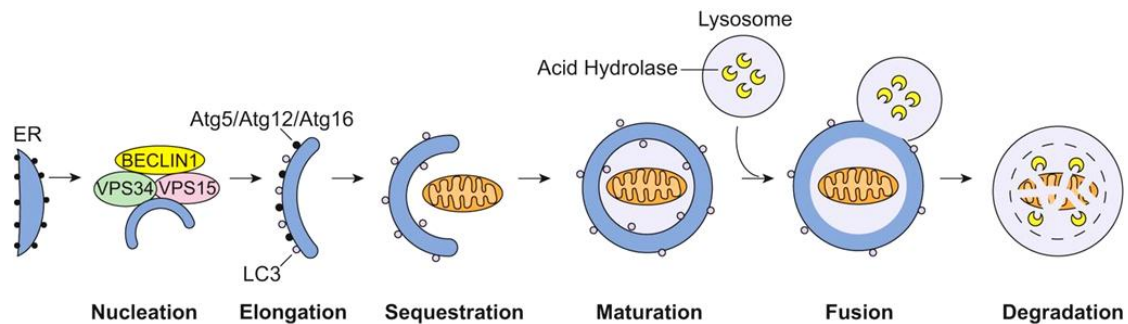
dute eta honek iskemia, diabetesa edo neurodegenerazioa bezalako giza gaixotasun desberdin asko eragiten ditu (Aufschnaiter *eta lank.*, 2016; Chan, 2006; Madrigal-Matute eta Cuervo, 2016). Mitokondrio akasdunek, ATPa modu ez eraginkor batean ekoizten dute, eta ROS kantitate handiak sortzeko arriskua daukate, zeinak, txeketzen ez bada, proteina eta lipidoen peroxidazioa eta mtDNA-n luzaroan iraun dezaketen kalteak eragin ditzakeen (Murphy *eta lank.*, 2011; Yakes eta van Houten, 1997). Kaltetutako mitokondrioetan mtDNAean mutazio kaltegarriak ere aurki daitezke eta honek mitokondrioaren aktibitate egokia arriskuan jar dezake ere (Youle eta Narendra, 2011). Mitokondrioetan kalte handiek organulua “zulatua” (ingelesez “leaky”) bilakatzea eragin dezakete; ondorioz proteina pro-apoptotikoak zitoplasmara askatzen dira, normalean oso ondorio larriekin (Tatsuta eta Langer, 2008).

Mitokondrioek gaixotasunen patogenesira zehazki nola laguntzen dutenaren inguruan eztabaida handia dago. Zenbait kasutan ez dago argi osasuntsu ez dauden mitokondrioen pilaketa gaixotasunaren kausa edo eragilea den. Hala ere, energia beharrian handia daukaten neurona bezalako zelulen gaixotasun mitokondrialen aurreko sentikortasunak eta mitokondrioen kalitate kontrolko geneetan gaixotasun espezifikoak eragiten dituzten mutazioak identifikatzeak, homeostasi mitokondrialaren erregulazioaren inguruko ikerketa asko aurrera eramatea eragin du (Aufschnaiter *eta lank.*, 2016).

Legami eta ugaztunetan, zelulek autofagiaren bidez mitokondrioak ezabatzen dituzte kalitate kontrolerako eta zelularen beharrian aldakorretara egokitzeko (Ashrafi eta Schwarz, 2013; Kiššová *eta lank.*, 2004; Tal *eta lank.*, 2007). Prozesu hau bereziki garrantzitsua da aurretik aipatutako ROS-en eraketa areagotuarekin eta mintzaren iragazkortasunarekin lotuta dauden mitokondrio kaltetuak era hautakorrean ezabatzeko (Youle eta Narendra, 2011). Prozesu zelular honek zahartzea atzeratzen duela uste da (García-Prat *eta lank.*, 2016) eta bere erregulazio ezegokia neurodegenerazioa, miopatiak eta minbizia bezalako giza egoera patologiko ugariarekin lotuta dago (Aufschnaiter *eta lank.*, 2016; Kubli eta Gustafsson, 2012; Lu *eta lank.*, 2013; Nixon, 2013).

Ugaztun zeluletan, mitofagia batez ere bidezidor makroautofagikoaren bidez gertatzen dela dirudi (Tolkovsky, 2009). Mitofagia lehen aldiz deskribatu zenean, autofagia ez-hautakorren bidezidorrarekiko ezberdintasuna nahiko lausoa zen. Mitokondrioen degradazio autofagikoan soilik parte hartzen duten proteinen aurkikuntzaren ostean (Kiššová *eta lank.*, 2004), azkar argitu zen mitofagia kalitate kontrol mekanismo berezia dela. Mitokondrio ez funtzionalak (edo batzuetan

erredundanteak), mintz bikoitzeko autofagosometan bahituz eta lisosometara eramanez, zelulak energi iturria diren organulu akasdu hauek eragin dezaketen edozein arazo neutralizatzeko gai dira (**1.23 Irudia**). Azken 10 urteetan emandako zelula bizidunen irudikapena bezalako tekniken garapenek, mitofagiaren dinamiken eta erregulazioaren inguruko ikerkuntzan aurrerapen izugarria ahalbidetu dute.



1.23 Irudia. Mitokondrioen autofagia hautakorren ikuspegi orokorra. Mitofagia, BECLIN 1/VPS34/VPS15 konplexuaren nukleazio pausuarekin hasten da, autofagosomaren eraketari hasiera ematen diona. Gero, ATG5/ATG12/ATG16 eta LC3k mintzaren elongazioan parte hartzen dute. Ostean, autofagosoma mitokondrio baten inguruan fusionatzen da, mintz bikoitzeko besikula helduaren barruan bahituz. Azkenik, autofagosoma lisosomarekin fusionatu eta mitokondrioa hidrolasa lisosomalen bidez degradatzen da. (Moyzis *eta* *lank.*, 2015-etik hartua).

Mitofagia, bai *in vivo* zein *in vitro*, hainbat sistema desberdinetan ikertua izan da eta mitokondrioen degradazioa ingurune eta garapen kausa ezberdinen ondorioz aktibatu daitekeela ikusi da. Gaur egun argi dago mitofagia ez dela mitokondrioen kaltetzearekin aktibatzen den defentsa mekanismo simple bat, zelularen kalitate kontrol eta moldaerarako ezinbesteko ekintza baizik.

1.3.1 Mitofagia Mekanismoak

1.3.1.1 Mitofagia Legamietan

Legamietan, mitofagia, lisosoma-moduko bakuolo azidikorako mitokondrioen garraioan eta degradazioan oinarritzen da. Legamiek, nitrogenoaren gabezia eta rapamizinarekin egindako tratamendua bezalako egoera askoren aurrean mitofagia aktibatzen dute (Bhatia-Kiššová *eta* Camougrand, 2010). Mitofagia, autofagia arruntaren bidezidorrarekiko ezberdinak diren eta estuki erregulatuak dauden mekanismoen bidez gauzatzen dela erakutsi da. Mitokondrioaren kanpo-mintzean (OMM, ingelesetik *outer mitochondrial membrane*) aurkitzen den Uth1p (ingelesetik *probable secreted beta-glucosidase UTH1*) bezalako proteinak baldintza konkretu

batzuetan mitofagia zuzenean erregulatzen zutela frogatutako lehenenak izan ziren (Kiššová *eta lank.*, 2004).

Ondorengoko ikerketa genetikoen bidez, mitofagiako ezinbestekoa den beste OMMko proteina bat, Atg32, identifikatu zen (Kanki *eta lank.*, 2009; Okamoto *eta lank.*, 2009; Sakakibara *eta lank.*, 2015). Mitokondrio akasduak autofagosomara eramateko, Atg32, autofagosoman dagoen Atg8rekin zuzenean lotzen da eta aurretik identifikatutako Atg11 egokitzailerik autofagiko hautakorrarekin ere elkarreragiten du (Kanki *eta Klionsky*, 2008). Interesgarria suerta daiteke, peroxisometan Atg32aren domeinu zitosolikoaren adierazpenak, pexofagia bidezko organulu hauen degradazioa eragiten duela (Kondo-Okamoto *eta lank.*, 2012). Honek, Atg32ak berak organuluaren autofagia aktibatu dezakeela iradokitzen du. Duela gutxi, proteina-aldamio garrantzitsua den eta Atg32rekin elkarreragiten duen Atg11k, mitokondrioaren fisiomakinaria mitokondriora errekrutatzen duela erakutsi da, horrela mitofagia erraztuz (Mao *eta lank.*, 2013)

1.3.1.2 Eritropoesian Zeharreko Mitofagia

Ugaztunen globulu gorrien garapenean (eritropoesian), osasuntsuak baina erredundanteak diren mitokondrioen ezabapena mitofagiaren adibide bitxi bat da. Globulu gorri helduetan, mitofagiaren bidezidor desberdin baten bidez, mitokondrioen guztizko ezabapen hautakorra gertatzen da, horrela globulu gorriak mitokondriorik gabe utziz (Kundu *eta lank.*, 2008). Eritroideen diferentziazio terminal arruntan zehar, OMMko NIP3-moduko proteina den NIXaren adierazpena altuagoa da (Aerbajinai *eta lank.*, 2003) eta hau beharrezkoa da mitokondrioak guztiz ezabatzeko (Sandoval *eta lank.*, 2008; Schweers *eta lank.*, 2007). NIXaren gabeziak eritroideen heltze akasduna eta anemia eragiten du saguetan (Sandoval *eta lank.*, 2008). Barde *eta lankideek*, NIXaren adierazpena, mitofagiako gene diren ULK1 *eta* Beclin 1 bezalako geneekin batera, diferentziazioan zehar proteina- *eta* RNA-bidezko kontrol transkripzionalen bidez erregulatzen dela erakutsi zuten (Barde *eta lank.*, 2013). NIXaren adierazpen altuak, lehenengo kofaktore transkripzionala den KRABari lotutako 1-proteina (KAP1, ingelesetik *KRAB associated protein 1*) bidezko NIX lotzen duen mikroRNAREN errepresioa behar du. Beraz, mikroRNA espezifikoaren gainadierazpenak edo KAP1aren *knockout*-ak, mitofagiaren inhibizioa *eta* anemia larria eragiten ditu saguetan (Barde *eta lank.*, 2013).

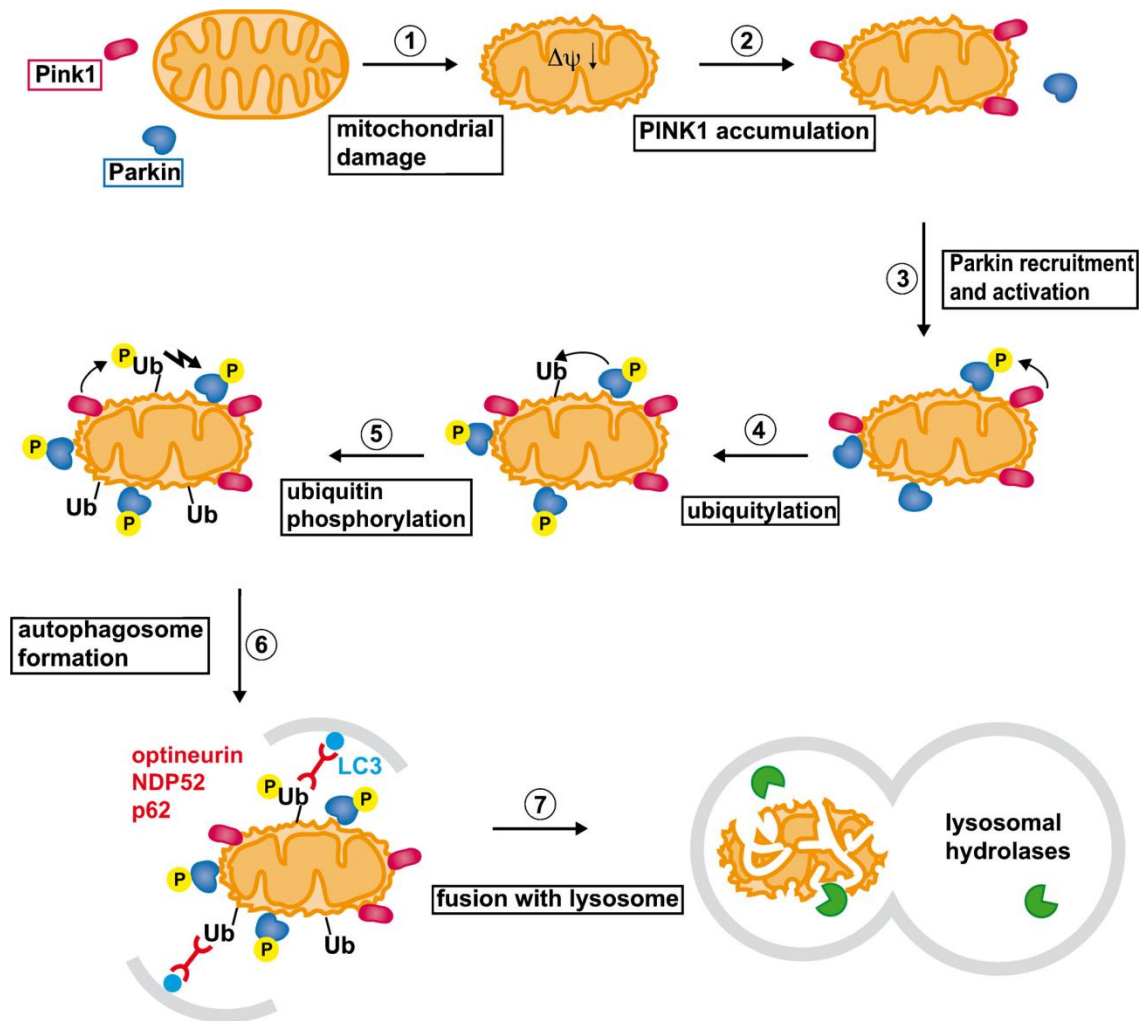
Legamien Atg32aren antzera, NIXek autofagosomaren bidezko mitokondrioen ezabapenean hautakortasuna esleitzen du. NIXek zuzenean, bere LIR motiboaren bidez, autofagosomako markatzaile diren LC3 eta GABARAP proteinek in elkarreragiten du (Novak *eta lank.*, 2010; Yoshii *eta* Mizushima, 2015), horrela mitokondrioren inguruan autofagosomen errekrutamendua eta eraketa ahalbidetuz. Autofagosomako osagaiekin zuzenean elkarreragiten duen arren, NIXek mintz mitokondrialaren depolarizazioaren eta ROSen eraketaren bidez ere mitofagia erregula dezake (Ding *eta lank.*, 2010). NIXek erregulatutako ROS eraketaren igoerak, mTOR inhibizioaren bidez autofagosomen eraketa areagotuz, mitofagiaren ezinbesteko pausua izango litzateke (Ding *eta lank.*, 2010).

1.3.1.3 PARK2 bidezko Mitofagia

Mutazioek E3-ubikitina ligasa den PARK2 (ingelesetik *E3 ubiquitin-protein ligase parkin*) proteinan (Kitada *eta lank.*, 1998) eta PTEN-eragindako ustezko 1-kinasan (PINK1, ingelesetik *PTEN-induced putative kinase 1*) (Valente *eta lank.*, 2004), Parkinson-en gaixotasunaren (PD, ingelesetik *Parkinson disease*) forma autosomiko errezesiboak sortzen dituzte. Mutazioak bi proteina hauek kodetzen dituzten geneetan, gaixotasun neurodegeneratibo familiarren arrisku faktore gisa identifikatu dira. Dena den, hasieran batean ez zegoen argi gaixotasun hauetan bi proteina hauek nola eragiten zuten. *In vivo* egindako ikerketek mutazio hauek funtzio mitokondrial ezegokia eragiten zutela erakutsi zutenean, arreta puntua mitokondrioren kalitate kontrolko eginkizun posible batean jarri zen (Gautier *eta lank.*, 2008; Hoepken *eta lank.*, 2007). PINK1, PARK2rekin genetikoki erlazionatzen zuten lehenengo ikerketak kontuan hartuta (Clark *eta lank.*, 2006; Exner *eta lank.*, 2007; Park *eta lank.*, 2006), oraindik ez dago argi mutazioek proteina hauetan nola eragiten duten mitokondrio akasdunen pilaketa. Narendra *eta* lankideek honi erantzun bat eman zioten aurkitu zutenean PARK2k, mitofagiaren bidez degradatuak izateko helburuarekin, normalean kaltetutako mitokondrioak lotzen zituela (Lazarou *eta lank.*, 2015; Narendra *eta lank.*, 2008; Rüb *eta lank.*, 2016). Fluoreszenteki markatutako PARK2 adierazten zuten zelula bizien irudikapenek, mitokondrio kaltetuetarako PARK2 zitosolikoaren errekrutatze dinamikoa erakutsi zuten. Ondoren, PARK2rekin inguratutako mitokondrio hauek degradatzeko autofagosomen barruan bahituak eta lisosometara garraiatuak dira. Interesgarria suertatzen da, PARK2 gainadierazten duten hainbat zelulek, depolarizazio mitokondrialak eragiten duten farmakoekin egindako tratamenduen bidez eragindako

mitokondrioen kalte orokorraren ostean, beraien mitokondrioen populazio osoa garbi dezaketela.

Mitokondrio osasuntsu batean, PINK1 inportatu eta mitokondrioaren barne-mintzan (IMM, ingelesetik *inner mitochondrial membrane*) azkar ezabatzen da presenilinari lotutako erronboide-moduko proteina (PARL, ingelesetik *presenilin-associated rhomboid-like protein*) proteasaren bidezko prozesamendu proteolitiko konstitutiboaren bidez (Deas *eta lank.*, 2011; Greene *eta lank.*, 2012; Jin *eta lank.*, 2010). Barneko mintz potentzialaren ($\Delta\Psi$) – ATParen eraketarako eta proteina mitokondrialen inportaziorako beharrezkoa dena – disipazioaren ostean, PINK1 OMMan kokatzen da bere bikotea den PARK2 erakartzeko (Lazarou *eta lank.*, 2015; Matsuda *eta lank.*, 2010; Narendra *eta lank.*, 2010a) **(1.24 Irudia)**. Mitokondrioaren mintz potentzialak PINK1 proteinaren lokalizazioan, prozesamenduan eta agian egonkortasunean ere eragina duen arren (Jin *eta lank.*, 2010), PINK1aren inportazio erreazioaren mintz potentzialarekiko menpekotasuna ez da erabatekoa, IMM edo matrizerako bidezidor kanonikoa jarraitzen duten beste pre-proteinetan bezala (Becker *eta lank.*, 2012). Azken honen, eta beste ikerketa batzuen emaitzek, barneko mintz potentzial baten aurrean, PINK1 polipeptidoa IMMAn partzialki txertatzen dela iradokitzen dute. Hau kanpo-mintzeko translokasa (TOM, ingelesetik *translocase of the outer membrane*) eta barne-mintzeko 23-translokasa (TIM23, ingelesetik *translocase of the inner membrane*) konplexuen bidezkoa da (Rüb *eta lank.*, 2016 artikuluan berrikusia). PINK1 eta PARK2ren arteko elkarrekintza beharrezkoa da PARK2ren fosforilaziorako eta bere buruarekin lotzeko. Hau bere ubikitina ligasa aktibitaterako garrantzitsua dela dirudi (Kane *eta lank.*, 2014; Kondapalli *eta lank.*, 2012; Lazarou *eta lank.*, 2012; Shiba-Fukushima *eta lank.*, 2012). Aipatzekoa da, PARK2ren aktibazioaren testuinguruan, PINK1ek ez duela soilik PARK2 fosforilatzen baita ubikitina ere (Kane *eta lank.*, 2014; Kazlauskaite *eta lank.*, 2014; Koyano *eta lank.*, 2014).



1.24 Irudia. Gaur egungo PINK1/PARK2en bidezko mitofagiaren eredia. Baldintza arruntetan, PINK1aren mailak oso baxuak dira. Estres mitokondrialak, mitokondrioaren $\Delta\psi$ jaitsiera edo galerarekin batera eman daitezkeen mitokondrioen kaltetzea eragingo luke (1). $\Delta\psi$ -aren faltan, PINK1 OMMan piltzen da (2). PINK1ek gehienetan zitosolikoa den PARK2 E3 ubikitina ligasa erakarri eta aktibatzen du, PARK2ren Ser 65 fosforilazioaren bidez (3). PARK2k ubikitina (Ub) zenbait OMMko proteinekin konjugatzen du (4). PINK1ek OMMko proteinei lotutako Ub fosforilatzen du. Eraturako fosfo-Ubk PARK2 are gehiago aktibatzen du (5). Bai Ub zein LC3 proteina autofagikoa (urdin argiz) lotzen dituzten proteina-egokitzailleek (gorriz), organuluaren bahiketa gauzaten dute mintz autofagosomiko baten barruan (lerro grisak) (6). Ondoren, autofagosoma lisosoma batekin fusionatzen da bere eduki guztia hidrolasa lisosomalen bidez degradatzeko (berdez; 7). (Rüb *eta lank.*, 2016-etik hartua).

PARK2 geneak kodetzen duen PARK2 proteina zitosolikoa, E3 ubikitina-proteina ligasa bat da (Winklhofer, 2014). Bereizmen handiko PARK2ren egitura kristalinoek, baldintza arruntetan entzima bere burua inhibizio egoera batean mantentzen duela erakutsi dute (Riley *eta lank.*, 2013; Trempe *eta Fon*, 2013; Wauer *eta Komander*, 2013). Bere errekrutatze mitokondrial eta aktibazioaren ostean, PARK2k OMMko hainbat iturri inespezifiko batean ubikitinatzen dituela uste da, horien artean daude voltai menpeko kanal anionikoa (VDAC, ingelesezik *voltage dependent anion channel*) (Geisler *eta lank.*, 2010), mitofusina 1 + 2 (Mfn1/2; fusio

mitokondriala gauzatzen duten GTPasak) (Gegg *eta lank.*, 2010; Tanaka *eta lank.*, 2010), eta garraio mitokondrialeko proteina-egokitzaila den Rho GTPasa mitokondriala (Miro, ingelesetik *mitochondrial Rho GTPase*), zeinak kinesina motore proteinak mitokondrioaren gainazalera ainguratzen dituen (Glater *eta lank.*, 2006; Wang *eta lank.*, 2011b). Zenbait itu hauen ubikitinazio eta degradazio proteasomikoak, erreakzio mitofagiko gehiago aktibatzen ditu organulu osoa markatuz autofagiaren bidezko ezabapenerako. Isolatze-mintzaren eraketa kaltetuta dagoen mitokondrioaren inguruan, hartzaile autofagikoen bidezkoa da. Hauek, beraien ubikitina lotzeko domeinuaren bidez, proteina ubikitinizatueta eta, LIR baten bidez, autofagosomen mintzetan dagoen LC3ra lotzeko gai dira (Pankiv *eta lank.* 2007). Gaur egun arte, PINK1/PARK2en bidezko mitofagian eginkizunen bat daukaten hartzaile desberdinak identifikatuak izan dira (Pickrell *eta Youle*, 2015; Yoshii *eta Mizushima*, 2015), horien artean aurkitzen dira SQSTM1/p62 (Geisler *eta lank.*, 2010), NBR1 (Kirkin *eta lank.*, 2009a), NDP52 (Lazarou *eta lank.*, 2015) eta optineurina (Wong *eta Holzbaur*, 2014).

Mitokondrio ez funtzionalen bereizgarritasun nagusia arnasketa kateak eratzen duen barne $\Delta\psi$ -aren galera da. Izan ere, zelulen tratamendua arnas katearen banaketa (ingelesez *uncoupling*) eragiten duen karbonil zianida m-klorofenil hidrazina (CCCP, ingelesetik *carbonyl cyanide m-chlorophenyl hydrazine*) protonoforoarekin normalean mitokondrioen disfuntzioa eragiteko erabiltzen da. Zeluletara CCCP gehitu eta 3 orduko inkubazioaren ostean, PINK1 endogenoaren mailak ia detektaezinak dira eta 12-16 orduren buruan maximo batera iristen dira. Baldintza hauen menpean, soilik luzera osoa duen polipeptidoa beha daiteke, eta PINK1 molekula guztiak mitokondrioekin ko-purifikatzen dira. Desakoplatzailea kendu ostean, PINK1 mailak erlatiboki azkar oso maila baxuetara bueltatzen dira (Matsuda *eta lank.*, 2010; Narendra *eta lank.*, 2010a). PINK1 potentzial ezegokiko mitokondrioetan pilatzeak, PINK1 kalte mitokondrialaren sentzore garrantzitsu bat dela ere ondorioztatzen garamatza (Rüb *eta lank.*, 2016 artikuluan berrikusia). CCCPrekin tratatutako giza zeluletan, $\Delta\psi$ -ren galeraren ondorioz eta mitokondrioaren autofagiaren bidezko ezabapena gauzatzeko, PARK2 zitosoletik mitokondrio kaltetueta ere translokatzeko zela ikusi zen (Narendra *eta lank.*, 2008).

Mitokondrioetan PINK1en pilaketak mitokondrioen beste akats batzuekin ere erlazionatua izan da, adibidez, ROS maila altuak (Priyadarshini *eta lank.*, 2013) edo tolestu gabeko proteinen pilaketak matrize mitokondrialean (Jin *eta Youle*, 2013). Hala ere, ziurrenik ROS tratamendua, estres proteotoxikoa, edo beste edozein motako kalte mitokondrialak, modu ez zuzen batean, CCCP desakoplatzaileak eragiten duen $\Delta\psi$ -ren antzeko galera bat eragingo lukete.

Ikerketa batek proposatu duenez, NIX garrantzitsua da mitokondrioen depolarizazio goiztiarrerako eta, PARK2 gainadierazten duten zeluletan, CCCPren tratamenduari aktibatzen den mitofagian zehar eginkizun bikoitza dauka (Ding *eta lank.*, 2010). NIXari lotutako depolarizazioak PARK2 mitokondriora errekrutatzea eragiten du, horrela p62 bidezko mitofagia ahalbidetuz (Ding *eta lank.*, 2010). Gainera, NIXek superoxidoen askapena areagotuz eta mTOR aktibitatea partzialki inhibituz, autofagiaren aktibazioan parte hartzen duela erakutsi da (Ding *eta lank.*, 2010). Eraitza hauek, NIXek mitofagian daukan funtzioa ez dela soilik zelula eritrikoetara mugatzen, eta NIXek PARK2ren bidezidor mitofagiko hautakorra baino lehenago mitofagian parte hartu dezakeela iradokitzen dute.

PARK2 ez da mitofagia gidatzen duen ubiquitina ligasa bakarra. NFkBren aktibatzailea den 1-ubikitina ligasa mitokondrialak (Mul1, ingelesetik *mitochondrial ubiquitin ligase activator of NFkB 1*) muskulu eskeletikoaren atrofian zehar mitofagia areagotzen du. Mul1en inhibizioak mitokondrio kopurua mantentzen du eta muskuluen atrofia eragiten duten estimuluean dauden saguetan muskuluen atrofia partzialki saihesten du (Lokireddy *eta lank.*, 2012). Azkenik, 78-glikoproteina (gp78), PARK2rekiko modu independente batean depolarizazioak aktibatutako mitofagia bideratzen duen duela gutxi identifikatutako beste E3-ubikitina ligasa bat da (Fu *eta lank.*, 2013). Interesgarria suerta daiteke, gp78 ERri asoziatua dagoela eta, mitokondrioaren depolarizazioaren ostean, bere ubiquitinazio aktibitateak LC3 erakartzen duela ERren gertutasunean dauden mitokondrioak ezabatzeko (Fu *eta lank.*, 2013).

1.3.2 Mitofagosomen Jatorria eta Aukeraketa

Mitofagia prozesuaren funtsezko pausuetako bat autofagosomaren bidez ezabatu beharreko mitokondrioen ezagutzea da. Autofagosomak kaltetutako mitokondrioetara zehazki nola errekrutatzen diren oraindik argitu gabe dago (Randow *eta Youle*, 2014). p62 proteina-egokitzailak bai ubiquitina zein LC3 lotu ditzake eta PARK2 bidez poli-ubikitinizatuak izan diren mitokondrioetan pilatzen dela ikusi da (Geisler *eta lank.*, 2010; Narendra *eta lank.*, 2010b). Dena den, p62k mitokondrioen pilaketan eginkizunen bat daukala dirudien arren (Narendra *eta lank.*, 2010b), p62 autofagosomak mitokondrioetan errekrutatzeke (edo alderantziz) ezinbestekoa den edo ez oraindik ez dago argi (Geisler *eta lank.*, 2010; Narendra *eta lank.*, 2010b). p62z gain beste bi proteina-egokitzailak autofagiko garrantzitsuak identifikatu dira PARK2ren substratu gisa mitokondrioen depolarizazioa gertatu ondoren: Tax1 lotzen duen 1-

proteina (TAX1BP1, ingelesetik *Tax1 binding protein 1*) (Newman *eta lank.*, 2012) eta kaltzioa-lotzeko eta *coiled-coil* domeinuak dauzkan 2-proteina (CALCOCO2, ingelesetik *calcium-binding and coiled-coil domain-containing protein 2*) (von Muhlinen *eta lank.*, 2012). Interesgarriki, dagoeneko erakutsi da CALCOCO2/NDP52k, LIR motibo ez-kanoniko baten bidezko LC3Crekin emandako elkarrekintza espezifikoaren bidez, bakterio inbaditzaileak autofagiarako aukeratzen dituela (von Muhlinen *eta lank.*, 2012; Randow *eta Youle*, 2014).

Legamietan Atg32rekin eta ugaztunen eritrozitoetan NIXekin aipatu den bezala, mitofagian autofagosomaren errekrutatzea ez da beti proteina egokitzailerik zitosolikoen menpe egongo. Izan ere, aurretik aipatu den bezala, FUNDC1 LC3ra lotzen da bere LIR motiboaren bidez eta elkarrekintza hau hipoxia baldintzetan FUNDC1en desfosforilazioaren ostean areagotzen da (Liu *eta lank.*, 2012). Orokorrean, hipoxiak aktibatutako mitofagiak beste mekanismo bat darama aurrera, zeinetan OMMko eta autofagosomako proteinen elkarrekintza erregulatuen eta zuzenen bidez mitokondrioak mitofagiarako aukeratuak diren.

Degradatu beharreko materiala aukeratzeko ez dira soilik proteina hartzaileak erabiltzen, zenbait lipidok ere giza makinaria autofagikoa mitokondrioetara bideratzeko hartzaile gisa dihardute. N-estearoil zeramida (Cer18), lipidatua eta autofagosomari lotuta dagoen LC3 (LC3-II) mitokondrioen mintzera ainguratzeko hartzaile gisa jokatzen duela proposatu da. Hau Cer18 eta LC3-II arteko elkarrekintza zuzenaren bidez gauzatuko litzateke (Sentelle *eta lank.*, 2012). Kardiopilinen kanporaketa mitokondrioaren kanpo-mintzara Atg8aren giza ortologoa den LC3rentzako seinalea dela proposatu da ere (Chu *eta lank.*, 2013; Maguire *eta lank.*, 2016). LC3k bai mitokondrioen ezagutzean zein autofagosomaren eraketan lagunduko luke, azkenik kaltetutako mitokondrioen ezabapena eraginez. Gai hau 1.4 Atalean sakontasun handiagorekin azaldua izango da.

Autofagosomaren eraketaren inguruko ikerketa gehienak autofagia mota ez-hautakorretan oinarritzen dira eta ez mitofagian *per se*. Duela gutxi artikulu batek, isolatze-mintz autofagikoa depolarizatuta dauden mitokondrioetan *de novo* eratu daitekeela proposatu du (Itakura *eta lank.*, 2012). LC3aren errekrutamenduaren aurretik, PARK2 bidezko mitofagiaren hasierako urratsetan ATG9A proteina autofagikoa eta autofagia aktibatzen duen ULK1 kinasa konplexuak kaltetutako mitokondrioetara erakartzen direla aurkitu zuten (Itakura *eta lank.*, 2012). Ikerketa lan gehiago beharrezkoa da mitokondrioen ezabapenerako eratzen diren autofagosomak, elikagaien gabezia eratzten diren autofagosomak ezberdinak diren jakiteko.

1.3.3 Dinamika Mitokondrialen bidezko Mitofagiaren Erregulazioa

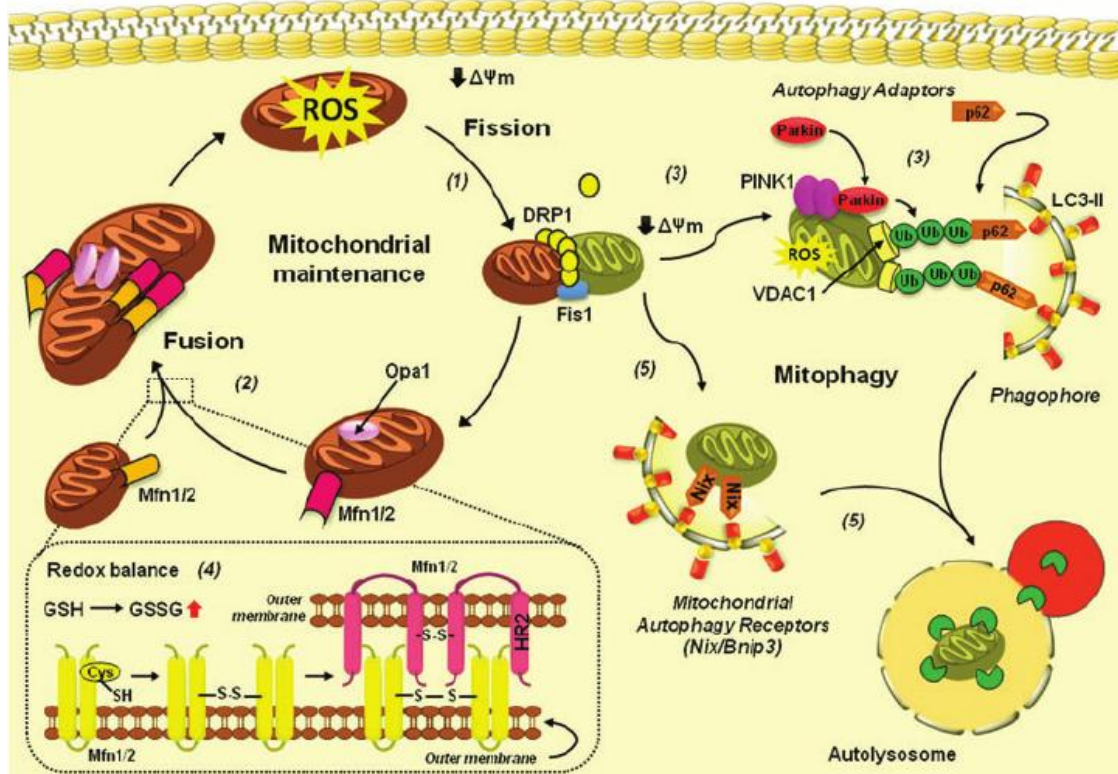
Mitokondrioak fusio, fisio eta zelulan zeharreko mugimenduaren bidez etengabe itxuraz aldatzen diren organulu oso dinamikoak dira. Dinamika hauek ezinbestekoak dira zelulen bizitza eta heriotzerako eta mitokondrioekin lotutako bidezidor zelular askorekin erlazionatuak izan dira, adibidez, kaltzioaren seinalizazioa, apoptosia eta ziklo zelularra, eta baita arazo neurologiko eta gaixotasun metabolikoekin ere. Gainera, fusio mitokondrialaren oztopaketa eta fisioaren sustapena, mitofagiaren aktibazioan ohikoak diren gertaerak dirudite (Hsu eta Shi, 2016; Ni *eta lank.*, 2015).

1.3.3.1 Mitofagian Zeharreko Fisio Mitokondrialaren Eginkizuna

Fisio mitokondrial, dinaminarekin lotuta dagoen Drp1 proteinak aurrera eramaten du (Smirnova *eta lank.*, 2001) eta OMMko proteinek diren fisio mitokondrialaren 1-proteina (Fis1, ingelesetik *mitochondrial fission 1 protein*) (James *eta lank.*, 2003) eta fisio mitokondrialaren faktorea (Mff, ingelesetik *mitochondrial fission factor*) (Otera *eta lank.*, 2010) erregulatzen dute (**1.25 Irudia**). Fis1 eta Mff ez bezala, Drp1 GTPasa, fisio mitokondrialeko guneetan era oso erregulatuan kokatzen den proteina zitoplasmatico bat da. GTParen hidrolisiak fisioa eragin baino lehen, Drp1aren aktibitatea Mff-k markatutako zatiketa-guneetan gertatzen den bere buruaren oligomerizazioaren menpekoa izango litzateke (Otera *eta lank.*, 2013). Drp1aren errekrutamenduaren aurretik, banaketa mitokondrialeko guneak izango direnak ER-tubulu bidezko konstriktzioaren bidez ere identifikatuak izan daitezke (Friedman *eta lank.*, 2011). Drp1 bidezko fisioak mitofagian daukan eginkizunaz gain, Drp1ek apoptosian eta zatiketa zelularrean funtzio garrantzitsuak betetzen ditu mitokondrioen fragmentazioa erregulatuz (Otera *eta lank.*, 2013).

Ugaztunen hainbat zelula motetan, mitofagia gertatzeko lehenengo fisio mitokondrial beharrezkoa dela onartzen da. Beste batzuek diotenaren arabera, ulergarria da 5 μm -tik gorako mitokondrioak 0.5 μm -ko diametroa duten autofagosomen bidez irentsiak izan baino lehen zatikatuak izatea (Gomes eta Scorrano, 2011). Twig eta lankideek, fisioak mintz potentzial baxua eta OPA1 maila murriztuak dituzten mitokondrio zatikatuak sor ditzakela frogatu zuten lehenak izan ziren (Twig *eta lank.*, 2008) (**1.25 Irudia**). Mitokondrio hauek, beraz, ez dute sare mitokondrial osasuntsuarekin berriro fusionatzeko gaitasunik (Youle eta van der Bliek, 2012). Sare mitokondrialetik kanporatuak izan ostean, mitokondrioak azkenean mitofagia bidez ezabatuak dira. Drp1 (K38A) dominante negatiboaren adierazpenak eragindako Drp1-bidezko fisioaren inhibizioak mitofagia inhibitzen zuela ikusi zen (Twig *eta lank.*, 2008), eta hau Drp1^{-/-} MEFetan PARK2 bidezko mitofagia gertatzen ez dela

erakusten duten ondorengo emaitzekin bat dator (Tanaka *eta lank.*, 2010). Duela oso gutxi, PINK1ek, normalean Drp1 inhibitzen duen mitokondrioetako PKAri eraginez fisio erreakzioa areagotzeko gaitasuna duela erakutsi da (Pryde *eta lank.*, 2016).



1.25 Irudia. Mitokondrioen fusioa, fisioa eta mitofagia. (1) Fisioak Fis1en antolaketa lokala eta DRP1 GTPasaren errekrutamentua behar du, mintzaren zatiketa eragingo duen fisio makinariaren mihiztadurarako. (2) Fusioa, kanpo-mintzean Mfn1/2 dinamina GTPasen bidez eta barne-mintzean atrofia optikoaren 1-proteina (Opa1, ingelesetik *optic atrophy 1 protein*)-ren bidez gauzatzen da, zeintzuek bata bestearen ondoan dauden mitokondrioak lotzen dituzten. (3) Fisioaren ostean, mitokondrioaren mintz potentzial murriztuak PINK1 eta PARK2 mitokondriora translokatzera eragiten du, non mintz mitokondrialeko proteinen ubiquitinazioa sustatzen den, adibidez, VDAC1rena, zeinak mitokondriora lotuko den p62 hartzaille autofagikoa erakartzen duen. (4) Glutation disulfuroaren (GSSG, ingelesetik *glutathione disulfide*) pilaketak Mfn1/2en zisteinen oxidazioa gauzatzen duela frogatu da, honek mintz en arteko fusioa errazteko Mfn1/2en arteko lotura eragiten duelarik. (5) Nix-ren menpeko autofagiak garapenean zeharreko mitokondrio "osasuntsuen" ezabaketan parte hartzen du. (Navarro-Yepes *eta lank.*, 2014-etik hartua).

Jakina da, fisio mitokondrial, fusiorako gaitasun murriztuaren ondorioz erraztua izan daitekeela, horrela mitokondrioen birziklapenaren kontrol dinamiko baterako aukera gehiago izanez. *Drosophila* eta ugaztun zeluletan, PARK2 bidezko mitofagian, PARK2ren translokazioak mitokondrio depolarizatuera Mfn-en ubiquitinazioa abiarazten du (Gegg *eta lank.*, 2010; Rana *eta lank.*, 2013; Ziviani *eta lank.*, 2010). PARK2 bidezko Mfn-en ubiquitinazioak, proteasomen menpeko beraien

degradaziora darama (Tanaka *eta lank.*, 2010), mitokondrio ez funtzionalak gainontzeko mitokondrioen saretik are gehiago isolatuz. Mfn-en proteasomen bidezko degradazioak II. motako AAA+ ATPasa den balosinadun proteinarene (VCP/p97, ingelesetik *type II AAA+ ATPase valosin-containing protein*) laguntza behar du (Tanaka *eta lank.*, 2010). OMMko proteinen PARK2 bidezko ubikitazioak, VCP mitokondrioetara erakartzea eragiten duela frogatu da (Kim *eta lank.*, 2013). Mutazioek VCPan, hainbat gaixotasun bateratu ditzakeen “gaixotasun multisistemiko degeneratiboa” eragiten dute. Gaixotasun horien artean aurkitzen dira albo esklerosi amiotrofikoa, Parkinson-en gaixotasuna eta hezurretako Paget-en gaixotasuna (Abramzon *eta lank.*, 2012; Spina *eta lank.*, 2013; Watts *eta lank.*, 2004). Gaixotasunekin lotutako VCPren forma mutatuaren gainadierazpenak, MEFetan PARK2 bidezko mitofagia saihesten du. Honek, VCPren forma mutatuaren eta Mfn-en iraunkortasunaren bidezko PINK1/PARK2 bidezidorraren blokeoak, gaixotasun multisistemiko degeneratiboa eragingo lukeela iradokitzen du (Kim *eta lank.*, 2013).

1.3.3.2 Fusioak Mitokondrioak Mitofagiatik Babesten ditu

Fusio mitokondrialaren makinaria ezinbesteko 3 proteinatan oinarritzen da: OMMko Mfn1 eta Mfn2 GTPasak (Santel *eta Fuller*, 2001) eta IMMko OPA1 proteina (Misaka *eta lank.*, 2002) (**1.25 Irudia**).

Ikerketa genetikoaren bidez, fusioa mtDNAren mantentzeko beharrezkoa dela (Chen *eta lank.*, 2010) eta gutxienez ATParen sintesi mitokondrialaren areagotzearekin batera gertatzen dela (Tondera *eta lank.*, 2009) frogatu da. Berriki deskribatu denez, elikagaien gabezia zehar mitokondrioak fusionatzen dira eta mitofagia ekiditen dute ATParen eraketa mantentzeko. Horrela, zelulek mantentzeko gabeziak aktibatutako makroautofagian zehar beraien mitokondrioak babes ditzakete sare mitokondrial handi bat mantenduz (Gomes *eta lank.*, 2011a; Rambold *eta lank.*, 2011). Badirudi mitokondrioak elikagaien gabezia zehar mitofagiatik babestuak direla, bai fusioaren inhibizioaren zein fusioaren mantentzeko bidez. Izan ere, mantentzeko gabezia mitofagia aktibatua izango litzateke OPA1 edo Mfn2 bezalako fusio faktoreak faltako balira (Gomes *eta lank.*, 2011a; Rambold *eta lank.*, 2011) eta bestalde, Drp1aren gabeziak, mitokondrioen birziklapena are gehiago eragotziko luke (Gomes *eta lank.*, 2011a).

Aurrerapenak egon diren arren, dinamika mitokondrialen eta mitofagiaren arteko elkargurutzamendua eta honek gaixotasunetan duen eragina guztiz ulertzeko, bide luzea geratzen da oraindik. Elikagaien gabeziak eragiten duen mitokondrioren

luzapena, falta den mantenugai motaren araberakoa da. Honek, dinamika mitokondrialen kontrol estua oraindik ongi zehaztu gabe dauden seinalizazio bidezidor konplexuetan oinarritzen dela iradokitzen du (Gomes *eta lank.*, 2011b). Sare mitokondrialak zelula mota batetik bestera aldatu egiten direla kontuan edukitzea ere garrantzitsua da.

1.4 Lipido Espezifikoen Eginkizuna Autofagian eta Mitofagian

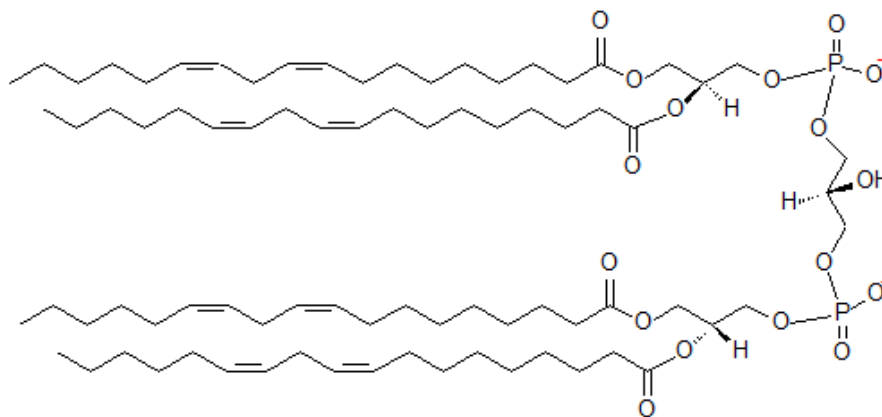
Ugaritzen doazen ebidentziek adierazten dutenez, autofagian zehar lipido batzuk autofagosomaren, autolisosomaren edo OMMaren osagai estruktural hutsak izan ordez, proteina autofagiko espezifikoen funtzioan eragin dezakete, elkarrekintza espezifikoen bidez edota bigeruzatuta lipidikoen ezaugarri fisikoak aldatuz (Salazar *eta lank.*, 2009; Morad *eta Cabot*, 2013; Sentelle *eta lank.*, 2012; Chu *eta lank.*, 2013; Chu *eta lank.*, 2014; Dany *eta Ogretment*, 2015; Li *eta lank.*, 2015; Shatz *eta lank.*, 2016).

1.4.1 Kardiolipina

CLak, bai prokariotoen zein eukariotoen mintzetan, kokapen ezberdinetan baina, aurkitu ziren antzinako fosfolipido mota baten parte dira: bakterioen mintz plasmaticoan osagai gehienekoak dira, baina eukariotoetan ia mitokondrioetan soilik ageri dira (Daum *eta Vance*, 1997; Hoch, 1992; Schlame, 2008). CLaren biosintesirako bidezidorrak ere ezberdinak dira: bakterioetan fosfatidilglizerolezko (PG, ingelesetik *phosphatidylglycerol*) bi molekuletatik biosintetizatzen dira, bestalde eukariotoetan PG batetik eta zitidina difosfato diazilglizerol (CSP-DAG, ingelesetik *cytidine diphosphate diacylglycerol*) batetik sintetizatzen dira (Tian *eta lank.*, 2012). Bakterio eta mitokondrioen CLren espezie molekularrak ere ezberdinak dira: lehenengo kasuan karbono kate laburrak eta aseak edo monoasegabeak diren CL espezieak dira ohikoak, mitokondrioetan berriz, kate luzeagoko eta poliasegabeako CLak dira nagusi (Schlame, 2008).

Eukariotoetan, CL mitokondrioetako glizerofosfolipido bereizgarria da eta zelula osasuntsuetan mitokondrioen hainbat funtzioetan parte hartzen du, horien artean aurkitzen dira organuluaren ultraegitura egokiaren mantenua, metabolismo energetikoa, proteinen inportazio prozesuak, eta dinamika mitokondrialak (Hsu *eta Shi*, 2016; Maguire *eta lank.*, 2016). Gainera, CLak mitofagiaren bidezidor autofagikoan ere parte hartzen du eta hau tesi lan honetan aztertua izan da (ik. 3. eta 4. Kapituluak).

CLaren egitura, bi lipido fosfatidiliko lotuta dauzkan glizerol batean oinarritzen da, beraz, 4 kate aziliko (gantz azido; FA, ingelesetik *fatty acid*) eta negatiboki kargatutako bi fosfato talde. Egitura hau fosfolipido arruntena baino elastikoagoa da, CLk bi karbono kiral eta gehienetan poliasegabeak diren lau kate aziliko dauzkalako, ugaztunetan behintzat (Ardail *eta lank.* 1990). Lau FA hondar guztiak berdinak badira, CL molekula simetrikoak dira; baina ezberdina den FA hondar bat edo gehiago agertzeak molekularen antolaketa simetrikoa desorekatzen du. Bihotza, muskuluak edo gibela bezalako zenbait ehunetan, lau C18:2 FAz osatutako CL simetrikoak dira ugariak (Schlame *eta lank.*, 2005) (**1.26 Irudia**). Mitokondrioen mintzetan dauden CLen egitura dimeriko eta kate aziliko poliasegabeak, egitura lamelar eta kurbadura negatiboaren artean aldatzeko gaitasunaren arduradunak izango litzateke, mitokondrioen barneko gangar tolestuak eraikitzeko adreilu perfektua bilakatuz (Acehan *eta lank.*, 2009; Schlame, 2013; Xu *eta lank.*, 2006).



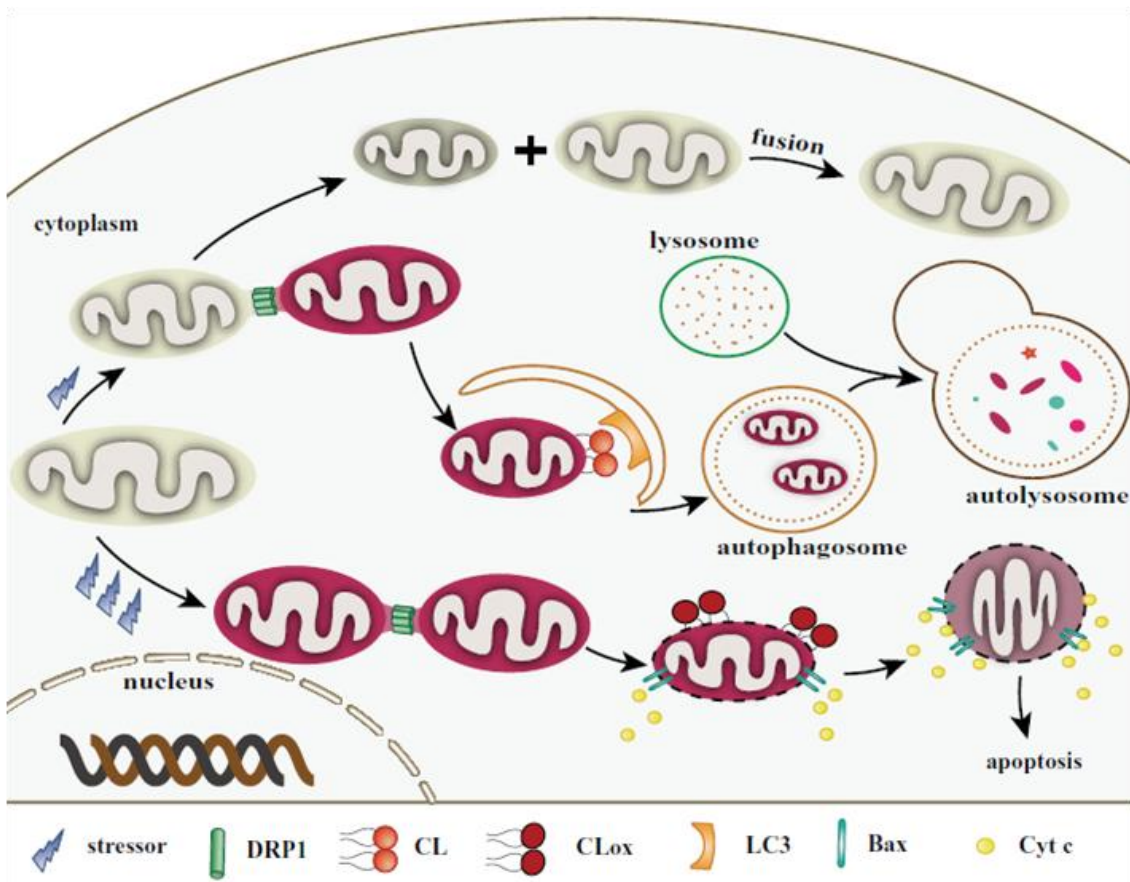
1.26 Irudia. Tetralinoleilcardiolipina (TLCL)-ren egitura (CL 18:2).
(<http://www.lipidhome.co.uk/lipids/complex/dpg/index.htm>-etik egokitua).

Mintz mitokondrialak glizerofosfolipido ugari izateagatik bereizten dira, bestalde esterolak eta esfingolipidoak kantitate txikian ageri dira (Daum *eta Vance*, 1997). OMM *eta IMM*aren arteko lipido konposaketa oso desberdina da (Zinser *eta Daum*, 1995; de Kroon *eta lank.*, 1997). OMM *eta IMM*ko glizerofosfolipido kantitate erlatiboari dagokionez, PC, PE *eta* fosfatidilinositolarentzako (PtdIns, ingelesetik *phosphatidylinositol*) adostasun bat dago. Hala ere, mitokondrioen mintz bakoitzean dagoen CL kantitatea zenbait eztabaiden jatorria izan da. CL gehiena ERTik datorren azido fosfatidikotik (PA, ingelesetik *phosphatidic acid*) sintetizatzen da IMMko matrizearen aldean entzima desberdinen aktibazioaren bidez. CLaren metaketa ERTik IMMaren barneko aldera gertatutako PAren garraioaren *eta* sintetizatu berria den

CLaren bi mintzetan zeharreko birbanaketaren araberakoa da (Osman *eta lank.*, 2011; Claypool *eta* Koehler, 2012; Tamura *eta lank.*, 2013). Gainera, CL sintasaren topografia – CL biosintesiaren azken urratsaren katalizatzailea – CLk IMMMan daukan asimetria baldintzatzen duen faktore nagusietako bat da.

Hasieran, CL ia IMMMan soilik aurkitzen zela uste zen, OMMko lipido kantitate guztiaren batzuekiko \approx %5 izanez. Hala ere, CLaren banaketa mintz mitokondrialetan zehar oso asimetrikoa dela eta mitokondrioaren kalte eta depolarizazioaren ostean asko aldatzen dela frogatu da: CL kantitate nabarmen bat OMMra translokatzeko da (Baile *eta lank.*, 2013; Gonzalez *eta* Gottlieb, 2007). Adibidez, Charleen T. Chu *eta* lankideek, masa espektrometria erabiliz, elektroio garraio kateko I-konplexuaren inhibitzailea den rotenonarekin eta mitofagia aktibatzen duten beste estimulu batzuekin tratatutako arratoien neurona kortikal primarioetan eta SH-SY5Y zeluletan, OMMko CLaren bai kantitatea zein espezieak handitu zirela ikusi zuten. Aipatzekoa da, rotenonaren tratamendu ez hilgarrietan, autofagia gauzatzeko nahikoak zirenak, ez ziren CLren peroxidazioaren produkturik detektatu (Chu *eta lank.*, 2013; Chu *eta lank.*, 2014 artikuluan berrikusia). Gainera, autofagiaren makinariak CL modu eraginkorragoan ezagutzen zuen, mono- eta diliso-CL bezalako CLren metabolitoak edota CLren oxidazioaren produktuak baino (Chu *eta lank.*, 2013). Honek, mitofagia bidezko kaltetutako mitokondrioen ezabapenerako CLaren oxidazioa ez dela beharrezkoa adierazten du (**1.27 Irudia**). Hau, estres oxidatiboa eta lipidoen peroxidazioa, kalte mitokondrial eta mitofagiarekin batera doazela dioten iritsi onartuekin bat ez datorrela dirudi (Kirkland *eta lank.*, 2002). Izan ere, mekanismo pro-apoptotikoetan, CLaren parte-hartzearen ezaugarri espezifiko nagusiak ez du soilik CL OMMMan agertzea behar, baizik eta baita bere oxidazioa ere (Kagan *eta lank.*, 2005).

CLak mintz mitokondrialetan duen lehentasunezko kokapenak ez du soilik IMM OMMrekin konparatuz CLaren banaketa hautakorra kontutan hartzen, baizik eta baita IMMaren kanpo monogeruza barne monogeruzarekiko CLz aberastuta egotearen aukera ere (Gallet *eta lank.*, 1997; Harb *eta lank.*, 1981). CLren birmoldaketaren erreakzio batzuk (Cao *eta lank.*, 2004) ERan gertatu litekeen arren, beraz CLaren mintzan zeharreko translokazioen beharizan fisiologikoarekin (Esposti *eta lank.*, 2001), funtzionamendu egokia daukaten mitokondrioetan, CL soilik IMMMan aurkitzen dela uste da, non barne monogeruzak kanpo monogeruza baino CL kantitate gehiago izango luke (Hovius *eta lank.*, 1990; Krebs *eta lank.*, 1979).



1.27 Irudia. Kardiolipinaren eginkizunak mitofagian eta apoptosian. (1) Estres baxuen eta erdi mailakoan menpean: 1-dinamina-moduko proteina (DNM1L/DRP1, ingelesetik *dynammin-1-like protein*) mitokondrioetan kokatzen da CLari lotuz. Mitokondrioak bi unitate alabetan banatzen dira. Mintz potentzial baxuena daukana ziur aski mitofagia bidez degradatzen da. LC3, kaltetutako mitokondrioak aukeratu litzazke OMMan CLarekin konbinatuz. Mintz potentzial altuena daukana, mitokondrio osasuntsu batekin fusionatuz konponduko litzateke. **(2) Estres apoptotiko/handi batean zehar:** ROS asko eraten dira. CL hidrogeno peroxidoaren laguntzaz (H_2O_2), c-zitokromoaren (Cyt c, ingelesetik *cytochrome c*) bidez oxidatua eta OMMra transferitua da. Mitokondrioak DRP1 eta fisioarekin lotutako beste proteina batzuen bidez ere banatuak izaten dira. tBIDaren (ingelesetik *truncated-BH3 interacting domain death antagonist*) aurrean, Bax (ingelesetik *BCL-2 associated X protein*) oligomerizazioaren bidez, mintz mitokondrialeko poroa sortzen da. Ondoren, Cyt c eta beste faktore apoptotiko batzuek zitosolera kanporatzen dira kaspasen aktibazioa eraginez (Li eta Lank., 2015-etik moldatua).

CLaren kanporaketak IMMaren barne monogeruzatik OMMren gainazalera, gutxienez 3 translokazio behar ditu:

- (i) IMMaren barne monogeruzatik kanpo monogeruzara,
- (ii) IMMaren kanpo monogeruzatik OMMaren barne monogeruzara, eta azkenik
- (iii) OMMren barne monogeruzatik kanpokora.

Fosfolipidoen 3-eskranblasa (PLS3, ingelesetik *phospholipid scramblase-3*), Nm23-H4, mitokondrioaren kreatina kinasa (MtCK, ingelesetik *mitochondrial creatine kinase*) eta tBIDek CL transferituz mitokondrioaren mintzaren berrituraketan laguntzen dute (Kagan *eta lank.*, 2006, 2014). PLS3, mitofagian eta apoptosian zeharreko OMMaren barne monogeruzatik kanpo monogeruzarako CLren translokazioaren arduraduna den entzima mitokondrial bat da (Chu *eta lank.*, 2013; Liu *eta lank.*, 2003; Van *eta lank.*, 2007). tBID, zeina proteina pro-apoptotikoen BH3-domeinu bakarreko azpitaldearen kideetako bat den, OMMko CLari elkartzuz mitokondrioarekin lotzen da (Kim *eta lank.*, 2004; Lutter *eta lank.*, 2000; Sorice *eta lank.*, 2004). PLA2, tBIDren bidez aktibatzen da, monolisokardiolipina (MLCL, ingelesetik *monolysocardiolipin*) eta dilisokardiolipina (DLCL, ingelesetik *dilysocardiolipin*) emanez (Kagan *eta lank.*, 2006). LisoCL eta CL kanpora garraiatzen dituen transmintz garraiatzaile gisa ere joka dezake. Gainera, tBIDek PLS3 gehiago aktibatzen du eta OMMan CL kopurua handitzen duen berrelikadura (ingelesezt *feedback*) ziklo positibo bat eratu dezake (He *eta lank.*, 2007; Kagan *eta lank.*, 2006; Liu *eta lank.*, 2008). MtCK eta Nm23-H4, biak basikoak diren mintz proteina periferikoak dira. MtCK oktamerikoa IMMan CLra eta OMMan VDAC proteinara lotzen da, mintz mitokondrialen zeharreko zubi gisa jokatuz (Epanand *eta lank.*, 2007b). Gainera, mitokondrioaren nukleosido difosfato kinasa den Nm23-H4k ere zubi baten moduan jardun dezake, IMM eta OMM aldi berean lotuz eta CLren transferentzia ahalbidetuz (Epanand *eta lank.*, 2007a; Schlattner *eta lank.*, 2013). Transazilasa nagusia den tafazina proteinak ere CLaren garraioan lagunduko luke. Tafazina IMMaren kanpo monogeruzan eta OMMaren barne mongeruzan aurkitzen da; beraz, mintzen arteko gunean dagoela kontsidera daiteke. *In vitro* soilik bigeruzan eratzten ez duten zenbait CLen kate azilikoak ordezkari ditzake (Schlame, 2013; Schlame *eta lank.*, 2012; Hsu *eta lank.*, 2015).

Aurretik aipatu bezala, Chu *eta lank.*ek, OMMko CL LC3ren hartzaile mitokondrial gisa jarduten duela eta honek mitofagia eragiten duela proposatu zuten (**1.27 Irudia**). Izan ere, elkarrekintza hau ekiditeak rotenonak aktibatutako mitokondrioen garraioa autofagosometara eta lisosometara inhibitzen zuen (Chu *eta lank.*, 2013). LC3-CL bidezidor hau, kalteak aktibatutako mekanismoen aurretik PINK1/PARK2 bidezidorrarekiko ezberdina izango litzateke, izan ere, LC3-CL bidezidorrari ez da mitokondrioen depolarizaziorik gertatzen. Egokitze-molekularren (ingelesezt *molecular docking*) bidez LC3k sistema autofagikoaren bidezko mitokondrioen ezabapenerako garrantzitsuak diren kardiolipina lotzeko guneak dituela proposatu den arren (Chu *eta lank.*, 2013), LC3-CL elkarrekintzaren oinarri molekularra oraindik ez dago guztiz zehaztua. Tesi lan honetan, teknika kuantitatibo biofisikoak eta

zelularrak erabili dira elkarrekintza honen mekanismo molekularrak aztertzeko eta OMMaren lipido bigeruzaren ezaugarri fisikoak aldatuz, eta ez soilik proteina-lipido elkarrekintza zuzen eta espezifiko baten bidez, CLak LC3Baren asoziazio mitokondriala eragiten duen ikertu da. Gainera, LC3B-CL elkarrekintzaren mekanismoak proteina espezifikoak edota Atg8aren giza ortologo guztientzat berdinak diren zehazteko, CL eta GABARAP, GABARAPL1 eta GABARAPL2ren arteko elkarrekintzak ere aztertuak izan dira.

CLak autofagiarekin lotutako beste proteina batzuekin ere elkarreragiten du, adibidez, aurretik aipatu den DRP1rekin, zeinak fisio mitokondrialean laguntzen duen (Frank *eta lank.*, 2001; Smirnova *eta lank.*, 2001) edo immunitatearekin lotutako M-GTPasa (IRGM, ingelesetik *immunity related GTPaseM*)-rekin, zeinak depolarizazio mitokondrialean, fisioan eta horren ondoriozko mitofagian eragiten duen.

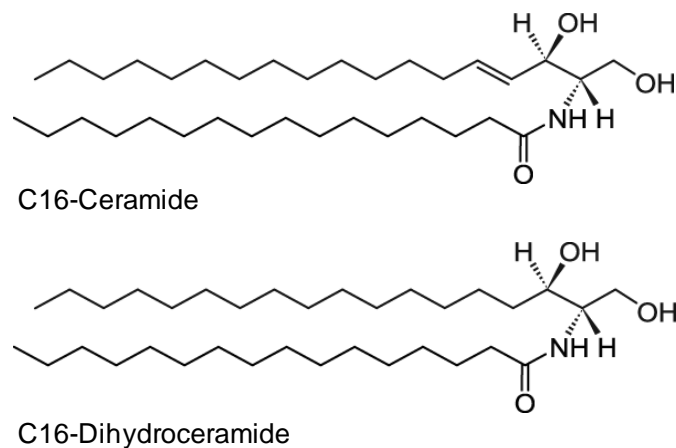
Azkenik, CLak Beclin 1 eta LC3rekin lotuz, autofagosomaren eraketan ere parte hartuko luke. Interesgarria suertatzen da, Beclin 1 proteinek, eboluzioan zehar kontserbatua izan den domeinu (ECD, ingelesetik *evolutionarily conserved domain*) baten bidez, CLan aberastuak dauden mintzekin elkarreragiteko lehentasuna izatea (Huang *eta lank.*, 2012). ECDaren azkeneko partean dauden hiru aminoazido aromatikok lotura gunea izan daitekeen atzamar hidrofobiko bat osatzen dute. ER eta mitokondrioaren fagoforoaren eraketarako mintzen iturri dira (Ktistakis *eta* Tooze, 2016 artikuluan berrikusia). Beclin 1 proteinek mitokondrioarekin lotura estu bat duenez, mintz mitokondriala egitura aurre-autofagosomikoa eratzeko erabiltzen duen lokarria izan daiteke. Mintz mitokondrialek egitura aurre-autofagosomikoaren elongaziorako ere eskuragarri egongo litzateke, izan ere, CLak LC3rekiko lotura estua ageri du eta azken honek berak fagoforoaren hazkuntzan parte hartzen du (Füllgrabe *eta lank.*, 2014).

1.4.2 Zeramidak

Esfingolipidoek, autofagia erregulatzen duten beste molekula talde bat osatzen dute, zeintzuk mintz biologiko ugarritan ageri diren. Horietako batzuk seinalizazio zelularrean bigarren mezulari gisa funtzio garrantzitsua betetzen dute. Zeluletan esfingolipido azpiespezie asko mintzaren egituraren, matrize extrazelularrekiko eta auzoko zelulekiko elkarrekintzetan, garraio besikularrean, eta fagosomak edo autofagosomak bezalako egitura espezializatuen eraketan eragina daukate (Grassmé *eta lank.*, 2007; Schenck *eta lank.*, 2007). Beraz, esfingolipidoak zelulen fisiologian eta minbizia edo Alzheimer bezalako hainbat gaixotasunen patologian zeresan handia

daukatela erakusten ari da (Grassmé *eta* Iank., 2007; Schenck *eta* Iank., 2007). Minbuzian, hainbat esfingolipido mota ohizkoak ez direla aurkitu da eta hauek kanpo ingurunekeo seinaleak zatiketa zelular eta biziraupen bidezidorrekin koordinatuz ditzakete (Ogretmen *eta* Hannun, 2004).

Esfingolipidoen metabolismoa, oso dinamikoa den prozesu bat da, zeinak zeramida, esfingosina *eta* esfingosina 1-fosfatoa (S1P, ingelesez *sphingosine 1-phosphate*) bezalako bigarren mezulariak sortzen dituen (Ogretmen *eta* Hannun, 2004; Spiegel *eta* Milstien, 2003). Atsedean egoeran dauden zelulek gehienetan esfingolipidoen metabolito maila oso baxuak dauzkate. Zeramida, esfingolipidoen metabolismoaren molekula zentrala da, zeinak zelula hazkuntzan, heriotza zelularrean, proliferazioan *eta* estresarekiko erantzunean eginkizunak dauzkan (Spiegel *eta* Milstien, 2003).



1.28 Irudia. C16-zeramida *eta* C16-dihidrozeramidaren egiturak.

(<https://avantilipids.com/>-etik hartua).

Zeramidak (N-azil-esfingosinak), *D-eritro*-esfingosinatik eratutako molekulak dira zeinen C2 amino taldera amida lotura baten bitartez gantz azido bat lotzen den. Zeramida fisiologiko gehienak azilikoa 16 *eta* 26 karbono bitartekoa da *eta* monoasegabea *edo* aseabe izan daiteke (**1.28 Irudia**). Zeramidak, batez ere erretikulu endoplasmatikokoan sintetizatzen dira. Esfingomielinaren biosintesia, berriz, Golgi aparatuan gertatzen da *eta*, neurri txikiago batean, mintz plasmatikokoan ere. Bi esfingomielina eraketa gune hauek garraio besikularraren bidez lotuta daude. Bi modu ezberdinetan sintetiza daitezke:

(i) *de novo* sintesia, serina *eta* palmitoil-CoA kondentsazioaren bidez 3-oxoesfinganina eratuz, azkenik dihidrozeramidaren sorrerara eramanez. Azken pausu

batean, dihidrozeramida oxidatu eta bere 4-5 posizioan *trans* lotura bikoitz bat sartuz, zeramida sortzen da;

(ii) esfingomielinasa azidoak (A-SMase, ingelesetik *acid sphingomyelinase*; konpartimentu azidoetan kokatuak) eta neutroak (mintz plasmaticoan eta mitokondrioetan kokatuak) deituriko esfingomielinaren hidrolisian parte hartzen duten entzimen aktibitatearen bidez sortua (Goñi *eta lank.*, 2012).

A) Zeramidak Bigarren Mezulari gisa

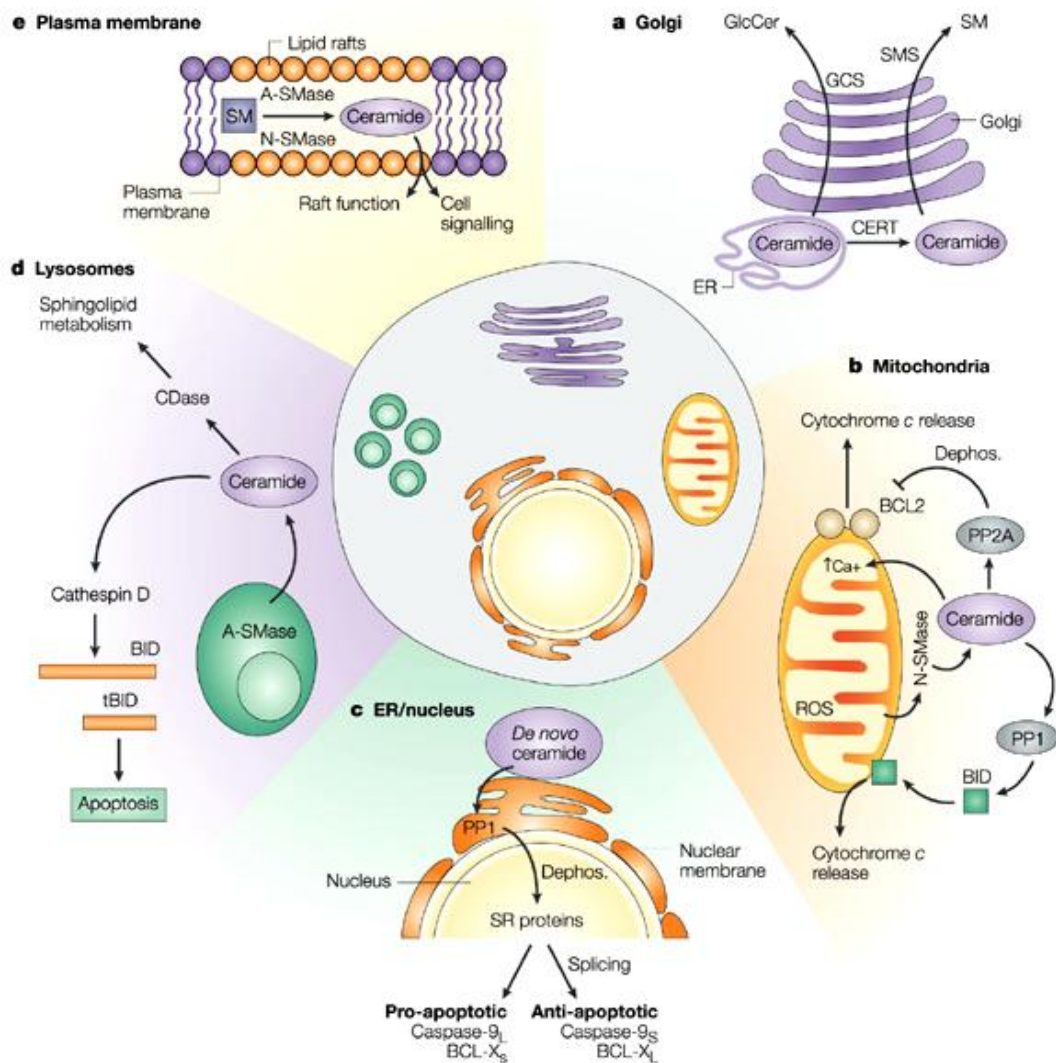
Zeramidak edo beraien produktu metabolikoek, estimulu extrazelularrek aktibatutako efektuak gauzatzen dituztela dirudi, horrela proliferazio zelularra, diferentziazio zelularra, autofagia eta apoptosia bezalako efektu ezberdinetara eramanez.

Gaur egun argi dago hazkuntza zelularra blokeatzen duten eragileek, zeramidaren eraketa aktibatzen dutela, esfingomielinasa bidez, *de novo* sintesiaren bidezidorrak aktibatuz edo zeramidasak bezalako zeramida metabolismoaren beste entzima batzuk erregulatuz ere. Aktibatzaile hauen artean aurkitzen dira $TNF\alpha$ (ingelesetik *tumor necrosis factor*), Fas ligandoa, γ -interferoia, 1-interleukina, oxido nitrikoa, daunoerrubizina, erradiazioa eta giza immunoeskasiaren birusaren (GIB/HIV, ingelesetik *human immunodeficiency virus*) infekzioa. Gainera, ebidentzia argiak iradokitzen du, zeramidak, agente horiek aktibatutako erantzun apoptotikoa eta ziklo zelularraren inhibizioa gauzatzen edo modulatzeko dituela: (i) zeramida pilatzeko gaitasuna duten molekulek, hazkuntzaren inhibizioa edo apoptosia eragiteko gai diren agente gehienak barneratzen dituzte eta ziur aski horietara soilik mugatzen da, (ii) zeramida endogenoaren aldaketak apoptosiaren gauzatze fasea hasi baino lehen (adibidez, kaspasen aktibazioa baino lehen) gertatzen dira, (iii) iragazkorrek diren zeramida analogoen gehikuntzak zenbait zelula lerrotan apoptosia aktibatzen du (Gangoiti *eta lank.*, 2010).

Zeramiden bidez kontrolatuak daudela diruditen molekula ituen identifikazioak, zeramidek seinaleen transdukzioan eginkizun garrantzitsu bat betetzen dutela dioen hipotesia indartzen du (**1.29 Irudia**). Jakina da zeramidek 1- eta 2A-proteina fosfatasak (PP1 eta PP2A, ingelesetik *protein phosphatase 1/2A*) aktibatzen dituztela, C-proteina kinasa alfa ($PKC\alpha$, ingelesetik *protein kinase C alpha*) desfosforilatzen eta inaktibatzen duten proteina fosfatasak, eta BCL-2 (ingelesetik *B-cell CLL/lymphoma 2*) desfosforilatzen dutenak (Ruvolo *eta lank.*, 1999). Gainera, zeramidek, zeramidak aktibatutako proteina kinasa (CAPK, ingelesetik *ceramide-activated protein kinase*) bat

aktibatzen dute, zeinak mitogenoen bidez aktibatzen den proteina kinasen (MAPK, ingelesetik *mitogen-activated protein kinase*) aktibazioa eragin (Zhang *eta lank.*, 1997) eta ziklo zelularra erregulatzen duen. Era berean, jakina da bai zeramidek zein molekula ituek, mitokondrioetan eta apoptosian funtzio garrantzitsua betetzen dutela. Adibidez, isolatutako mitokondrioak zeramidekin tratatzen direnean, mitokondrioaren arnas kateko III. konplexua inhibitzen dela ikusi da (Gudz *eta lank.*, 1997). Horretaz gain, bai isolatutako mitokondrioetan zein zeluletan zeramidek ROS eraketan laguntzen dute (García-Ruiz *eta lank.*, 1997). Gainera, zeramidasak mitokondrial bat identifikatua izan da (El Bawab *eta lank.*, 2000), zeramiden metabolismorako bidezidor mitokondrial bat dagoela sendoki iradokitzen duena, eta mitokondrioen funtzionamenduan eta batez ere apoptosiaren erregulazioan eginkizun garrantzitsua edukiko lukeena.

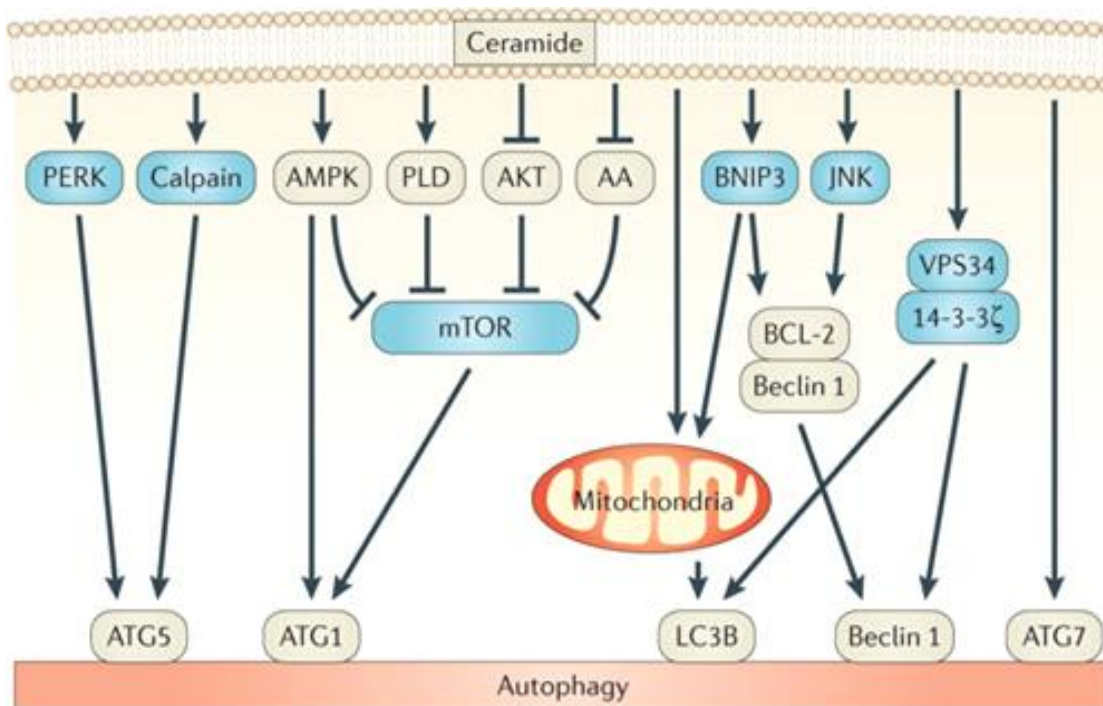
Zeramidatik zeramida-1-fosfatoa, esfingosina eta S1P sor daitezke. Zeramida desazetilatu da esfingosina sortzeko, zeina esfingosina kinasen (SphK, ingelesetik *sphingosine kinase*) bidez fosforilatua den S1P sortzeko (Spiegel *eta Milstien*, 2003). Zelulek zeramida, esfingosina eta S1P mailen arteko oreka dinamiko bat mantentzen dute. Gaur egun onartua da, zeramida, esfingosina eta S1P estres zelularren aurreko erantzunetan kontrako efektuak dauzkatela (Spiegel *eta Milstien*, 2003; Ogretmen *eta Hannun*, 2004). Aurretik aipatu den bezala, hainbat zitokina, hazkuntza-faktore, farmako eta estresak, *de novo* sintesiaren edota esfingomielinaren hidrolisiaren bidez zeramida endogenoaren mailen igoera eragiten dute (Goñi *eta lank.*, 2012; Salazar *eta lank.*, 2009) eta zeramida mailen igoera honek hazkuntza zelularren gelditzea eta apoptosia eragingo lituzke (Hannun *eta Luberto*, 2000). Adibidez, zenbait minbiziaren aurkako farmakok apoptosia aktibatzen dute zeramidaren *de novo* sintesia areagotuz (Garzotto *eta lank.*, 1998, 1999). Gainera, beste hainbat kanpo estimulak, batez ere hazkuntza eta biziraupen faktoreek, SphK aktibatuz, S1P mailen igoera eta aldibereko zeramida mailen jaitsiera eragiten dute. Beraz, zeramida, esfingosina eta S1P arteko oreka dinamikoak zelulak bizi edo hiltzen diren zehazten lagunduko luke (Spiegel *eta Milstien*, 2003). Honek "erreostato esfingolipidiko" (ingeleseztan "*sphingolipid rheostat*") baten proposamena ekarri zuen, zeinaren arabera metabolito antagoniko hauen kantitate erlatiboa zelularen patua zehazten funtsezkoa den (Cuvillier *eta lank.*, 1996).



1.29 Irdia. Zeramidaren seinalizazioaren bidezidor konpartimentalizatuak. ERan *de novo* sortutako zeramida COL4A3BP/CERT (ingelesetik *collagen type IV α -3-binding protein*) bidez Golgi mintzetara (a) garraiatua da SMaren sintesirako. Zeramida glukosilzeramida (GlcCer, ingelesetik *glucosylceramide*) ere bilaka daiteke Golgiko glukosilzeramida sintasa (GCS, ingelesetik *glucosylceramide synthase*)-ren bidez, baina hau ez da CERT-menpekoa. Mitokondrioetan (b), zeramida esfingomielinasa neutroaren (N-SMase, ingelesetik *neutral sphingomyelinase*) bidez sortzen da, zeina ROS bidez aktibatua izan daitekeen. Hemen sortutako zeramida PP1 eta PP2A aktiba ditzazke. Ondoren, PP2A, BCL-2 eta AKT (ingelesetik *RAC- α serine/threonine-protein kinase*) bezalako proteina anti-apoptotikoak desfosforilatu eta inaktiba ditzazke, horrela apoptosira eramanez. PP1ek ere pro-apoptotikoa den BID (ingelesetik *BH3 interacting domain death agonist*) proteinan eragin dezake. ER/nukleoan (c), *de novo* sintetisatutako zeramidak PP1 aktiba dezake, zeinak BCL-X-ren (ingelesetik *B-cell lymphoma-extra*) moztitsasketa alternatiboa zuzentzen duten serina-arginina (SR) proteinen desfosforilazioa eragiten duen. Lisometan (d), zeramida A-SMase-ren ekintzaz sortzen da. Hemen, zeramidak katepsina D aktibatu eta proteina pro-apoptotikoa den BID proteinaren aktibazioa zuzen dezake, tBIDera moztuz eta apoptosira eramango duen 9- eta 3-kaspasaren aktibazioa eraginez. Zeramidaren sorrera mintz plasmatikoa (e) *raft* lipidikoak izenez ezagutzen diren mintzaren azpi-konpartimentu espezifikoean gerta daiteke. Hemen sortutako zeramidak, *raft*-etan pilatutako Fas bezalako hartzaileen bidezko seinalizazio bidezidor espezifikoeak eragin ditzake. CDasa, zeramidasa (ingelesetik *ceramidase*). SMS, esfingomielina sintasa (ingelesetik *sphingomyelin synthase*). (Ogretmen eta Hannun, 2004-etik hartua).

Zeramidak ez du soilik apoptosian parte hartzen, baizik eta erantzun zelular autofagikoan ere. Minbizi-zelula lerro gehienetan, autofagia zelularen kate luzeko dihidrozeramiden eta zeramiden mailen igoerarekin lotuta dago, nahiz eta gero eta argiago dagoen dihidrozeramidak autofagiaren aktibatzaile nagusiak direla (Morad *eta lank.*, 2013). Gainera, duela gutxi, Corcelle-Termeau eta lankideek Niemann-Pick A eta B motako gaixotasunekin lotutako esfingomielina pilaketak, ATG9Aren garraioa eta mintz autofagiko goiztiarren heltzea eta ixtea eragozten dituela erakutsi dute (Corcelle-Termeau *eta lank.*, 2016).

Zeramidek AKT inhibi dezakete, hau mTOR aktibitatearen erregulazio negatibora eta autofagiaren aktibiziora darama (**1.30 Irudia**). Zenbait minbizi-ereduetan, dihidrozeramidak eta zeramidak autofagia aktibatu dezakete elikagaien garraiatzaileak diren proteinen erregulazio negatiboaren bidez, zeina mTOR aktibitatearen erregulazio negatiboarekin ere lotuta dagoen (Pattinre *eta lank.*, 2009). Gainera, zeramidak eta dihidrozeramidak mTOR inhibitu dezakete mTOR aktibitatearen erregulatzailea den D-fosfolipasa (PLD, ingelesetik *phospholipase D*) inhibituz (Venable *eta lank.*, 1996).



1.30 Irudia. Zeramidak bideratutako erantzun autofagikoaren bidezidorrak. Zeramidak aktibatutako erantzun autofagikoetan parte hartzen duten osagai intrazelular nagusiak erakusten dira. (Morad eta Cabot, 2013-tik hartua).

Gainera, BNIP3, mitofagiaren aktibatzaile bat, BH3-domeinu bakarra duten proteina familiako kidea da, eta glioma gaiztoetan zeramidak aktibatutako autofagia BNIP3ren transkripzioaren aktibazioarekin batera gertatzen da (Daido *eta lank.*, 2004). Honek LC3ren adierazpena igo eta Beclin 1-BCL-2 konplexuak eragozten ditu (**1.30 Irudia**), gertaera hauek FoXO3 gauzatutako seinalizazioen aktibazioaren bidez gidatuko litzateke. AMP-menpeko proteina kinasa (AMPK, ingelesetik *AMP-dependent protein kinase*), mTORaren beste erregulatzaile bat, funtsezko sentsore energetiko bat da zeinak, ATG1 (ULK1 izenez ere ezagutua) fosforilazioaren bidez, zeramidak erregulatutako autofagiaren aktibazioan laguntzen duen (Peralta eta Edinger, 2009). Zeramidak, erantzun autofagikoak eragin ditzake VPS34 (PI3KC3 izenez ere ezagutua) proteina konplexura lotuz, zeinak VPS34ren disoziazioa eta ondoriozko aktibazioa eragiten dituen. III. motako PI3K honek, endozitozi eta autofagia bezalako garraio besikularreko prozesuak gauzatzen ditu; bere aktibazioa autofagia hasteko beharrezkoa da (Pozuelo-Rubio, 2011).

Velasco-ren ikerketa-taldeak arratoi eta saguetan egindako kanabinoideen administrazioak zenbait tumore xenografoen ereduaren hazkuntza gelditzen duela erakutsi du. Beraz, konposatu hauek minbiziaren aurkako farmako potentzialen familia berria kontsideratzen dira. Kanabinoideen minbiziaren aurkako ekintza, gutxienez neurri batean, minbizi-zelulen heriotza aktibatzeke duten gaitasunean oinarritzen da (Salazar *eta lank.*, 2009; Patsos *eta lank.*, 2010). Proposatutako autofagia bidezko minbizi-zelulen heriotzaren aktibazioaren ereduaren, THCK (ingelesetik *Δ9-tetrahydrocannabinol*), kanabisaren osagai psikoaktibo nagusia eta anandamida kanabinoide naturalaren analogo bat, ERan esfingolipidoen *de novo* sintesiaren suspertzea eta ondoriozko ER estresarekin-lotutako seinalizazio bidezidor baten aktibazioa eragiten ditu. Bidezidor honen aktibazioak autofagia eragiten du eta THCren ekintza pro-apoptotiko eta anti-tumoralerako ezinbestekoa da. THC tratamenduaren efektu horiek hobeto ulertzeko asmoz, tesi lan honetan zeramida eta dihidrozeramida mailen igoerak mintzaren ezaugarrietan nola eragiten duen aztertu da. Hurrengo atala zeramidak mintzetan daukan jokaeraren inguruko ikerketetan oinarrituko da.

Beste aldetik, duela gutxi C18-zeramidak autofagosomari lotutako LC3 formarekin zuzenean elkarrengaitan duela eta honek mitofagia hilgarria eragiten duela erakutsi da (Sentelle *eta lank.*, 2012; Dany eta Ogretmen, 2015). Kasu honetan, zeramidak aktibatutako heriotza zelular autofagikoa giza minbizi-zeluletan, prozesu autofagiko hautakor baten bidez gertatzen da eta apoptosiarekiko independentea da. Eredu molekularren bidez aurreikusitako LC3Bren zeramida lotzeko guneak, 1-zeramida sintasak gauzatutako mitokondrioen hautaketa honetarako ezinbestekoak

direla erakutsi da (Sentelle *eta lank.*, 2012). Tesi lan honen testuinguruan, zeramidak baita kardiolipinak ere hartzaile gisa betetzen dituzten funtzio berriak LC3 mintz mitokondrialetara ainguratuz, eta degradaziorako-materialaren aukeraketa honetarako beharrezko mekanismo espezifikoak ikertu dira.

B) Zeramidaren Jokaera Mintzean

Zeramidaren presentzia lipido bigeruzan, aldaketak eragiten ditu mintzaren ezaugarrietan (karga, jariakortasuna, iragazkortasuna). Zeramidan aberatsak diren domeinuen eraketa, esfingomielinasa aktibitatearen efektu garrantzitsu bat da. Domeinu hauek, sintetizatutako zeramiden albo banaketaren ondorioz eratzen dira. Domeinuen eraketaren arrazoiak oso ezberdinak izan daitezke, baina ziur aski garrantzitsuena zeramidak (eta esfingolipidoak orokorrean) buru polarraren mailan hidrogeno loturen sare handi bat eratzeko daukaten gaitasuna da. Glizerofosfolipidoek hidrogeno hartzaile gisa soilik jardun dezaketen bitartean, esfingolipidoek (adib. esfingomielina eta zeramidak) beraien hidroxilo eta amida taldeen bidez, hartzaile eta baita emaille gisa ere jardun dezakete (Goñi eta Alonso, 2009). Zeramidak, domeinuak eratuz alboz banatzeko daukaten gaitasuna prozesu biofisikoetan funtsezkoa da, eta eragin fisiologiko garrantzitsuak izan ditzake. Proposatua izan da, zelularen kanpoko seinale batek (adibidez ligando bati lotutako Fas) mintz plasmaticoan esfingomielinasa (SMasa, ingelesetik *sphingomyelinase*) aktibatzen duenean, entzimaren produktu gisa zeramida agertzeak, *raft*-en berrantolaketa eragiten duela makrodomeinu handiagoak eratuz non Fas pilatzen den. Hartzaileen pilaketak zelulan beste efektoreak aktibatzen dituela uste da (Goñi eta Alonso, 2009).

Zeramidak, mintz-ereduen baita mintz zelularren iragazkortasun hesiak ere aldarazteko gaitasuna du. Ruiz-Argüello eta lankideak, bigeruzan zeramiden sorrera bai liposomen zein mamu-eritrozitikoaren edukiaren askapena eragiten duela behatu zuten lehenengoak izan ziren (Ruiz-Argüello *eta lank.*,1996). Ondorengo ikerketek, zeramidak mintz mitokondrialak kaltetzeko gai zirela ikusi zuten, ondorioz, c-zitokromoaren askapena eraginez. Askapena eragiten duen mintzaren berregituraketa prozesuan, zeramidaren bi ezaugarri izango litzateke garrantzitsuak. Bata, zeramidek bigeruzan kurbadura negatiboa, mintz zelularretan normalean aurkitzen den kurbadura motaren aurkakoa, eragiteko duten gaitasuna. Bestea, zeramidan aberatsak diren domeinuetan banatzeko joera. Zeramidan aberatsak eta urriak diren domeinuen arteko interfasea, ziur aski askapena gertatzen deneko zonaldea da.

Aurretik aipatu den bezala, tesi lan honetan zeramida mailen igoerak eragiten dituen eta heriotza zelular autofagikora eramango luketen mintzaren ezaugarrien aldaketak aztertu dira. Helburu honetarako burututako esperimenduetan, zeramidak eta dihidrozeramidak proportzio ezberdinetan gaineratuak izan dira eta beraien mintza desorekatzeko gaitasuna ikertua izan da.

Laburbilduz, zeramidak autofagiaren erregulazioan daukaten eginkizuna azaltzeko mekanismo ezberdinak proposatuak izan dira: (i) zeramiden mailen igoerarekin lotutako heriotza zelular autofagikoa, (ii) zeramidak aktibatutako autofagia seinalizazio molekula ezberdinen aktibazio edo inhibizioaren bidez, eta (iii) zeramida eta LC3 arteko elkarrekintza zuzenak eragindako mitofagia hilgarria. Hala ere, esfingolipidoen eta autofagiaren arteko elkarreragitearen inguruko ikerketa gehiago beharrezkoak dira, izan ere, erlazio hauek hobeto ulertzeak neurodegenerazioan, infekzioan, minbizian eta beste gaixotasun batzuetan autofagiaren kontrol estrategiko berriak garatzen lagunduko luke.

1.5 Helburuak

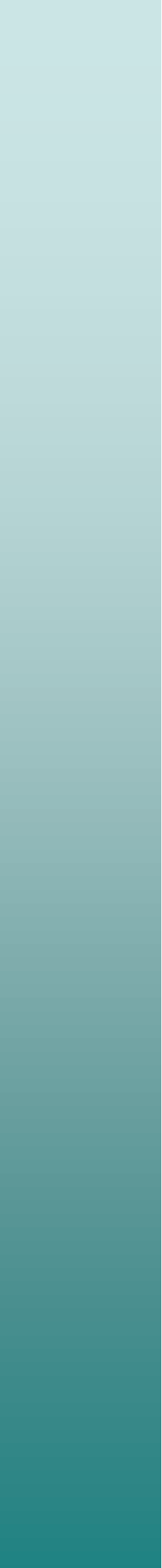
Ikerketa lan honek, autofagosomak kaltetuak dauden mitokondrioak nola ezagutzen dituen eta prozesu hori bideratzen duten mekanismo molekularren ulermen hobea lortzea du helburu. Kardiolipinaren kanporaketak mitokondrioaren kanpoinzera, mitofagian zehar LC3arentzako seinale gisa jokatzen duela proposatu da. Zeramidak ere prozesu honetan LC3arentzat degradatu beharreko materialaren hartzaile gisa jarduten duela eta autofagia ez-hautakorren bidezko heriotza zelularrean laguntzen duela proposatu da. Proteina-lipido elkarrekintza hauen mekanismo molekularrak eta eginkizun espezifikoak, eta lipido hauen eragina mintzaren ezaugarrietan, gure interesaren oinarria dira bai mitofagian zein bidezidor autofagiko ez-hautakorretan. Alderdi hauen ezagumenduan aurrerapausuak ematearren, teknika biofisiko kuantitatiboak, mintz ereduen bidezko metodoak erabiliz, eta biologia zelularreko teknikak erabili dira.

Tesi lan honen helburu espezifikoak ondorengoak dira:

- Atg8aren zenbait giza ortologoek kardiolipinarekiko elkarrekintzaren *in vitro* ikerketa fisikoak burutzea, eta mintzean elkarrekintza horien ondorio estruktural eta funtzionalak zehaztea.
- Mitofagian zeharreko mitokondrioen aukeraketan, aipatutako elkarrekintzaren eginkizuna giza zelula lerro ezberdinetan ikertzea.
- THCK gauzatutako minbizi-zelulen heriotza autofagikoan mintzen jokaera aztertzea, eta mitofagia hilgarrian eta ondorengo tumoreen supresioan zeharreko LC3B-zeramida elkarrekintzaren mekanismo molekularrak ikertzea.

2. Kapituluua

Teknika Esperimentalak



2. Kapituluua:

Teknika Esperimentalak

2.1 Biologia Molekularreko Metodoak

Biologia molekularreko teknikek, DNA sekuentziak isolatu, purifikatu eta aldatzea ahalbidetzen digute (Sambrook eta MacCallum, 2001). Tesi lan honetan, DNA errekonbinanteko teknika arruntak erabiliz, giza Atg proteina desberdinen DNA konplementarioak (cDNA, ingelesetik *complementary DNA*) bakterioen adierazpen bektoretan klonatu, eta hondar espezifikoek proteinaren funtzioan daukaten eragina aztertzeko asmoz, beraien sekuentzietan mutazioak ere gauzatu dira.

2.1.1 DNA Anplifikazioa eta Klonazioa

Intereseko cDNA klonatzeko helburuarekin, polimerasaren kate-erreakzioaren (PCR, ingelesetik *polymerase chain reaction*) bidez, DNA zati bat anplifikatu eta bere kabuz erreplikatzeko gai den egitura genetiko batean txertatzen da, gehienetan plasmido batean, normalean bektore gisa ezagutzen dena. Prozesua pausu hauetan banatu daiteke: 1) PCR bidezko anplifikazioa, 2) Liseriketa, 3) DNAREN arazketa, 4) Ligazioa, 5) Transformazioa, 6) Erauzketa eta 7) Klon positiboaren aukeraketa (**2.1 Irudia**).

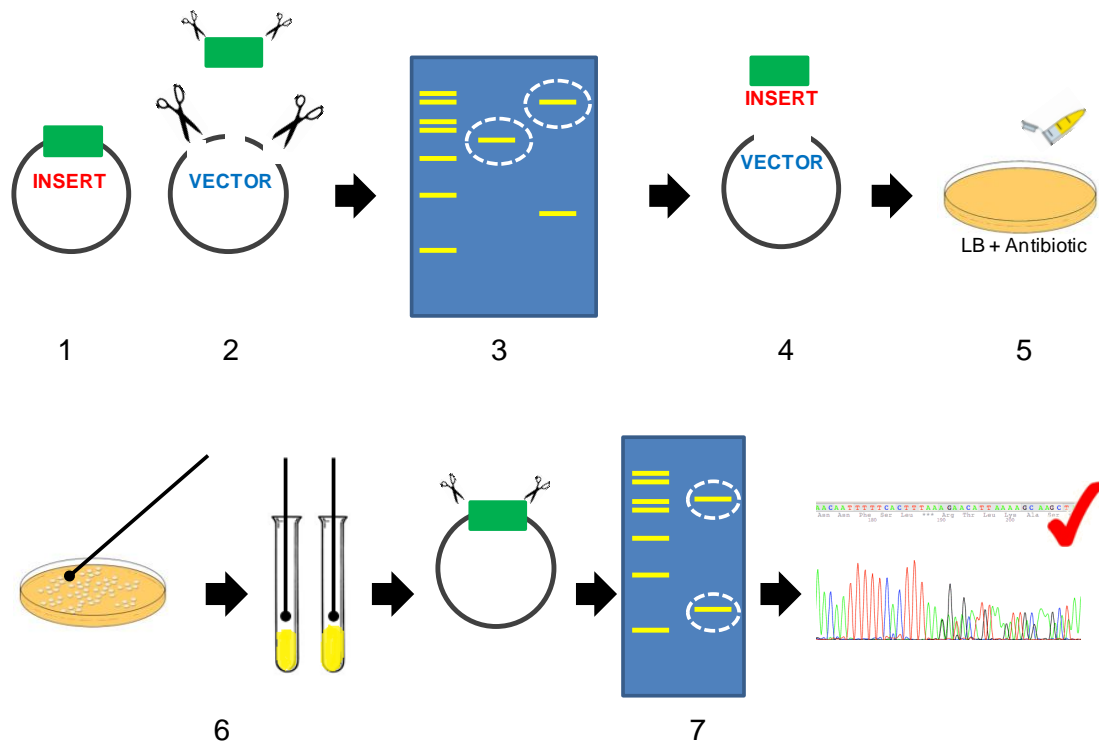
1. PCR bidezko Anplifikazioa eta Arazketa

Intereseko cDNA, MastercyclerPro termozikladorea (Eppendorf AG, Hamburgo, Alemania) eta PfuTurbo DNA polimerasa erabiliz (Agilent Technologies, Santa Clara, CA, EEBO) anplifikatu zen, fabrikatzaileak zehazten dituen PCR baldintza orokorrak erabiliz. Bektore ituetan ligazioa ahalbidetzeko, errestrikzio-gune egokiak zeuzkaten aurreranzko eta atzeranzko hasleak, Sigma Aldrich-ek (San Luis, Misuri, EEBO) sintetizatu zituen. PCR produktuaren arazketa,

QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, EEBB) sistema komertziala erabiliz burutu zen.

2. Liseriketa

PCR produktua baita bektore itua ere, errestrikzio entzima berdinekin (New England Biolabs, Mississauga, Ontario, Kanada edo Roche, Basel, Suitza) moztu ziren 20 µL-ko erreakzio batean 37°Ctan eta ordu batez. Bektorea bere buruarekin ligatzea ekiditeko, *shrimp* fosfatasa alkalinoa (USA corp, Cleveland, OH, EEBB) erabiliz desfosforilatu zen aurretik errestrikzio entzimen bidezko liseriketarako erabilitako erreakzio nahasturan, 37°Ctan eta ordu batez.



2.1 Irudia. Klonazioaren pausu orokorrak. (1) PCR amplifikazioa, (2) Liseriketa, (3) Arazketa, (4) Ligazioa, (5) Transformazioa, (6) DNA erazketa eta (7) Klon positiboen aukeraketa.

3. Liseriketa Produktuen Arazketa

PCR edo liseriketa bidez lortutako DNA zatiak erazi eta arazketa burutzeko, agarosazko gelean egindako elektroforesia erabili zen. Gela, %1 agarosa (w/v, Bionline, Londres, UK) 1x TAE idargetzailean (gehienetan agarosaren 1.5 g 1x TAE-ren 150 mL-tan) urtuz prestatu zen. Nahasketa, homogeenoa geratu arte, irakite puntara arte berotu zen. Nahastura homogeenoa lortu ondoren, gela gutxi gora-behera 60°Ctara hozten utzi zen. Behin hoztuta, gelaren 100 mL-ko,

etidio bromuroaren (Bio-Rad, Hercules, CA, EEBC) 10 mg/ml-ko soluzio baten 2.5 µL gaineratu ziren, gela erretilu batean putzuak markatzen dituen orrazi batekin jarri aurretik. Gela giro tenperaturan 30 minutuz mantendu ostean, orraziak kendu eta MiniPlus (HU10) elektroforesi andel horizontal batean (Anachem, Luton, EB) TAE indargetzailearekin estali zen.

DNA laginak, 5x karga tindatzaile soluzioan (Thermo Fisher Scientific, Waltham, MA, EEBC) edo 10x DNA karga indargetzailean, 4:1 (v/v) eta 9:1 (v/v) diluitu ziren, hurrenez hurren. DNA zatien tamaina kalkulatzeko, laginak 1 kb-eko DNA markatzailea den *GeneRuler*-en (Fermentas, Waltham, MA, USA) 5 µL-rekin batera kargatu ziren. Elektroforesia, EC105 indartzaile (Thermo Fisher Scientific) batekin 100 boltiotan gauzatu zen, gutxienez 25 minutuz. UV transargitzaile bat erabiliz, DNA bandak argi UVaren bidez ikusi ziren eta irudiak Olympus Camedia C-5060 kamera digital bat erabiliz lortu ziren. Klonazioa egiteko, intereseko DNA bandak purifikatuak izateko erauziak izan ziren. Geletan laginen DNA kantitatea bisualki kalkulatu ahal izateko ligazioa egin aurretik, DNA markatzaile eta laginen kantitate jakinak erabili ziren. DNA produktuak gel baten bidez purifikatu ziren, QIAquick Gel Extraction Kit (Qiagen) sistema komertziala erabiliz, eskuliburuan zehazten den moduan. Kit hau, DNAREN arazketa burutzeko, DNAREN gatz kontzentrazio altuetan silika-gelezko mintz-zutabeekiko daukan afinitateaz baliatzen da.

4. Ligazioa

DNA zatiak bektore lineal eta desfosforilatuetan txertatzeko, ligazio erreakzioa erabili zen. Bektore hauek, aurretik errestrikzio entzimen bidez moztuak izaten ziren 5' mutur bateragarria izateko. 20 µL-ko ligazio erreakzioak, 1x Ligasa indargetzailean disolbatutako T4 DNA ligasaren (Roche) unitate baten presentzian, 16°Ctan eta gauean zehar burutu ziren.

5. *E.coli* Zelula Kompetenteen Transformazioa

Ligazio produktuekin egindako zelulen transformaziorako, *E. coli* bakterio kompetenteak izotzean urtu ziren eta ligazio nahasturaren (0.5 µL-tik 1 µL-rako DNA plasmidoduna) 20 µL *E. coli* DH5-α zelula kompetenteen 50 µL edo 100 µL-tara gaineratu ziren. Lehendabizi zelulak 20 minutuz izotzean inkubatu ziren eta ostean 42°Ctan 60 segundotako bero-txoke erreakzio bat burutu zen. Zelulen indarberritzea ahalbidetzeko, nahastura berehala izotz balde batera eramana eta 5 minutuz inkubatua izan zen. Bakterioak indarberritzeko, 1 mL *Luria-Bertani* (LB) medioarekin 45 minutuz 37°Ctan irabiatuz inkubatu ziren. Azkenik, zelulak 8000 x

g-tan minutu batez mahai-zentrifugatzaille batean zentrifugatu, eta jalkina LB medioaren 50 μ L-tan berresekitu eta antibiotiko egokidun agar plaka batera gaineratu zen. Gauean zehar 37°Ctan egindako inkubazioan, soilik bektore honen kopia bat edo batzuekin transformatutako bakterioak kolonietan haziko dira agar plaka hauetan.

6. *E. coli*-tik egindako DNA Plasmidikoaren Erauzketa

Gehienetan DNA plasmidikoa (1 μ g/mL), *E. coli*-tik eta QIAprep Spin Miniprep kitaren (Qiagen) eskala txikiko arazketa teknika erabiliz erauzi zen. Gauean zehar hazitako 5 mL-ko bakterio-kultiboari, lisi alkalinoa eta horren osteko DNA arazketa gauzatu zitzaion, fabrikatzailearen argibideak jarraituz. DNA plasmidikoa luzaroan erabilia izango bazen -20°Ctan gorde zen.

7. *Klon Positiboen Aukeraketa*

Miniprep sistema erabiliz erauzitako bektore plasmidikoetan intereseko intsertoaren presentzia baieztatu zen, errestrikzio entzima egokiak (2. pausuan deskribatzen den moduan) eta elektroforesia erabiliz. PCR anplifikazioan zehar barneratutako edozein mutazio, sekuentziazio bidez (Secugen S.L, Madril) baztertu zen. DNA kontzentrazioa espektrofotometria bidez zehaztu zen, laginaren absorbantzia 260 nm-tan (A_{260}) neurtuz, eta Nanodrop™ espektrofotometro batean (Thermo Fisher Scientific) araztasuna konprobatu zen (A_{260}/A_{280} ratioa kontuan hartuta, ratioa >1.8 izatea helburutzat izanez).

2.1.2 Mutagenesi Guneratua

In vitro egindako mutagenesi guneratua oso teknika baliotsua da proteina baten aminoazido bakoitzak proteinaren egitura eta funtzioari egiten dion ekarpena zehazteko. Aurreko 2 hamarkadetan zehar, azpi-klonaziorik gabeko mutazio guneratuak burutzeko oso eraginkorrak eta sinpleak diren metodoak garatu dira (Fisher eta Pei, 1997). Tesi lan honetan, intereseko proteinetan mutazio puntualak burutzeko, Stratagene Quikchange Site-Directed Mutagenesis Kit-a (Agilent Technologies) erabilia izan da.

Prozesuaren oinarria, helize bikoitzeko DNA (dsDNA, ingesetik *double-stranded DNA*) hasierako molde gisa eta intereseko mutazioa daukaten oligonukleotido sintetikoak diren bi hasleen erabileran datza (**2.1 Taula**).

Hasleak		Sekuentzia
LC3B ^{R10,11A}	Aurreranzkoa	5'-GAAGGTGGCGGCCTGCTTGAA-3'
	Atzeranzkoa	5'-TTCAAGCAGGCCGCCACCTTC-3'
LC3B ^{Q15C}	Aurreranzkoa	5'-CGGACATCTTCTACTCTGCATTCTGAAGGTG CGGCGCTGCT-3'
	Atzeranzkoa	5'-CCATCTTCATCTTTCTCACACTCATACACCTCT GAGATT-3'
LC3B ^{S101C}	Aurreranzkoa	5'- AGCAGCGCCGCACCTTCTGAATGCAGAGTAGAAGA TGTCCG-3'
	Atzeranzkoa	5'-AATCTCAGAGGTGTATGAGTGTGAGAAAGATGAA GATGG-3'
GABARAP ^{R14,15A}	Aurreranzkoa	5'-CTTCTCGCCCTCAGAGGCGGCCTTCTCGAAC GGATGC-3'
	Atzeranzkoa	5'-GCATCCGTTTCGAGAAGGCCGCCTCTGAG GGCGAGAAG-3'
GABARAP ^{K47A}	Aurreranzkoa	5'-AGAAGGCACCAGGTATTTTCGCTTTGTCCAGGTC-3'
	Atzeranzkoa	5'-GACCTGGACAAAGCGAAATACCTGGTGCCTTCT-3'
GABARAP ^{K46,47A}	Aurreranzkoa	5'-ATAGGAGACCTGGACGCAGCGAAATACCTGGT-3'
	Atzeranzkoa	5'-ACCAGGTATTTTCGCTGCGTCCAGGTCTCCTAT-3'
GABARAP ^{K46,47,48A}	Aurreranzkoa	5'-GCACCAGGTATGCCGCTGCCTCCAG-3'
	Atzeranzkoa	5'-CTGGAGGCAGCGGCATACCTGGTGC-3'
GABARAP ^{S16C}	Aurreranzkoa	5'-CTCGCCCTCACAGCG GCGCTTCTC-3'
	Atzeranzkoa	5'-GAGAAGCGCCGCTGTGAGGGCGAG-3'
GABARAP ^{S88C}	Aurreranzkoa	5'-CCCATTGTGGCACAGGTGGGTGGAAT-3'
	Atzeranzkoa	5'-ATTCCACCCACCTGTGCCACAATGGG-3'

2.1 Taula. Mutagenesi guneratua burutzeko erabilitako oligonukleotidoen zerrenda.

Hasle oligonukleotidikoak, bakoitza bektorearen kontrako harizpi baten osagarria da eta PfuTurbo DNA polimerasa (Agilent Technologies) erabiliz, temperatura zikloetan burutzen den PCR bidez luzatuak dira. Hasle oligonukleotidikoaren eransketa

plasmido mutatua sortzen du. Temperatura zikloen ostean, produktua *DpnI*-rekin tratatzen da. *DpnI* endonukleasa (sekuentzia itua: 5'-Gm6ATC-3'), DNA metilatu eta hemimetilatuekiko espezifikoa da eta jatorrizko DNA moldea liseritzeko eta mutazioak dauzkan DNA sintetizatu berria aukeratzeko erabiltzen da. Ondoren, intereseko mutazioak dauzkan bektore moztua, DH5- α *E.coli* zelula kimiokonpetenteak transformatzeko erabiltzen da.

Ondorengo protokoloan (**1. Protokoloa**), LC3B eta GABARAPen mutazio puntualak sartzeko tesi lan honetan erabilitako prozedura orokorra azaltzen da. Hala ere, erreakzioak huts egin zuen kasu konkretuetan, protokoloa aldatu egin zen hasle kontzentrazioa jaitsiz, DNA moldearen kontzentrazioa igoz, edo PCR hibridazio temperatura jaitsiz.

1. Protokoloa. Mutagenesi Guneratua

1. Hasle mutagenikoak, Stratagene-ren web-bidezko hasleen diseinurako programa (<http://www.stratagene.com/cprimerdesign.com>) erabiliz diseinatu ziren, zeinak gutxienez 78°Cko urte temperatura daukaten eta mutazioa sekuentziaren erdigunean zentratzen duten hasleak diseinatzen dituen. Mutazio generatuak sartzeko diseinatutako hasle guztiak Sigma-Aldrich-ek sintetizatu eta purifikatu zituen.

2. Osagaiak:

- 0.5 μ L Aurreranzko haslea (2.5 pmol/ μ L)
- 0.5 μ L Atzeranzko haslea (2.5 pmol/ μ L)
- 0.5 μ L DNA moldea (10 ng/ μ L)
- 0.5 μ L 40 mM dNTPak (10 mM bakoitza)
- 0.5 μ L Pfu Turbo Polimerasa (2.5 U/ μ L)
- 5 μ L Polimerasa indergetzailea (5X)
- 17.5 μ L H₂O esterila

(Protokoloak hurrengo orrialdean jarraitzen du)

3. PCR amplifikazio baldintzak:

- 5 min 95°Ctan
 - 18 aldiz errepikatu
 - 30 seg 95°Ctan
 - 1 min 55°Ctan
 - 2 min 72°Ctan
 - 7 min 72°Ctan
4. *DpnI* bidezko liseriketa, erreakzio nahasturari *DpnI* (20 U/μL)-aren 0.25 μL gaineratuz eta 37°Ctan ordu batez inkubatuz egiten da.
 5. *DpnI*-aren inaktibazioaren ostean (80°C 20 min), erreakzioaren azken produktua zelula konpetenteak transformatzeko erabiltzen da. Gehienetan, DH5-α *E. coli* zelula kimiokonpetenteen 50 μL-tara produktu honen 1 μL gehitzen da.
 6. Azkenik, 5 kolonia aukeratu, beraien DNA *miniprep* sistemaren bidez (GeneJET Plasmid Miniprep Kit, Thermo Fisher Scientific) amplifikatu eta mutazioaren presentzia egiaztatzeko baita PCRak eragindako edozein mutazio baztertzeko ere sekuentziatu egiten dira (Secugen S.L.).

2.2 Proteinen Adierazpena eta Arazketa

2.2.1 Proteinen Adierazpena

LC3B, LC3B^{GA}, GABARAPL2 eta GABARAP proteinak GST pGEX-6P1 bektorean klonatuak (I. Tanida doktoreak eman zizkigun, National Institute of Infectious Diseases, Tokio, Japonia), eta zegozkien forma mutanteak, *E. coli* BL21 (DE3) zelulak transformatzeko erabili ziren. Proteinak, glutation S-transferasa (GST)-isatsa duten fusio-proteinak bezala adierazi ziren. Lehenengo, zelulak *E. coli* BL21 (DE3) glizerol stock batetik hartuak, LB-anzipilina (Amp) mediodun 100 mL-ko edukiontzi batera mugitu ziren, eta irabiagailu orbital batean eta 37°Ctan gauean zehar inkubatu ziren. Inkubazioaren ostean, kultiboa eskalatu zen gauean zehar inkubatutako kultibo asearen 10 mL, LB-Amp medioaren 1L-ra pasatuz, eta nahastura 37°Ctan 600 nm-tako uhin luzerara neurtzen den dentsitate optikoa (OD₆₀₀, ingelesetik *optical density at 600*

nm) 0.7-0.8 bitartekoa izan arte inkubatu zen. Ondoren, proteina errekonbinantearen adierazpena hasteko, 0.5 mM isopropil- β -D-1-tiogalaktopiranosidoa (IPTG) gehitu zen. Proteinen adierazpen maila eta proteolisirako sentikortasun desberdintasunak direla eta, proteina bakoitzarentzako indukzio eta adierazpen denbora desberberdinak optimizatu ziren (**2.2 Taula**). IPTG gehitu ostean, laginak irabiagailu orbital batean adierazitako denboretan inkubatu eta zentrifugazioaren ostean (4500 x g 15 min, 4°C) bakterioak batu ziren. Azken zentrifugazio hau, Beckman Coulter zentrifugatzailean eta JLA-9.100 errotorea erabiliz (Beckman Coulter, Brea, CA, EEBB) burutu zen. Gainjalkina baztertu eta jalkina arazketa pausuetarako -80°Ctan gorde zen.

Proteina	Adierazpen baldintzak
LC3/GABARAPL2	3 ordu 37°Ctan
GABARAP	4 ordu 20°Ctan

2.2 Taula. Proteinen adierazpen baldintzak.

2.2.2 GST-dun Proteinen Arazketa

Arazketa prozesuarekin hasteko, zelulen apurketa eta lisatuaren garbiketa pausuk jarraitu ziren. Bakterio-jalkinak apurketa-indargetzaile egokian berresekitu ziren [10 mM PBS, 20 mM Tris-HCl, pH 7, 150 mM NaCl eta prestatu berriko zenbait osagai osagarriak: 1 mg/mL liozima (Sigma-Aldrich), 1 mM ditiotreitola (DTT, Sigma-Aldrich), bakterio proteasen inhibitzaileak (Roche) eta 2.5 μ g/mL DNAasa (Sigma-Aldrich)]. Ondoren, liozima bidezko bakterioen horma zelularraren apurketa ahalbidetzeko, nahastura 4°Ctan 45 minutuz irabiagailu orbital batean inkubatu zen. Gero, zelulak izotzean (gehiegizko beroketa saihesteko) sonikatu ziren, 40 ziklo, 10 seg on/10 seg off. Liseriketa zelularraren ostean lortutako esekidura, Beckman Coulter zentrifugatzailea eta JA-25-50 errotorea (Beckman Coulter) erabiliz 30000 x g-tan, 30 minutuz 4°Ctan zentrifugatu zen hondakin zelularrak baztertzeko. Jalkina baztertu zen eta gainjalkina 0.45 μ m and 0.2 μ m-ko filtroak erabiliz filtratu zen, eta azkenean izotzetan mantendu zen arazketarekin jarraitzeko.

Indargetzailea	Indargetzailearen konposaketa	
Garbiketa indargetzailea	1	PBS1x, pH 7.4 + 1 mM DTT
	2	PBS1x, 50 mM Tris-HCl, pH 8.7 + 1 mM DTT
	3	PBS1x, 50 mM Tris-HCl, pH 8.7, 500 mM NaCl + 1 mM DTT
PreScission indargetzailea	50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA + 1 mM DTT	

2.3 Taula. GST-dun proteinen arazketan erabilitako indargetzaileak.

GST-dun LC3B, GABARAPL2 eta GABARAP proteinak, afinitate kromatografiaren bidez purifikatu ziren *Glutation Sefarose 4B* erretxina (GE Healthcare, Buckinghamshire, EB) erabiliz. 4 L-ko bakterio-kultibo bakoitzarentzat, erretxinaren 1 mL behar zen. Lehenengo, erretxina 3 aldiz zentrifugazio bidez garbitu zen ur distilatuaren 10 erretxina-bolumen erabiliz, eta gero, beste 5 aldiz lisi indargetzailea erabiliz. GST glutationera lotzea ahalbidetzeko, bakterio-gainjalkina erretxinarekin nahastu eta nahastura 3 orduz eta 4°Ctan irabiagailu orbital batean inkubatu zen. Fluxu grabitazionaleko zutabe bat (Bio-Rad) proteinak loturik zeuzkan erretxinaz bete zen, eta eluzioaren lehenengo lagina (FT, ingelesetik *flow-through*) baztertu zen. Ostean, zutabea, **2.3 Taulan** adierazitako indargetzaile bakoitzaren 10 erretxina-bolumen erabiliz garbitu zen. Gero, prestatu berriko DTTaren 1 mM zeukan PreScission indargetzailearen 2 erretxina-bolumen erabiliz, PreScission proteasaren (GE Healthcare) 50 µL (stock-a: 2000 unitate/mL) gehitu ziren. Proteasa bidezko mozketa egoki baterako, lagina 15mL-ko Falcon hodi batera pasatu eta 4 orduz 4°Ctan irabiagailu orbital batean inkubatu zen. PreScission indargetzailea erabiliz, moztutako proteina fluxu grabitazionaleko zutabetik eluitu zen eta 4mL-ko 2 frakzio hartu ziren. Horren ostean, PreScission indargetzailearen beste 4 mL erretxinadun zutabera gehitu ziren eta gauean zehar 4°Ctan inkubatzen utzi zen. Gero, 4mL-ko beste 2 frakzio eluitu ziren eta SDS-PAGE gel baten bidezko bereizketa eta Coomassie urdin tindatzailea erabiliz frakzioak aztertu ziren. Proteinetan aberastuak ziren frakzioak kontzentratuak eta -80°Ctan %20 glizerolean gorde ziren. Proteina guztiak detergentarik gabeko bakterioen erauzkin disolbagarrietatik purifikatu ziren eta >%90 puruak ziren.

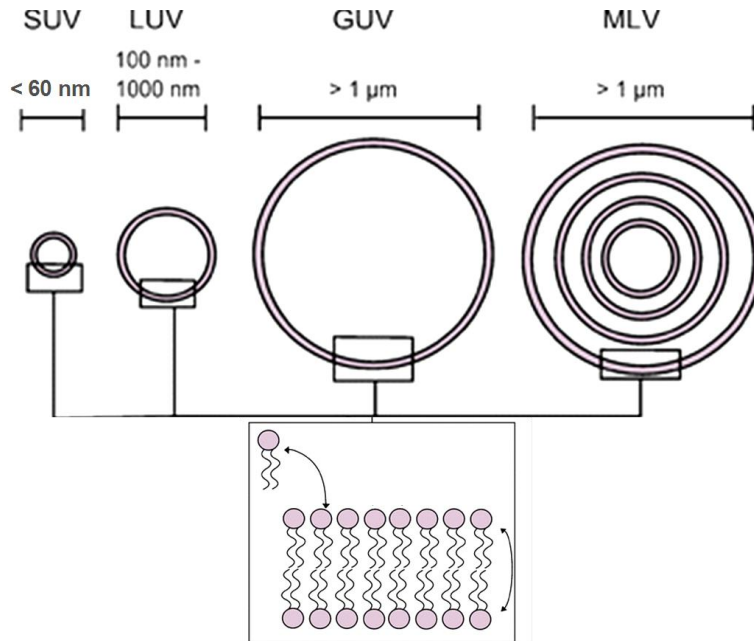
2.3 Mintz-Ereduen Sistemak

Bai ikuspuntu estruktural zein funtzionaletik mintz biologikoez daukaten konplexutasunak, beraien ikerketarako zailtasun handia suposatzen du. Hori dela eta, lipido puruen, lipido nahasturen, eta berreraikitako lipido-proteina sistemen ezaugarriak ikertzeko, hainbat mintz-ereduen sistemak garatu dira. Sistema bakarra ez denez egokia beraien ikerketan erabiltzen diren teknika guztietarako, mintz-eredu ezberdinak egotea beharrezkoa da. Sistema eredu hauek horrela taldekatu daitezke: (1) monogeuzak, (2) bigeruzak lauak, eta (3) liposomak edo lipido-besikulak (Gennis, 1989). Tesi lan honetan, horietako bi erabiliak izan dira: (i) lipido-besikulak edo liposomak, eta (ii) lipido monogeruzak. Aurrerago, beraien erabilera nagusi eta prestaketa prozeduren inguruko azalpen zehaztua emango da.

2.3.1 Lipido-Besikulak (Liposomak)

Liposoma bat soluzio urtsu bat inguratzen duen bigeruzak konfigurazioa daukan lipido-egitura bat da. Itxura zilindrikoa duten lipidoek soluzio urtsuetan sakabanatzean liposomak era espontaneo batean sortzen dira, beraz mintz-eredu lipidikoen iturri egokia dira. Erabiltzen den prozesamenduaren arabera, liposomak bigeruzak edo lamela bakarra edo zenbait bigeruzak eduki ditzazkete, lan honetan erabilitako eredu sistema nagusia lamela bakarreko besikulak izanik (**2.2 Irudia**). Bi besikula motak eta teknika biofisiko desberdinak erabiliz ikertzeak, mintz prozesu gehienek ulermenerako ezinbesteko informazioa ematen du.

Liposomak luzaroan erabiliak izan dira fusioa, fisioa, disolbagarritasuna, edukien askapena, lipidoen flip-flop mugimendua, lipido-proteina lotura, proteina-proteina lotura eta beste hainbat gertaera neurtzeko. Interesgarria den erabilera bat, tratamendu medikoetan farmakoen administratzearako liposomak erabiltzea da. Farmakoak liposomen barruan enkapsulatuz, albo efektuak murriztuak izan daitezke eta leku ituan farmakoen askapen mailakaturik eta espezifikoa lortu daiteke (Gregoriadis, 1978; Gregoriadis, 2008). Gaur egun, minbiziaren aurkako tratamenduan liposomak farmakoen garraiatzaile gisa erabiltzearen inguruko ikerketa asko daude (ik. Allen eta Cullis, 2013; Noble *eta* *lank.*, 2014 eta Yingchoncharoen *eta* *lank.*, 2016, minbizien aurkako tratamenduan erabiltzen diren metodologia eta liposomen erabilerean inguruko duela gutxiko berrikustapenetarako).



2.2 Irudia. Fosfolipidoen antolaketaren laburpena tamaina desberdineko besikuletan (SUV, LUV, GUV eta MLV). Ez da eskalazko marrazkia.

2.3.2 Lamela Anitzeko Besikulak (MLVak)

60. hamarkadaren hasieran, Alec Bangham hematologo britaniarrak eta lankideek, ezagutzen diren lehenengo liposomen karakterizazioa burutu zuten Cambridgen, lezitinaren dispersioak behatuz mikroskopia elektronikoa erabiliz (Bangham eta Horne, 1964). Berak adierazi zuen bezala, lamela zentrokideak zuten “esperulita” batzuk eratzen ziren. Besikulak bai eskuz irabiatuz zein sonikazioz eratzen ziren, azken kasuan lamela gutxiagoko besikulen eraketa eraginez. Gaur egun, besikula hauek, lamela anitzeko besikulak (MLVak, ingelesezik *multilamellar vesicles*) bezala ezagutzen dira, eta gehienbat, lipido-faseen azterketarako lipidoen ikerketa biofisikoetan erabiltzen dira. Ikerketa hauetan, ekorketa kalorimetria diferentziala (DSC, ingelesezik *differential scanning calorimetry*), NMR edo X-izpi bidezko difrakzioa bezalako teknikak erabili ohi dira. Lipidoak hidratatu eta lagina nahasketa bortxe batean irabiatu ostean, MLVak berez sortzen dira, zeintzuek ur geruza fin batez banandutako 7-10 bigeruzak zentrokide izan ohi duten. Besikulen tamaina heterogeneoa da, 100 eta 5000 nm tartekoak (**2.2 Irudia**).

MLVen eraketa, liposomen eraketa metodo guztietatik sinpleena eta azkarrena da. Lamela anitzeko besikulak sortzeko tesi lan honetan erabilitako protokolo zehaztua deskribatzen da (**2. Protokoloa**).

2. Protokoloa. MLVak

1. Lipido puruen stock-ak prestatzeko, hautsetan dauden lipidoak kloroformo/metanol-ean (2:1 v/v) disolbatzen dira intereseko kontzentrazioan.
2. Disoluzio organikoan dagoen stock-atik, nahi den lipido kantitatea beirazko saio-hodi batera pipeteatzen da.
3. Disolbatzaile organikoa nitrogeno gas fluxu baten bidez lurruntzen da.
4. Disolbatzaile organikoa erabat ezabatzeko, lagina desgasifikatzaile batean 2 orduz hutsean mantentzen da. Horrela, disolbatzailerik gabeko lipido film lehor bat lortzen da beirazko saio-hodiaren behealdean.
5. Azkenean, lipidoa hidratatzeko, soluzio urtsu baten nahi den kantitatea pipeteatzen da, zeina lipido fasearen trantsizio tenperaturaren gaineko tenperaturan egon behar den. Ondoren, lipidoa saio-hodiaren behealdetik askatzeko, hodia nahasketa bortox batean sendoki irabiatzen da. Tenperatura igoz, lipidoa azkarrago hidratatzen da eta, lipido nahastura batekin besikulak prestatzean, lipido guztiak fase fluidoan mantentzen direnez besikulen lipido konposaketa homogeneoago bat lortu daiteke. Gehienetan, MLVak prestatzeko, metodo hau nahikoa izan ohi da.

2.3.3 Lamela Bakarreko Besikula Handiak (LUVak)

MLVak modu erraz batean eta azkar prestatzen dira, baina muga handi bat daukate. Besikulak agente disolbatzaile batekin tratatzen direnean, edota proteina bat besikula bati lotzean proteinaren efektua aztertzen ari garenean, soilik kanpo bigeruzaren lipidoekin izandako elkarrekintzen efektuak jaso daitezke. Honek, emaitzen analisia eta interpretazioa zailtzen du. Muga honi aurre egitearren, gehienetan lamela bakarreko besikulak erabiltzen dira (**2.2 Irudia**). Lamela bakarreko besikulak, beraien tamainaren arabera sailkatzen dira. Bataz besteko diametroa 60 nm baino txikiagoa dutenak lamela bakarreko besikula txikiak (SUVak, ingelesetik *small unilamellar vesicles*) izenez ezagutzen dira, bataz besteko diametroa 100 eta 500 nm bitartekoa daukatenak lamela bakarreko besikula handiak (LUVak, ingelesetik *large unilamellar vesicles*) bezala, eta mikretako diametro handiagoak daukatenak, lamela bakarreko besikula erraldoiak (GUVak, ingelesetik *giant unilamellar vesicles*) bezala. Tamainaz gain, prestateta metodoan ere beraien artean ezberdintzen dira.

Lamela bakarreko besikula handiek, besikula txikiek ez bezala, kurbadura estres txikia daukate, eta beraz, lipido espezie desberdinen nahasturaz osatutako LUVak prestatzean, gehienetan egonkorrak diren lipido-besikuletan bi monogeruzen arteko lipidoen banaketa homogeenoa bat lortzen da. Modu honetan, LUVak, ikerketa gehienak burutzeko sistema eredu oso egokiak dira. LUVak, gehienetan, intereseko poro tamainako polikarbonatozko filtro porotsuetan zehar egindako MLV esekiduren estrusio mekanikoaren bidez prestatzen dira (Mayer *eta lank.*, 1986). **3. Protokoloan** protokolo zehaztu bat deskribatzen da.

3. Protokoloa. LUVak

1. MLV prestateta protokoloaren 1-5 pausuak (**2. Protokoloa**).
2. Besikuletan lamela kopurua murrizteko asmoz, MLV soluzioari 10 izozte/urtze ziklo burutzen zaizkio. Hau, besikulak nitrogeno likidoan minutu batez mantenduz, eta ondoren, besikula osatzen duten lipidoetatik trantsizio tenperatua altuena daukan lipidoaren tenperaturaren gainetik dagoen ur bainu batean 3 minutuz mantenduz lortzen da. Azkenik, hurrengo zikloa hasi baino lehen, esekidura nahasketa bortex batean irabiatzen da.
3. Besikulak 10 aldiz estrusionatzen dira [LIPEX Liposome Extrusion System (Transferra Nanosciences, Burnaby, Kanada)] intereseko diametroa (gehlenetan 0.1 eta 0.4 μm bitartekoa) daukaten polikarbonatozko filtroak erabiliz, horretarako presio altuko estrutzaile bat (nitrogenozko gas fluxua) erabiltzen delarik. Besikulak tenperatura altu batean mantentzea beharrezkoa den kasuetan, estrutzailea ur bainu batera konekta daiteke.
4. Besikulen esekiduraren kontzentrazioa, Fiske-Subbarow metodoa erabiliz kalkulatu da (**6. Protokoloa**), zenbait lipido galdu daitezkeelako prozesuan zehar.
5. Azkenik, argiaren dispertsio dinamikoa (DLS, ingelesetik *dynamic light scattering*) erabiliz, besikulen tamaina egiaztatzen da (ik. 2.3.7 Atala).

2.3.4 Lamela Bakarreko Besikula Txikiak (SUVak)

Beraien diametro txikia dela eta (60 nm baino txikiagoa), SUVek kurbadura estres handia daukate, zeinak kanpo monogeruza, barne monogeruzarekin

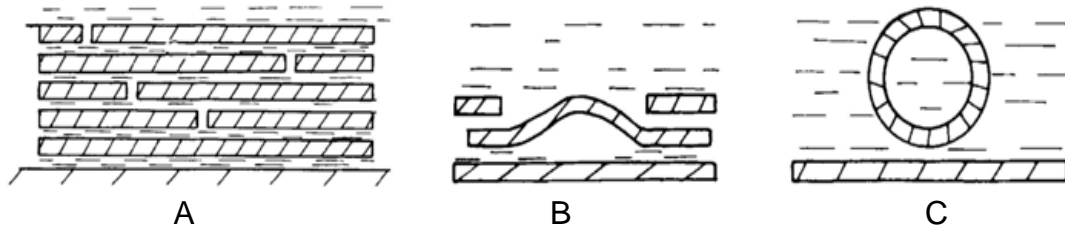
konparatuz, lipidoz aberastea eragiten duen (Szoka eta Papahadjopoulos, 1980). Beraien kurbadura estresak, besikula hauek mintz-eredu egokia izatea eragiten du, adib. mintzen arteko fusioa ikertzeko (Nieva eta *lank.* 1989). Izan ere, *in vivo* mintz zelularrak estres baldintza hauen menpean egongo litzateke. Lamela bakarreko besikulak, gehienetan MLV esekiduren sonikazio bidez prestatzen dira. Hau funtsean bainu sonikazioz edo sonikazio zunda baten bidez egiten da, azken kasuan sonikaziorako zunda MLV esekiduran barneratzen da. Horrela, MLV handiak, lamela bakarreko besikula txikiak murrizten dira. Lan honetan, lamela bakarreko besikula txikiak zunda baten bidezko sonikazioz (MSE Soniprep 150, MSE, Crawley, EB) prestatuak izan dira. Beraz, SUVak prestatzeko protokoloa MLVen protokoloaren berdina da, baina azkenean besikulen 20 minutuko sonikazio bat eginez (10 seg on/ 10 seg off) izotzean. Azkenik, SUV lagina 14500 rpm-tan 10 minutuz zentrifugatzen da, pilaketak edo sonikazio zundaren aztarna metalikoak kentzeko. SUVen eraketa zuzenean ikusi daiteke besikulen soluzioa gardena bihurtzen delako.

2.3.5 Lamela Bakarreko Besikula Erraldoiak (GUVak)

Gaur egun, lamela bakarreko besikula erraldoiak, mintzaren heteroogenitatearen inguruko ikerketan eredu sistema oso erabiliak dira. Elektroformakuntza bidez prestatutako GUVen tamaina, 5 eta 100 mikra artekoa da, bestela esanda, zelula batekin alderagarria. Honek, banakako besikulen mikroskopia zuzena egitea ahalbidetzen du. Besikula erraldioen sorrera, 1969an John P. Reeves eta Robert M. Dowben-ek deskribatu zuten lehen aldiz, lipido film lehorrak soluzio urtsuetan jarriz lipidoen fase trantsizioaren tenperaturaren gaineko tenperaturetan 24 orduz (Reeves eta Dowben, 1969). Besikula erraldoiak berez sortzen baziren ere, lipido totalaren ekoizpena oso baxua eta tamaina banaketa oso heterogeneoa zen.

1986an, Miglena I. Angelova eta Dimiter S. Dimitrov-ek besikula erraldoiak sortzeko metodo berri bat garatu zuten, metodo hau lipido film lehorrek soluzio urtsu batean eremu elektrikoaren menpe jartzean oinarritzen zen, eta gaur egun gehienetan erabiltzen den prozedura da (Angelova eta Dimitrov, 1986; Angelova eta *lank.*, 1992) (**2.3 Irudia**). Hasiara batean plazaratu zen prozeduraren antzera, soluzio urtsua fase trantsizio tenperatura altuena daukan lipidoaren tenperaturaren gainetik mantentzen da, baina kasu honetan metodoa askoz azkarragoa da eta tamaina homogeenoko besikula gehiago lortu daitezke. Eremu-elektrikoaren menpean egindako besikulen sorrera, lipido konposaketaren, soluzio urtsuaren indar ioniko eta pHaren, eta boltai elektriko eta frekuentzia baldintzen menpekoea da (Bagatolli, 2003). Bereziki, oso indar

ioniko baxuko indargetzaileak soilik erabili daitezke prozedura honen bidez GUVak prestatzeko. Hala ere, metodoa asko garatu da, eta gaur egun, baita gatz soluzio fisiologikoetan mintz zelularretatik abiatuta besikula erraldoiak eratzea ere posiblea da (Montes *eta* Iank., 2007).



2.3 Irudia. GUVen elektroformakuntzaren eskema. Ereku elektrikoak lipido filmak (A) haztea/fusionatzea (B) eta bata bestetik banatzea (C) eragiten du GUVak sortuz. (Angelova *eta* Dimitrov, 1986-tik moldatua).

Tesi lan honi dagokionez, besikula erraldoien erabilera garrantzitsu bat mikroskopia konfokala erabiliz banakako besikulen lipidoen albo banaketa ikertzea da. Helburu honekin, besikulak prestatzeko 2 prozedura ezberdin erabiliak izan dira: (i) esekiduran dauden besikula erraldoiak, eta (ii) platinozko hari batera itsatsitako besikula erraldoiak mikroskopiazko neurketa zuzenak egiteko. Bi prozeduren laburpen orokor bat ematen da. Saio konkretu bakoitzaren indargetzailea, eremu-elektroa edo mikroskopia baldintzak, dagokion kapituluaren Materialak *eta* Metodoak atalean zehazten dira.

2.3.5.1 GUVak Esekiduran

Esekiduran aurkitzen diren GUVak eratu ostean behaketa-ganberetara mugitzen dira (4. **Protokoloa**). Beraien behaketarako, dentsitate altuko sakarosa soluzio bat besikuletan enkapsulatzen da, horrela, besikulak soluzio isoosmotikodun ganberetara pasatzean ganberen behealdera jalkitzen dira *eta* irudikatuak izan daitezke.

4. Protokoloa. GUVak Esekiduran

1. Lipidoen stock puruak, komertzialki eskuragarri dauden lipidoak kloroformo/metanolean (2:1 v/v) diluituz 2 mM-ko kontzentrazioan prestatzen dira. Besikulak mikroskopio konfokal fluoreszente batean behatu ahal izateko, lipidoen stock-era zunda-fluoreszente baten nahi den portzentajea (%0.5 gutxi gora-behera) gaineratzen da.
2. Prozedura honetarako diseinatuak izan diren ganberak (Industrias Técnicas ITC, Bilbo) daukaten platinozko elektrodo ezberdinen gainazalera stock soluzioaren 4 μ L gaineratzen dira. Ganbera bakoitzak 4 putzu eta platinozko 2 elektrodo dauzka.
3. Ganbera hutsa egiten duen desgasifikatzaile batean sartzen da 2 orduz disolbatzailea guztiz kentzeko, eta ostean, ur bainu baten bidez nahi den tenperaturan 15 minutuz orekatzen da.
4. Gero, platinozko hariak aurretik intereseko tenperaturan orekatutako 300 mM-eko sakarosa soluzio batekin estaltzen dira.
5. Elektroformakuntzan zehar lurrunketa saihesteko, ganberaren putzuak porta baten bidez estaltzen dira.
6. Platinozko elektrodoak sorgailu batera konektatzen dira eta gutxi gora-behera 2 orduz intereseko eremu elektrikoa (870 mV) ezartzen da. Horrela, sakarosa enkapsulatua duten besikulak sortzen dira. Prestakuntzan zehar, tenperatura nahasturako lipidoetatik fase trantsizio tenperatura altuena duen lipidoaren tenperaturaren gainetik mantentzen da momentu oro.
7. Besikulak elektrodoetatik askatzea eragiteko, ezarritako frekuentzia (10 Hz) ordu 1 eta 30 minutuz jaitsi egiten da.
8. Eremu elektrikoa eta ur bainua deskonektatzen dira eta besikulak 30 minutuz orekatzen uzten dira.
9. Ondoren, besikulen esekidura behaketa-ganberetara pasatzen da, zeintzuk behi-serumaren albuminarekin (BSA, ingelesetik *bovine serum albumin*) (10 mg/mL) aurre-tratatuak izan diren eta soluzio isoosmotiko urtsu bat edo sakarosarik gabeko indargetzailea daukaten. Horrela, enkapsulatutako sakarosaren dentsitate handiagoa dela eta, besikulak jalkitzen dira, eta honek, fluoreszentiako mikroskopia konfokalaren bidezko neurketa zuzenak egitea ahalbidetzen du.

2.3.5.2 GUVak Hari batera Itsatsita

Mikroskopia bidezko neurketa zuzenak egiteko erabilitako GUVak, etxeke ganberetan eratzen dira eta platinozko hari batera itsatsita mantentzen dira (**2.4 Irudia**). Ganbera zuzenean mikroskopiopon jartzen da.

Platinozko hari batera itsatsitako GUVen prestaketa metodoa, esekiduran dauden besikula erraldioen prestaketa prozeduraren oso antzekoa da, baina 2 desberdintasun nagusiekin: (i) mikroskopia zuzena egiteko Luis A. Bagatolli-k (Odense, Danimarka) emandako ganbera berezia erabiltzen da, eta (ii) besikulak nahi den soluzio urtsu edo indargetzailean prestatzen dira, sakarosaren enkapsulazio beharrianik gabe (**5. Protokoloa**).



2.4 Irudia. Platinozko hari batera itsatsitako GUVak prestatzeko erabiltzen den etxeke ganbera eta politetrafluoroetilenozko (PTFE) putzu zirkularrak.

5. Protokoloa. GUVak Hari batera Itsatsita

1. Lipidoen stock puruak, komertzialki eskuragarri dauden lipidoak kloroformo/metanolean (2:1 v/v) diluituz prestatzen dira 0.2 mM-ko kontzentrazioan. Besikulen behaketa zuzena egiteko fluoreszentiako mikroskopia konfokala erabiliz, lipidoen stock-era zunda-fluoreszente baten nahi den portzentajea (%0.3 gutxi gora-behera) gaineratzen da.
2. Metodo honetarako diseinatuak izan diren PTFE-zko putzu zirkularretara itsatsita dauden platinozko elektrodo ezberdinen gainazalera, nahi den stock soluzioaren 5 μ L gehitzen dira.

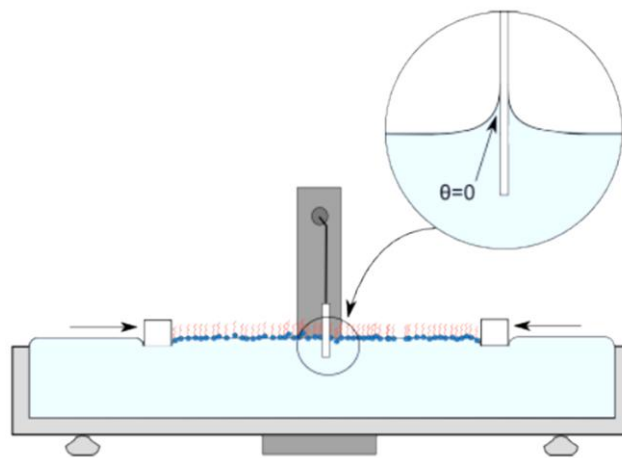
(Protokoloak hurrengo orrialdean jarraitzen du)

3. Disolbatzailearen edozein aztarna ezabatzeko, unitate zirkularrak hutsa egiten duen desgasifikatzaile batean 2 orduz mantentzen dira.
4. Gero, unitateak etxeko ganberaren putzu espezifikoetan sartzen dira (**2.4 Irudia**), zeinetara lehenago itsasgarri epoxidiko baten bidez beirazko estaldura bat itsatsi zaion.
5. Ondoren, ganbera nahi den tenperaturan orekatzen da 15 minutuz ur bainu bat erabiliz.
6. Gero, platinozko hariak aurretik dagokion tenperaturan orekatutako indargetzailearekin estaltzen dira, eta elektroformakuntzan zeharreko lurrunketa saihesteko neurri egokiko estalkiak jartzen dira.
7. Ganbera, platinozko elektrodoen bidez sorgailu batera (TG330 function generator, Thurlby Thandar instruments, Huntington, EB) konektatzen da eta dagokion eremu-elektrikoa ezartzen da 2 orduz, boltaia eta frekuentzien protokolo zehaztu bat jarraituz, adib.: 6 min 0.08 V/ 20 min 1.0 V/ 90 min 3.0 V.
8. Pausu honen ostean, eremu-elektrikoa eta ur bainua deskonektatzen dira eta besikulak 30 minutuz orekatzen uzten dira.
9. Azkenik, ganbera mikroskopiora mugitzen da eta platinozko harira itsatsitako besikulen behaketa zuzena burutzen da fluoreszentiako mikroskopia konfokala erabiliz.

2.3.6 Lipido Monogeruzak

Lipido-lipido edota lipido-proteina elkarrekintzak ikertzeko erabiltzen den beste eredu sistema bat ur-aire interfasean dauden molekula bakarreko filmetan oinarritzen da eta lipido monogeruza gisa ezagutzen dira. Besikulekin konparatuz, sistema honen abantaila garrantzitsu bat da ez daudela ez kurbadura ezta heterogenitate efekturik. Gainera, gainazaleko presioaren, lipido konposaketaren eta paketatze molekularren erabateko kontrola dago momentu oro. Bere desabantaila nagusia, lipido monogeruzetatik eskuratutako informazioa mintz biologikoen bigeruzetara estrapolatzearen ziurgabetasuna da. Hala ere, lipido-lipido elkarrekintzen jokaeraren inguruko informazio zehatza eskura daiteke eta hau zelula kultiboetan *in vivo* neurketak egitean ezinezkoa da.

Monogeruza bat lipido soluzio bat, aurretik prestatutakoa lipidoak kloroformo purua edo kloroformo/metanol bezalako soluzio organiko oso lurrunkorretan diluituz, soluzio urtsu baten gainean sakabanatuz sortzen da. Beraien natura anfipatikoa dela eta, lipidoak berez antolatzen dira, beraien buru hidrofiliakoak urarekin kontaktuan eta kate azilikoak airera begira jarriz. Behin eratuta dagoela, monogeruzan neurketa desberdinak burutzen dira bere gainazaleko presioaren kontrol zehatzan oinarrituz Langmuir balantza bat erabiliz. Hau normalean Wilhelmy metodoa erabiliz egiten da, non aire-ur interfasean kokatzen den eta kontrako muturretik elektro-balantza batera loturik dagoen zunda batek uraren gainazaleko tentsioaren edozein aldaketa neurtzen duen, adib. lipido monogeruzak eragiten dituenak (**2.5 Irudia**).



2.5 Irudia. Langmuir balantza bat non aire-ur interfasean lipido monogeruza bat dagoen. Eskualde handitua: Wilhelmy plakaren ukipen-angelua interfasean. (NIMA Technology, EB-tik hartua).

Kapitulu honen 2.7.2 atalean, Langmuir balantzaren oinarritutako lipido monogeruzaren inguruko metodoak, erabilipenak eta aspektu metodologikoak sakontasun handiagoarekin deskribatuak izango dira.

2.3.7 Argiaren Dispertsio Dinamikoaren (DLS) bidezko Besikulen Tamainaren Neurketa

Besikulen tamainaren neurketak argiaren dispertsio kuasi-elastikoa (QELS, ingelesetik *quasi-elastic light scattering*) teknikaren bidez egin ziren, normalean argiaren dispertsio dinamiko (DLS) gisa ezagutzen dena eta partikulak soluzioan daukaten mugimendu Browndarrarekin lotuta dagoena. Soluzioaren eskualde txiki batean fokatuta dagoen laser bat erabiliz, fokatutako eskualdea zeharkatzen duten lagineko partikulek dispertsatutako argiaren intentsitatearen fluktuazioak neurtzen dira.

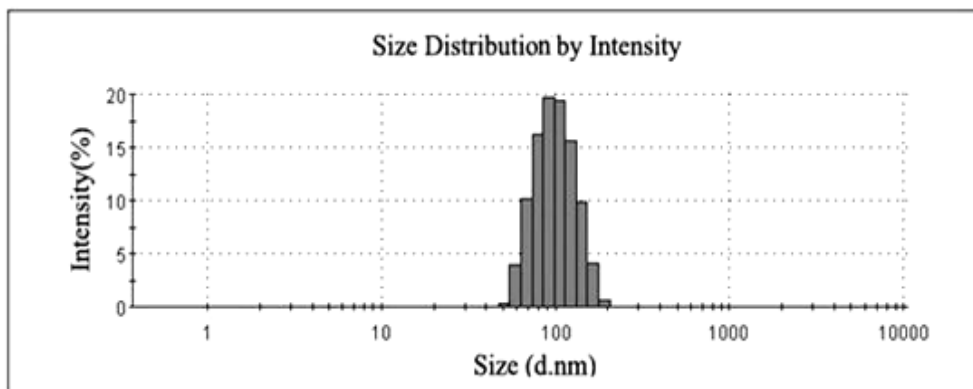
Partikulek, gure kasuan besikulak, argia dispertsatzen dute eta hau Stokes-Einstein ekuazioaren bidez, besikularen erradio hidrodinamikoarekin (R_h) erlazionatzen da.

$$D = k_B T / 6 \pi \eta R_h$$

1 Ekuazioa. Stokes-Einstein ekuazioa.

non: D difusio koefizientea, k_B Boltzmann-en konstantea = $1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}$, T tenperatura, η medioaren biskositatea eta R_h partikula esferikoaren erradio hidrodinamikoa diren.

Dispertsatutako argiak korrelazio funtzio bat ematen du, zeinetatik, algoritmo batzuk erabiliz, soluzioan dauden besikulen tamainaren banaketa lor daitekeen (**2.6 Irudia**). Balio hau laginaren tamainaren heterogeneotasunaren inguruko informazioa ematen duen polidispertsitatearen indize (PDI, ingelesetik *polydispersity index*) batekin batera doa. PDI 0 eta 1 bitartekoa izan daiteke, non zerotik gertu dauden balioak soluzio monodispertso homogeneoak adierazten dituen.



2.6 Irudia. 100 nm-tako polikarbonatozko filtroak erabiliz prestatutako PC besikulen populazio baten tamainaren banaketa adierazten duen grafikoa.

Tesi lan honetan, neurketak 0.6 nm-tik 6 μm -ra bitarteko detekzio ahalmena daukan Malvern Zeta-Sizer Nano ZS (Malvern Instruments, Worcestershire, EB) baten bidez egin ziren. Laginaren 60 μL (lipidotan 0.4 mM inguru) kubeta akriliko arruntetan jarri ziren, giro tenperaturan eta 5 mW-ko He-Ne laser bat ($\lambda = 633 \text{ nm}$) erabiliz argi iturri bezala. Besikulek dispertsatutako argia, laserrarekiko 173^o-tara kokatutako fotomultiplikatzailen baten bidez detektatu zen eta emaitzak aparatuaren programa komertzialarekin aztertu ziren.

2.4 Fosfolipido Saioa (Fiske-Subbarow Metodoa)

Fosfolipidoen saioa burutzeko, ongi ezarrita dagoen eta fosforo inorganikoaren kuantifikazioan oinarritzen den metodoa erabili zen. Metodo hau, 1925ean Cyrus H. Fiske eta Yellapragada Subbarow-en hasierako prozeduran oinarritzen da (Fiske eta Subbarow, 1925), geroago eraldatua izan zena (Bartlett, 1959 and Böttcher *eta lank.*, 1961). Laburki, metodo hau fosfolipidoak hidrolizatzean datza, lipidoen fosfato taldea errektibo espezifikoekin elkarrengaitzeko libre geratu arte. Horrek kontzentrazioaren arabera soluzioa koloreztatuko du (**6. Protokoloa**).

6. Protokoloa. Fosfolipido Saioa (Fiske-Subbarow Metodoa)

1. Laginak daukan lipido kontzentrazio zehatza ezartzeko beharrezkoa izango den fosforo kalibrazio kurba bat egin behar da. Beirazko hodian bi aleetara, 1 mM NaH_2PO_4 soluzio batetik fosforoaren 0, 25, 50, 75 eta 100 nmol pipeteatzen dira hurrenez hurren.
2. Lagina beirazko hodi ezberdinetara pipeteatzen da (gutxienez hiru ale). Pipeteatutako kantitateak, fosforo lipidikoaren teorikoki 50 nmol izateko kalkulatu dira, kalibrazio kurbaren erdigunean kokatua egongo dena. Soluzio organikoetan bolumenak neurtzean, Hamilton ziringak erabiltzen dira.
3. Hodi bakoitzera, %60 azido perklorikoaren (HClO_4) 500 μL pipeteatzen dira. Hodiak nahasketa bortex batean sendoki irabiatuak eta 45 minutuz 205°Ctan dagoen beroketa plaka batean sartzen dira. Horrela, fosfolipidoen hidrolisiaren bidez fosfato inorganiko librea lortzen da.
4. Hodiak hartu, giro tenperaturan hozten dira, eta ondorengo soluzioak gaineratzen zaizkie:
 - 4 mL 1X amonio heptamolibdato soluzioa $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}]$.
 - 500 μL 10% (v/v) azido askorbikoa (hodiak bortex baten laguntzaz irabiatzen diren bitartean gaineratua).

Fosfato inorganikoak molibdatoarekin erreakzionatzen du, zeinak jarraian azido askorbikoarekin erreakzionatzen duen hori-koloreko soluzio bat emanez.

(Protokoloak hurrengo orrialdean jarraitzen du)

5. Hodiak irakiten dagoen ur bainu batean sartu eta 6 minutuz bertan mantentzen dira. Denbora tarte horretan, soluzioaren kolorea urdin bilakatzen da fosforo kantitatearen arabera.
6. Azkenik, hodiak uretan hoztu eta lagin bakoitzaren absorbantzia 812 nm-tan neurtzen da. Lan honetan, Amersham Biosciences-eko (Piscataway, NJ, EEBB) Ultrospec 500 pro espektrofotometro bat erabili zen horretarako.
7. Laginen fosfolipido kontzentrazioa kalkulatzeko, absorbantzia estandarra fosforo kontzentrazioaren aurka irudikatzen da eta lortutako puntuak lerro zuzen batera doitzen dira. Laginaren kontzentrazioa kalkulatzeko kurbaren malda eta laginaren absorbantzia erabiltzen dira.

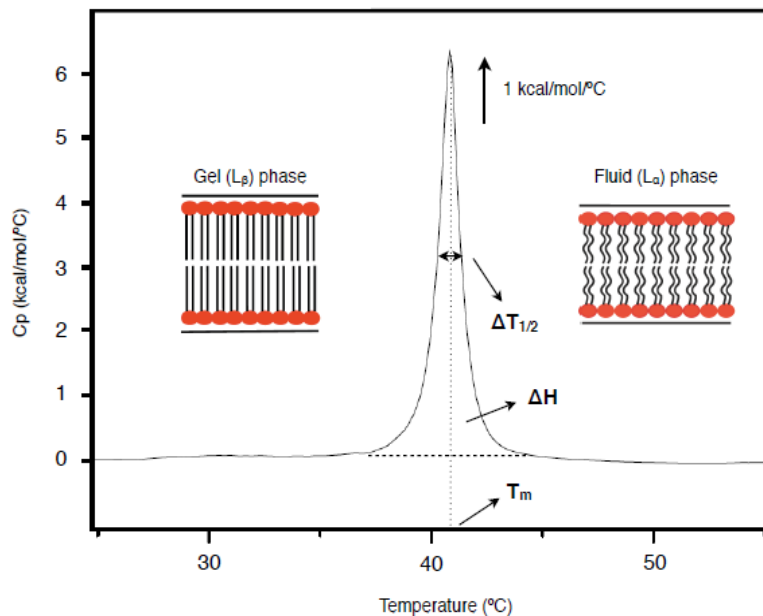
2.5 Ekorketa Kalorimetria Diferentziala (DSC)

DSC, lipido-fase trantsizioak neurtzeko teknika indartsua da. Ganbera adiabatiko batean ematen den temperatura igoera kontrolatu baten ondorioz gertatzen diren 2 gelaxken arteko bero aldaketetan oinarritzen da. Modu honetan, fase trantsizio termotropikoen parametro termodinamikoak zehaztu daitezke. DSC teknika lipidoen ezaugarri fisikoak neurtzeko askotan erabiltzen da. Honetaz gain, teknika honen bitartez ere proteinen desnaturalizazioa, proteina-ligando lotura edota proteinen aldaketa konformazionalak ikertu daitezke. Tesi lan honek DSCaz baliatuko da lipido domeinuen eraketaren inguruko informazioa eman dezaketen lipido-fase trantsizioak aztertzeko.

Bi gelaxketan, erreferentzia bat (gehienetan saioaren indargetzailea) eta MLV eran dauden lipidoak bolumen berdinean gehitzen dira hurrenez hurren (soluzioak beti ondo desgasifikatzen dira). Ondoren, gelaxkak ixten dira eta presio altu konstante batera eramanak dira, likidoen irakitea eta ponpen eraketa saihesteko. Bi gelaxkek Peltier sistema bat daukate laginak berotzeko eta hozteko temperatura aldaketa kontrolatuetan, eta bi gelaxken arteko temperatura ezberdintasuna neurtzen duen sistema baten bidez konektatuta daude. Beroketan zehar, gelaxketako temperatura berdin igotzen da laginak eraldaketa fisiko bat jasan arte. Eraldaketa honetan, energia askatu edo xurgatu dezake, eta ondorioz, laginaren gelaxkaren temperatura erreferentziazko gelaxkarekiko aldatu egiten da. Temperatura ezberdintasun honen aurrean, bi gelaxketan temperatura bera mantentzeko asmoarekin, sistemak lagina

dagoen gelaxkari beroa eman edo kentzen dio, aldaketa fisikoa endotermikoa edo exotermikoa izatearen arabera, hurrenez hurren. Parametro hau, kalorimetroak bero-ahalmen (c_p , ingelesez *heat capacity*) gisa adierazten du, energia x temperatura⁻¹ unitateetan. Kasu honetan, beroa energia forma da. Tesi lan honen testuinguruan, lipido-fase trantsizioak endotermikoak dira, eta gehienetan, lipidoen kontzentrazio molarrekin normalizatzen dira, ondorioz, c_p -a gehienetan kcal/mol°C edo kJ/mol°C unitateetan (energia termikoa jouletan adierazten bada) ematen da.

Lagin baten ekorketaren emaitza termograma izenez ezagutzen da, zeina c_p vs temperatura adierazten duen grafiko bat den. Lipido-fase trantsizioen termogramek, normalean maximo bat daukate, zeinetatik 3 parametro nagusi eskura daitezkeen: lipido-fase trantsizio temperatura (T_m), zabalera lipido-fase trantsizioaren altueraren erdian ($\Delta T_{1/2}$) eta lipido-fase trantsizio entalpia (ΔH). T_m gehienetan trantsizioaren maximoari dagokio, bestela esanda, trantsizio simetrikoen erdialdeari. $\Delta T_{1/2}$ °Ctan ematen da eta trantsizioaren kooperatibitatea edo lipido molekulen arteko kooperatibitatearen inguruko informazioa ematen du, kooperatibitate handia daukaten fase trantsizioek $\Delta T_{1/2}$ baxuagoa erakusten dutelarik. ΔH k, fase trantsizioa emateko beharrezkoa den bero kantitatea neurtzen du, eta fase trantsizio tenperaturetan zeharreko c_p -aren integrazioaren bidez lortzen da. **2.7 Irudiak** esfingomielinaren endoterma arrunt bat eta honi dagozkion parametro termodinamikoak adierazten ditu.



2.7 Irudia. Palmitoilesfingomielina (pSM) puruaren DSC termograma. Fase trantsizio endotermiko nagusia eta dagozkion parametro termodinamikoak erakusten dira.

Lipido nahasturetan, DSC bidez normalean neurtzen diren lipido-fase trantsizioak L_{β} - L_{α} eta L_{α} -alderantzizko lipido-fase hexagonala (H_{II}) trantsizioak dira. Laginen prestakuntzarako, ekorketa egiteko eta emaitzen analisirako protokolo labur bat deskribatzen da (**7. Protokoloa**).

7. Protokoloa. MLVen Ekorketa Kalorimetria Diferentziala

1. Aurretik desgasifikatutako (gutxienez 30 minutu) saioaren indargetzailea: 20 mM PIPES, 150 mM NaCl, 1 mM EDTA (pH 7.4) kalorimetroaren bai erreferentzia zein laginaren gelaxkan kargatzen da. Lan honetan, VP-DSC sentikortasun handiko ekorketa mikrokalorimetroa (MicroCal, Northampton, MA, EEBB) erabili zen.
2. Laginaren baldintza berdinetan indargetzailearen erreferentziako termograma lortzeko, beroketa ekorketa bat egiten da.
3. Termograma bukatu ondoren, laginaren gelaxkako indargetzailea, indargetzaile berdinean MLV eran dagoen lipido laginarengatik ordezkutzen da, zeina aurretik ondo desgasifikatu den.
4. Ondoren, gutxienez 3 beroketa ekorketa burutzen dira seinale konstante bat eskuratzen den arte, parametro termodinamiko espezifikoak kalkulatzeko.
5. Lagina hartu eta bere kontzentrazioa fosforo lipidikoa neurtuz kalkulatu da.
6. Emaitzen analisirako [kalorimetroaren ORIGIN 7.0 (MicroCal) programa erabiliz], laginaren hirugarren ekorketari indargetzailearen erreferentziako termogramaren seinalea kentzen zaio, eta sortzen den termogramaren base lerroa doitzen da. Laginaren kontzentrazioa normalizatzen da eta parametro espezifikoak zehazten dira.

2.6 Proteinen Analisia

2.6.1 Proteina Kontzentrazioaren Neurketak

Proteina kontzentrazioa, BCA (ingelesetik *bis-cinchoninic acid*) proteina saioa (Thermo Fisher Scientific) baita 280 nm-tan egindako absorbantziaren neurketak erabiliz ere kalkulatu zen.

2.6.1.1 BCA Saioa

BCA kolore sorrera eragiten duen erreakzioa, proteinaren aminoazido sekuentziaren 4 aminoazido (zisteina edo zistina, tirosina, eta triptofanoa) baldintzatzen dute. Hala ere, peptidoen ardatz nagusiak ere kolore sorreran eragina du, proteinen osaketa ezberdintasunak eragindako aldakortasuna gutxitzen duelarik. Lehenengo pausuan, Biuret erreakzioa bezala ezagutua, ingurune alkalino batean ematen den kobreaken kelazioak proteinarekin, urdin argi koloreko konplexu bat eratzen du. Bigarren pausuan, BCAk lehenengo pausuan erreduzitutako katioiarekin (kuprosoa) erreakzionatzen du.

BCA-kobre konplexua uretan disolbagarria da eta proteina kontzentrazioaren igoerarekiko lineala den absorbantzia dauka 562 nm-tan. Saioa ohiko protokoloari jarraituz eta aurre-diluitutako BSA proteina saio-estandarrek erabiliz gauzatu zen.

2.6.1.2 Absorbantzia 280 nm-tan

Proteinak eraztun aromatikoak duten aminoazidoak izateak, soluzioan argi ultramorea xurgatzea ahalbidetzen du, absorbantzia maximoa 280 nm-tan dutelarik. Egitura sekundario, tertziario, eta kuaternarioek guztiek absorbantzia eragina dute, beraz, pHa edo indar ionikoa bezalako faktoreek absorbantziaren espektroa alda dezakete. Lambert-Beer ekuazioa jarraituz eta iraungipen koefiziente teoriko bat erabiliz, proteinen kontzentrazioa kalkula daiteke.

$$A = \epsilon c l$$

2 Ekuazioa. Lambert-Beer ekuazioa.

non A absorbantzia, ϵ iraungipen molarraren koefizientea, c kontzentrazioa eta l kubetaren pasu optikoa den.

2.6.2 Proteinen Elektroforesia

Proteinen banaketa %12.5 edo 15 (w/v) akrilamida geletan, sodio dodezil sulfatozko poliakrilamidako elektroforesi gelak (SDS-PAGE) erabiliz egin zen. Laginak β -merkaptotanol osagarri moduan duen Laemmli karga indargetzailearekin (6x) nahastu eta 90°Ctan 5 minutuz berotu ziren. Masa molekularren markatzaile gisa, proteina estandarrek (tindatu-gabekoak eta aurre-tindatuak) (Bio-Rad) erabili ziren. Elektroforesia, 3.028 g Tris, 14.41 g glizina, 1 g SDS eta H₂O 1 L daukan indargetzailean eta 150 V-tan burutu zen. Proteinen behaketa azkar baterako, gela gutxienez 10 minutuz 0.1% (v/v) Coomassie Distiratsu Urdina R-250, %40 (v/v)

metanola eta %10 (v/v) azido azetikoak daukan soluzio batekin tindatu zen. Atzeko-planoko tindaketa %10 (v/v) azido azetikoarekin garbituz ezabatu zen eta irudiak Gel Doc™ EZ Imager (Bio-Rad) baten bidez hartu ziren.

2.6.3 Western Blotaketa

Proteinak SDS-PAGE getetik, nitrozelulosazko mintzetara (Bio-Rad) transferitzeko, Trans-Blot® (Bio-Rad) ekipoa erabili zen. Gelari Trans-Blot filtro paperez eta nitrozelulosazko mintz batez egindako sandwich bat egin zitzaion eta sandwicha transferentzia indargetzailearekin (2.25 g glizina, 5.81 g Tris, pH 8, 0.375 g SDS, 200 mL MetOH eta H₂O 1L arte) hidratatu zen. Proteinen transferentzia 22 V-tan 30 minutuz eta giro tenperaturan egiten zen. Proteina transferitu ondoren, mintzak esne gaingabetuaren %5a duen fosfato gatz indargetzailearekin (PBS, ingelesetik *phosphate buffered saline*) blokeatu eta antigorputz primarioarekin (1:1000) eta errefau peroxidasa (HRP, ingelesetik *horseradish peroxidase*) batekin konjugatutako antigorputz sekundario espezifikoarekin (1:5000tik 1:10000ra) inkubatu ziren. Mintzen garbiketa egin ostean, SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) errektiboekin, Curix 60 prozesadore baten bidez proteinak detektatu ziren. Laginaren seinalearen intentsitatea GS-800 dentsitometro (Bio-Rad) baten bidez kuantifikatu zen.

2.7 Proteina-Lipido Elkarrekintzak

Proteina eta mintz biologikoen arteko elkarrekintzak zehazten dituzten mekanismo molekularrak ulertzeko, mintza lotzeko prozesuaren afinitatea eta espezifikotasuna eta proteinak bigeruz lipidikoan hartzen duen topologia edo konformazioa ikertuak izan behar dira.

Mintz proteinen bereizmen handiko egiturak lortzeko, X-izpi bidezko kristalografia edo elektroien kristalizazioa erabil daitezke. X-izpi bidezko kristalografiaren kasuan, lipidoek askotan ez diote kristalizazio prozesuari aurre egiten, eta ko-purifikatu eta kristalizatuak izatekotan, kristalean ez dira behar bezain ordenatuak agertzen beraien karakterizazio zehatza egiteko (Palsdottir eta Hunte, 2004). Hori dela eta, lipido inguru batean dauden mintz-proteinen egitura zehazteko bi dimentsioko (2D) kristalen bereizmen atomikoarekin, elektroien kristalizazioa gero eta gehiago erabiltzen da (Raunser and Walz, 2009). Kristalak lortu ezin diren egituren kasuan, NMR espektroskopia (Aisenbrey *eta* *lank.*, 2013), Fourier-en transformatuaren

bidezko espektroskopia infragorria (FT-IR, ingelesetik *Fourier-transform infrared spectroscopy*) (Subirade *eta lank.*, 1995), edo elektroien erresonantzia paramagnetikoa (EPR, ingelesetik *electron paramagnetic resonance*) (Marsh, 2008), proteina-lipido elkarrekintzen inguruko informazio baliagarria eman dezaketen teknikak dira. Mintz proteinak ikertzeko, dinamika molekularren simulazioak ere erabil daitezke (Lindahl and Sansom, 2008). NMR edo X-izpitan oinarritutako metodoetan, proteina eta lipidoen mugimendu eta elkarrekintzak denbora- eta espazio-bereizmen handiarekin eta zehaztasun atomikoan monitorizatu daitezke. Duela gutxi, horrelako *in silico* metodo bat erabili da, proteina baten mintz zeharreko domeinuaren bidezko esfingolipido bakar baten ezagutze molekularra ikertzeko (Contreras *eta lank.*, 2012).

Aipatutako bereizmen handiko teknikez gain, proteina eta mintzen arteko elkarrekintzak gidatzen dituzten mekanismo molekularrak ikertzeko, beste hainbat saiakuntza fluorometriko, zentrifugazio bidezko eta mikroskopikoak ere eskuragarri daude (Contreras *eta lank.*, 2011; Zhao *eta* Lappalainen, 2012). Saio hauetako bakoitzak informazio mota ezberdina ematen digu eta muga espezifikoak dauzka.

Tesi lan honetan, lipidoen dot plapaketa saioak, gainazaleko presioaren neuketak eta sakarosa gradiente bidezko liposomen flotazio zentrifugazioa erabili izan dira LC3B, GABARAPL2 *eta* GABARAPek autofagiarekin lotutako zenbait lipidoekin lotzeko daukaten ahalmena aztertzeko. Proteina espezifiko baten mintzarekiko elkarrekintzak ikertzerako orduan hainbat faktore eduki behar dira kontuan. Faktore hauen artean aurkitzen dira: (i) besikularen lipido konposaketa, (ii) proteina:lipido ratioa, (iii) mintzaren kurbadura, (iv) pHa *eta* (v) indargetzailearen gatz kontzentrazioa.

2.7.1 Proteina-Lipido Gainjartze Saioa (Lipidoen Dot Plapaketa)

Proteina-lipido gainjartze (PLO, ingelesetik *protein-lipid overlay*) saioak lipidoetara lotzen diren proteinen lipido ligandoak identifikatzea ahalbidetzen du (Dowler *eta lank.*, 2002). Saio hau erabiliz, PH (Lemmon *eta lank.*, 1995), endosoma goiztiarraren 1-antigenoa (EEA1, ingelesetik *early endosome antigen 1*) (FYVE) (Stenmark *eta lank.*, 1996), *eta* PX homologia (Xu *eta lank.*, 2001) domeinuak dauzkaten hainbat proteinen lipidoak lotzeko espezifitateak ikertu dira. Kasu askotan, PLO saioen bidez zehaztutako proteinen espezifitateak beraien lipido ligandoekiko, proteina-lipido elkarrekintzak aztertzeko metodologia konplexuagoak erabiliz lortu diren emaitzekin bat datozela ikusi da.

Aurretik aipatu den bezala, PLO batean lortutako emaitzak kualitatiboak dira. Loturaren konstante kuantitatiboak lortzeko, gainazaleko plasmoien erresonantzia (SPR, ingelesetik *surface plasmon resonance*) edo kalorimetria bidezko balorazio isotermikoa (ITC, ingelesetik *isothermal titration calorimetry*) (Swamy eta Sankhala, 2013) bezalako teknikak gomendatzen dira. Hala ere, azken teknika hauek nahiko nekezak dira eta ekipamendu askoz garestiagoa beharrezkoa da. Beste lotura saio batzuen aurrean PLO saioak daukan abantaila nagusia, aldi berean hainbat proteinek lipido natural askorekiko dauzkaten lotura espezifitateak iker daitezkeela da. Mota honetako saiotik lortutako emaitzak, proteina ezberdinek lipido(ar)ekiko daukaten afinitate erlatiboaren indikatzaile kualitatibo onak dira. Gainera, PLO saiotik lortutako lehen emaitzak immunoplapaketa arrunt baten moduan daude, zeintzuk era semikuantitatibo batean interpreta daitezkeen irudien analisirako programak erabiliz.

Tesi lan honetan, lipidoen dot plapaketa **8. Protokoloan** deskribatzen den Dowler *eta lank.*, 2002-etik moldatutako prozedura jarraituz egin zen.

8. Protokoloa. Proteina-Lipido Gainjartze Saioa

1. Lipido ezberdinen stock soluzioak 2:1:0.8 MeOH:CHCl₃:H₂O-an disolbatzen dira. Beharrezkoa izatekotan, stock-aren 1:3 diluzio segidak prestatzen dira.
2. Lipido tantak (0.5 µL) Hybond C nitrozulosazko (GE Healthcare) mintzera gaineratzen dira eta ordu batez giro tenperaturan lehortzen uzten dira.
3. Proteina immunoplapaketa bidez detektatzeko, lehenengo, ordu betez eta giro tenperaturan esne gaingabetuaren %5a daukan PBSa erabiliz, nitrozulosazko mintza blokeatzen da, horrela elkarrekintza inespezifikoak saihesten dira.
4. Mintza beste ordu betez inkubatzen da proteinarekin PreScission indargetzailean 37°Ctan.
5. Nitrozulosazko mintza, 4 aldiz PBSarekin garbitzen da, 10 minutuz bakoitza, eta esne gaingabetuaren %5a daukan PBSan diluitutako antigorputz primarioarekin, 1:1000 diluzioan, inkubatzen da gauean zehar 4°Ctan.

(Protokoloak hurrengo orrialdean jarraitzen du)

6. Mintza, PBSarekin 4 aldiz garbitzen da eta jarraian esne gaingabetuaren %5a daukan PBSan diluitutako HRP-antigorputz sekundarioarekin (Santa Cruz Biotechnology, Dalas, TX, EEBB), 1:5000 diluzioan, ordu betez giro tenperaturan inkubatzen da.
7. PBSarekin 10 minutuko 4 garbiketa egin ostean, proteina kimiolumineszentzia bidez detektatzen da. Proteina bandak digitalizatu eta Quantity One programa (Bio-Rad) erabiliz dentsitateak neurtzen dira.

2.7.2 Langmuir Balantza

Beraien ezaugarri anfipatikoa dela eta, lipidoak ur gainazal baten gainean sakabanatzean beraien buru polarrak ur gainazalarekin kontaktu zuzenean eta kate aziliko hidrofobikoak airerantz begira orientatuak dituzte. Modu sinple honetan, aire-ur interfasean molekula bakarreko lipido film bat sortzen da, beste hitz batzuetan esanda, lipido monogeruza bat (ik. 2.3.6 Atala). “Langmuir filmak” eta “Langmuir balantza” terminoek, horrelako egituren prestaketa eta karakterizazioari erreferentzia egiten diete. Izen horien jatorria, kimikari eta fisikari amerikarra zen Irving Langmuir-ek arlo honen inguruan egindako ikerketa askoren ondoriozkoa da (Langmuir, 1917). Gainazalen kimikan egindako lanagatik, Langmuirrek kimikako Nobel saria jaso zuen 1932.urtean.

Langmuir balantzaren bidezko neurketak, lipido-lipido eta lipido-proteina elkarrekintzen karakterizaziorako iturri garrantzitsu dira. Besikulen edo bigeruzan lipidikoen erabilerarekin ez bezala, Langmuir balantzak, albo presioa, molekulen paketatzea eta lipido konposaketa estuki kontrolatzeko abantaila dauka. Besikulekin konparatuz, kasu honetan ez daude kurbadura edo fluktuazio efekturik. Gainera, teknikaren egungo garapenari esker, posiblea da monogeruzan zunda-fluoreszenteak erabiliz mikroskopia neurketa zuzenak egitea, edo lipido monogeruza indar atomikoko mikroskopia bidez aztertze euskarri solidoetara pasatzea. Lan honetan, Langmuir balantzan oinarritutako metodoak lipido-proteina elkarrekintzak ikertzeko erabili dira.

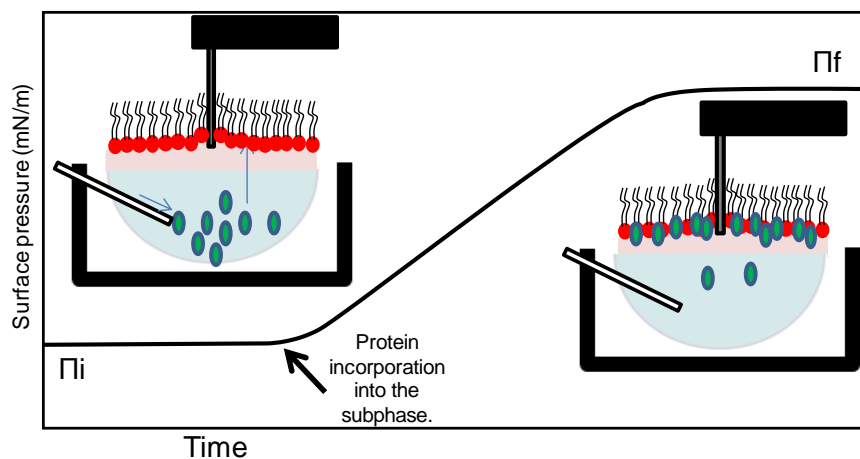
2.7.2.1 Gainazaleko Presioaren Neurketak

Langmuir balantzaren bidezko metodoak, kontrako muturretik elektro-balantza

batera loturik dagoen zunda baten bidezko uraren gainazaleko tentsioaren neurketan oinarritzen dira. Metodoa, Ludwig Wilhelmy-tik datorren “*Wilhelmy plate*” izenez ezagutzen da. Wilhelmy-k, 1863an, lehen aldiz gainazaleko tentsioa neurtzeko metodo bat garatu zuen (Wilhelmy, 1863). Wilhelmy-ren xafla oso fina da eta gehienetan platinoz edo kartoiz egina dago, baina kuartzo, beira edo mika bezalako beste material batzuk ere erabili izan dira. Aurretik azaldu den bezala, Wilhelmy-ren metodoak gainazaleko tentsioa neurtzen du, zeina gas-likido interfasean dagoen gehiegizko kohesio-energia libre den. Ur molekula inguruko molekulen indarren eraginpean daude, zeinak norabide guztietara dauden erakarpen indar berdinen eraginez ur soluzioaren gehiengo partean orekatuak dauden. Dena den, gas-likido interfasean, interfasean dauden ur molekula ez daude norabide guztietako erakarpen indarren menpean, eta ondorioz, gehiegizko kohesio-energia bat sortzen da, zeina gainazalaren energia libre gisa ezagutzen den. **2.8 Irudian** adierazten den bezala, gas-likido interfasean partzialki barneratuta dagoen zundak detektatzen dituen indarren arabera, gehiegizko energia hau indar/luzera gisa gainazaleko tentsioarekin lotu daiteke, zeina aldi berean gainazaleko presioekin lotu daitekeen. Zundak detektatzen dituen indarrak ondokoak dira:

Indar totala = zundaren pisua + zundaren flotagarritasuna + gainazaleko tentsioa

3 Ekuazioa. Langmuir balantzaren zundak detektatutako indarrak.



2.8 Irudia. Lipido monogeruza batean proteinen txertaketa prozesuaren irudikapen eskematikoa. Lipido monogeruza saio baten prestaketa. Proteina gehitu aurretik, monogeruzaren hasierako presioa nahi den baliora doitzen da (π_i). Proteina monogeruzan txertatzen da gainazaleko presioa igoaraziz orekara heldu arte (π_f).

Neurketak egiten hasi baino lehen, zundak detektatzen duen tentsioa zerora doitzen da, honela pisuaren indarra baztertzen delarik. Zundaren flotagarritasunak, zundaren barneraketaren ondorioz lekuz mugituak izan diren ur molekulek egiten duten indarra adierazten du, hau ere, neurketak dirauen bitartean zunda sakontasun konstantean mantenduz baztertzea lortzen da. Beraz, indar totala gainazaleko tentsiora murriztu daiteke, zeina ondorengo ekuazioan azaltzen den:

$$F = 2(w + t)(\gamma)(\cos \Theta)$$

3 bis Ekuazioa. Indar totalaren ekuazio murriztua.

non: w, t = zundaren pisu eta lodiera, γ = gainazaleko tentsioa, Θ = ukipen-angelua.

Zunda soluzio urtsuan barneratzean, menisko bat sortzen da, zero graduko ukipen-angelu bat sortuz, **2.5 Irudian** adierazten den bezala. Horrela, ekuazioa honako hau izango litzateke:

$$\gamma = \text{Indarra/Perimetra}$$

4 Ekuazioa. Gainazaleko tentsioa.

edo indarra/luzera zeina dina/cm unitateetan ematen den = mN/m.

Ur molekulek, beraien molekulen arteko erakarpen indar handiak direla eta, ezagutzen den gainazaleko tentsio altuenetariko bat eragiten dute, 72.8 mN/m 22°Ctan eta 1 atm-tan. Molekula anfiptikoen presentziak aire-ur interfasean, ur molekulen kohesio-energia galarazten du, uraren gainazaleko tentsioa jaitsiaraziz. Jaitsiera hau gainazaleko presio (π) gisa ezagutzen da, unitate berdinekin baina aurkako zeinuarekin.

$$\pi = \gamma_o - \gamma$$

5 Ekuazioa. Gainazaleko presioa.

Azkenik, Langmuir balantzarekin egiten diren neurketa guztiak gainazaleko presioaren aldaketetan oinarritzen dira. Normalean, oso lurrunkorra den disolbatzaile organiko batean [gehienetan kloroformo purua edo kloroformo/metanola (2:1 v/v)] dagoen lipido soluzio bat, soluzio urtsu baten gainazalean sakabanatzen da (aire-likido interfasea). Disolbatzaile organikoa lurruntzen den bitartean, lipidoak beraien kabuz antolatzen dira buru polarrak soluzio urtsurantz eta kate hidrofobikoak airerantz

orientatuz. Lipidoa eskuragarri dagoen azalera zehar sakabanatzen doan bitartean, uraren gainazaleko tentsioa gutxitzen da, hau da, gainazaleko presioa handitzen da. Horrela, adibidez lipido-proteina elkarrekintzak ikertzeko, lipido monogeruza bat prestatzen da nahi den lipido kantitatea aire-ur interfasean sakabanatuz gainazaleko presio kontrolatu batean. Ondoren, proteina azpifasean gaineratzen da eta proteinaren txertaketa edo adsortzioak monogeruzan eragiten dituen gainazaleko presioaren aldaketei dagokionez, lipido-proteina elkarrekintza aztertzen da (**2.8 Irudia**). Proteina batek monogeruzan txertatzeko daukan ahalmena ikertzeko, bere presio kritikoa (π_c) zehaztea oso erabilgarria da. Presio kritiko hau, gainazaleko presioaren igoera ($\Delta\pi$) balioa zero deneko hasierako gainazal presioaren (π_i) balioa da. Kontuan edukitzeko da, mintz zelularrek 30 mN/m inguruko bataz besteko gainazaleko presioa daukatela proposatu dela (Janmey eta Kinnunen, 2006; Marsh, 1996), dena den balio hau asko alda daiteke.

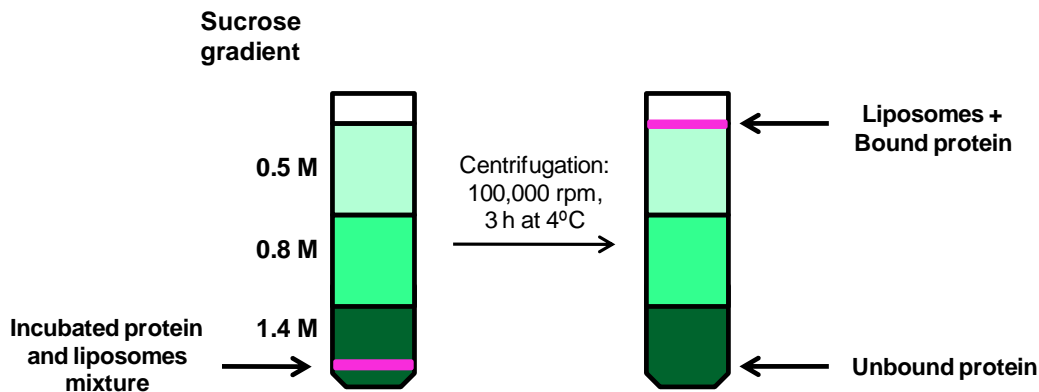
Langmuir filmak prestatzeko metodologiaren inguruko berrikustapen bat Cruz eta Pérez-Gil, (2007)-an aurki daiteke. Tesi lan honetan esperimendu bakoitza burutzeko erabilitako metodoaren baldintza zehatzak, dagokion kapituluaren Materialak eta Metodoak atalean azalduko dira.

2.7.3 Sakarosa Gradiente bidezko Liposomen Flotazio Zentrifugazioa

Liposomen flotazio saioak asko erabiltzen dira mintzetara lotzen diren proteinen afinitatea eta lipido espezifikotasuna ikertzeko (Landeta *eta lank.*, 2011; Yethon *eta lank.*, 2003). Liposomen flotazio esperimentuan, besikulak eta hauetara lotutako proteinak dentsitate gradiente bidezko zentrifugazioa erabiliz bereizten dira. Orokorrean, lamela bakarreko edo lamela anitzeko besikula handiak ikerketa hauek egiteko mintz-eredu sistema hoberenak dira. Askotan erabiltzen den eta sentikorragoa den beste teknika bat besikulen jalkitzea da, baina mintzekin elkarreragiten duten hainbat proteinek duten pilatzeko edo oligomerizatzeko joera dela eta, nahiz eta liposomarik ez egon proteina hauek jalkitzen dira. Egoera horretan, proteina horien mintzarekiko lotura ezin da besikulen jalkitze saioen bidez modu fidagarri batean aztertu. Kasu horietan, proteinen oligomerizazio edo pilaketarekiko horren sentikorrak ez diren liposomen flotazio saioak erabili daitezke.

Liposomen flotazio saioan, lehenengo pausua proteinak liposomekin [detekziorako Rodamina-PE (Rho-PE)-ren %0.05 mol daukatenak] 37°Ctan inkubatzea da. Gero, lagina 1.4 M sakarosara doitzen da PreScission indargetzailean sakarosa stock soluzio bat erabiliz, eta jarraia ez den sakarosa gradiente baten (erdiko geruza

0.8 M, goiko geruza 0.5 M sakarosa) behealdean kargatzen da. Lagina 3 orduz 4°Ctan eta 100000 rpm-tan zentrifugatu zen Beckman Optima TLX benchtop ultrazentrifugatzailea eta TLA-120.2 errotorea (Beckman Coulter) erabiliz. Ultrazentrifugazioan zehar, beraien dentsitate baxuagoa dela eta, liposomek eta liposometara lotutako proteinek gradientearen goiko frakziora flotatzen dute, eta proteina askea, berriz, azpian geratzen da (**2.9 Irudia**). Behealdetik hasiz, 250 µL-ko alikuotak hartu ziren (guztira 4 lagin) eta 6X karga indargetzailearen 50 µL gaineratu ziren. Gero, liposomak dauzkan goiko frakzioa (T), rodaminaren fluoreszentiak adierazten zuen bezala, eta proteina askea daukan beheko frakzioa (B) (25 µL), Coomassie Urdinez tindatutako SDS-PAGE eta western plapaketaren bidez aztertuak izan ziren.



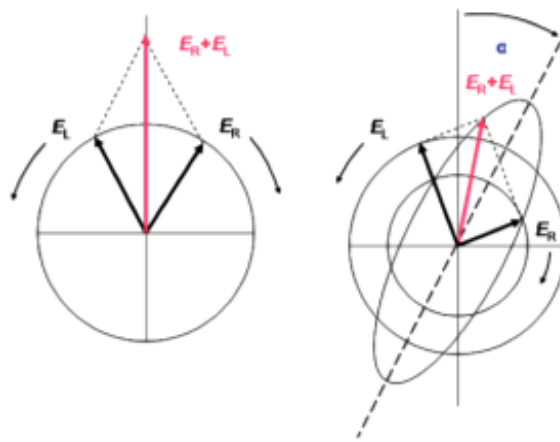
2.9 Irudia. Sakarosa gradiente bidezko zentrifugazio saio baten irudikapen eskematikoa. Lehendabizi, sakarosa gradiente bat prestatzen da behealdean lagina daukan saio-hodian. Zentrifugazioaren ostean, liposomek eta liposometara lotutako proteinek flotatu egiten dute, eta proteina askea, berriz, beheko frakzioan geratzen da.

2.8 Dikroismo Zirkularreko (CD) Espektroskopia

Dikroismo zirkularrak (CD, ingelesetik *circular dichroism*), proteinen egitura aztertzea ahalbidetzen du bereizmen gutxirekin, bai soluzio urtsuetan zein mintz-eredu sistemetan. Teknika honekin, X-izpi kristalografia edo NMR bidez ez bezala, hondar espezifikoaren informazioa lortu ezin daitekeen arren, peptido eta proteinen egitura sekundarioaren eta tolesturaren ezaugarriak indargetzaile fisiologikoetan azkar zehazteko tresna bikaina da.

Espektroskopia teknika honek, zirkulari polarizatua dagoen argiaren ezkerreko eta eskuineko osagaien xurgapenaren ezberdintasunak neurtzen ditu (Martin eta Schilstra, 2008). Linealki polarizatuta dagoen argia, fase eta anplitude berdina baina

aurkako norantza daukaten eta zirkularki polarizatuak dauden 2 argi izpiren gainjartzea bezala deskriba daiteke. Laginean zehar pasa ondoren, eskuin eta ezkerreko osagaiak xurgatuak ez badira (edo maila berean xurgatu badira), osagaien konbinazioak planoarekiko polarizatutako erradiazioa birsortuko luke. Hala ere, argi hau optikoki aktiboak diren molekula kiralak (aminoazidoak bezala) dauzkan lagin batetik igarotzen denean, zeintzuek zirkularki polarizatutako 2 osagaiantzat absorbantzia ezberdinak dauzkaten, gehiago xurgatu den osagaiaren anplitudea besteara baino txikiagoa izango da, eta hori dela eta, ondoriozko erradiazioa eliptikoki polarizatu egongo da (2.10 Irudia).



2.10 Irudia. CD efektuaren jatorria. (Ezkerraldea) Linelaki polarizatuta dagoen argia, anplitude eta fase berdina daukaten eta zirkularki polarizatuta dauden aurkako argien gainjartze bezala ikus daiteke. **(Eskuinaldea)** Eskuinaldeko eta ezkerreko osagai polarizatuaren xurgapen ezberdinak eliptizitatera (CD) darama. (Kelly *eta* Iank., 2005-etik moldatua).

Aurretik azaldu den bezala, CD, eskuinaldera (AR) eta ezkerredera (AL) zirkularki polarizatutako argiaren absorbantzia ezberdintasun gisa neurtzen da.

$$\Delta A = A_L - A_R$$

6 Ekuazioa. Dikroismo zirkularra.

CDa ardatz erdi-nagusiaren eta erdi-txikiaren erlazioa bezala definitzen da. Erlazio hau, eliptikotasun (θ) bezala ezaguna den angeluaren tangentea da. Angelu hau orokorrean oso txikia denez, θ -ren tangentea θ -ren berdina da eta xurgapenarekin hurrengo adierazpenaren bidez erlazionatzen da:

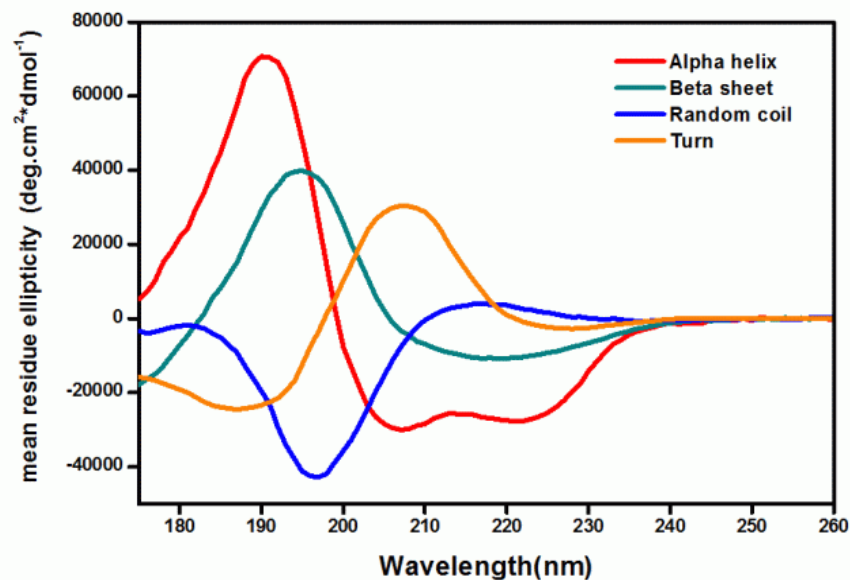
$$\theta = 32.982(\Delta A)$$

7 Ekuazioa. Eliptikotasuna.

Proteina konkretu baten CD espektroa, θ -a uhin luzeeraren aurka grafikatuaz lortzen da.

2.8.1 Proteinen Egitura Sekundarioaren Determinazioa

Lotura peptidikoa, ultramore urruneko espektroen (170 nm-tik 250 nm-ra) absorbantzia eragiten duen kromoforo nagusia da. Gainera, lotura peptidikoek proteinetan duten antolaketa espezifikoak, espektro ezaugarri bereizgarriak dauzkaten argia xurgatzen duten unitate isolatuak sortzen ditu. Beraz, alfa-helizea, beta-orria eta *random-coiled* egitura bakoitzak, CD espektroan itxura eta magnitude bereizgarri bat sortzen du (2.11 Irudia).



2.11 Irudia. Egitura sekundario mota ezberdinekin lotuta dauden ultramore urruneko espektroak. Egitura sekundario ezberdinek CD espektro bereizgarriak daukate: alfa proteina helikoidalek, 222 nm eta 208 nm-tan banda negatiboak daukate eta 193 nm-tan banda positibo bat. Ondo zehaztutako beta-orri antiparaleloak dauzkaten proteinek, 218 nm-tan banda negatiboak daukate eta 195 nm-tan banda positiboak. Azkenik, egitura desordenatuek, 210 nm-tik gora oso eliptikotasun baxua daukate eta 195 nm-tik hurbil banda negatiboak.

Tesi lan honetan, ultramore urrunean egindako CD espektroskopia erabili izan da LC3B, GABARAPL2 eta GABARAP proteinen batz-besteko egitura sekundarioaren estimazio kuantitatiboa lortzeko eta baita proteina mutanteen egitura egokia zen aztertzeko ere. Neurketak, termikoki kontrolatutako Jasco J-810

espektropolarimetroan (Jasco Spectroscopic Co. Ltd., Hachioji, Japonia) egin ziren, 37°Ctan eta 1 mm-ko pasu optikoa duen kuartzozko kubeta erabiliz. Espektroak, 0.2 nm-ro, 50 nm/min-ko abiaduraz, 260 nm-tik 200 nm-ra eta 2nm-ko banda zabaleraz egin ziren. Espektro finalak 20 espektroen arteko batez bestekoa eginez kalkulatu ziren. Batez besteko honi indargetzailearen seinalea kendu, eta ondoren erakusten den ekuazioa erabiliz, hondarren batez besteko eliptikotasun molarra $[\theta]$ kalkulatu zen.

$$[\theta] = \theta/10cln \text{ (deg.cm}^2\text{.dmol}^{-1}\text{)}$$

8 Ekuazioa. Eliptikotasun molarra.

non: θ eliptikotasuna (miligraduak), c proteina kontzentrazioa (M), l kubetaren pasu optikoa (cm), eta n proteinaren lotura peptidiko kopurua den.

2.9 Fluoreszentzian Oinarritutako Espektroskopia Teknikak

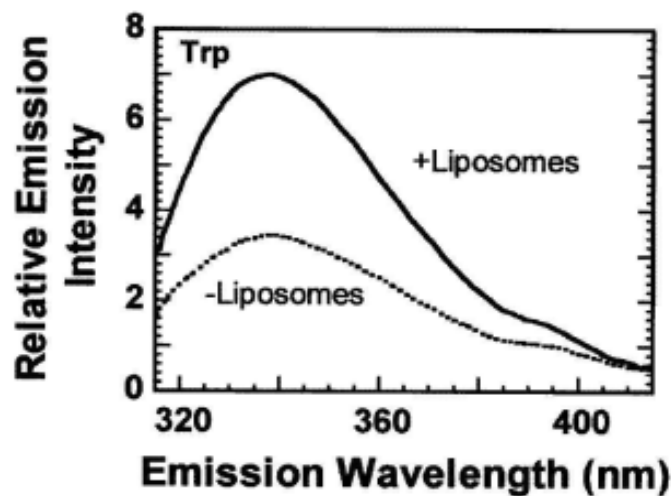
Fluoreszentzia, espektroskopia tekniken artean sentikorrenerikoa da eta mintzean ematen diren fusioa, agregazioa edo iragazkortzea bezalako gertaerak denbora errealean aztertzea ahalbidetzen duen teknika da. Aurretik erabilia izan da entzima hidrolitikoan (SMasa eta PLC) aktibitate fusogenikoa ikertzeko (Goñi *eta lank.*, 2003).

Fluoreszentzian oinarrituriko espektroskopia teknikek, beraien sentikortasun intrintsekoa, denbora-tarte egokia eta perturbazio-efektu minimoak direla eta, lipido-proteina elkarrekintzen hainbat alderdi ikertzeko ere erabilgarriak dira (Johnson, 2005). Oreka egoeran egindako fluoreszentzian oinarrituriko espektroskopia neurketek (adib. fluoreszentziaren intentsitatea, espektro aldaketa, eta indargabetze edo *quenching* saioak), sisteman gertatzen ari diren elkarrekintza molekularren informazioa ematen dute eta proteina konkretu baten mintz batekiko elkarrekintza aztertzeko erabiliak izan daitezke. Gainera, fluoreszentzian oinarritutako beste metodo batzuek, adibidez, TR-FRET (ingelesezik *time-resolved fluorescence resonance energy transfer*), elkarrekintza horien dinamiken inguruko informazioa ere eman dezakete.

2.9.1 Triptofanoaren Fluoreszentzia Intrintsekoan Oinarritutako Espektroskopia

Eraztun aromatikoaren fluoreszentzia intrintsekoa, proteina-lipido elkarrekintzak ikertzeko erabil daiteke. Triptofanoaren (Trp) fluoreszentzia da gehien erabiltzen dena. Proteinen triptofanoaren fluoreszentzia intrintsekoaren aldaketak, aldaketa

konformazionalak gertatzean edo ligando bati lotzean askotan gertatzen dira. Orokorrean, triptofanoa ingurune hidrofobiko batean dagoenean, bere fluoreszentiaren intentsitatea handitu egiten da, eta medio urtsu batean dagoenean gutxitzen da (**2.12 Irudia**). Beraz, proteinak mintza lotzeko gainazalean dagoen edo konformazio aldaketa batekiko sentikorra den triptofano bat badauka, triptofanoaren fluoreszentzia erabil daiteke agente kaotropikoek, mintzarekiko elkarrekintzak edo ligandoarekiko loturak, beste batzuen artean, eragindako aldaketak zehazteko (Ostolaza eta Goñi, 1995; Bakás *eta lank.*, 1996; Soloaga *eta lank.*, 1998; Lakowicz, 2007; Kraft *eta lank.*, 2009; Zhao *eta* Kinnunen, 2002).



2.12 Irudia Triptofanoaren fluoreszentzia intrinsekoa. O perfringolisina toxinarean triptofanoaren fluoreszentzia intrinsekoan ematen diren aldaketak kolesterola daukaten liposometara lotu ostean. (Heuck *eta lank.*, 2000-tik hartua).

Trp neurketak egiteko, lagineko osagaiak (proteinak edo proteinak gehi liposomak), ordu betez 37°Ctan inkubatu ziren. Inkubazioaren ostean, fluoreszentzia espektroak QuantaMaster espektrofluorimetroan (Photon Technology International, Birmingham, NJ, EEBB) erregistratu ziren. Kitzikapenaren uhin luzera 295 nm-koa zen eta banda zabalera 5 nm-koa, Trp-aren emisio espektroak 310 nm-tik 450 nm-ra neurtu ziren, 1 nm/seg-eko abiaduraz. Kontrol egokiekin eta sistema optiko eta uhin luzeerarekiko menpekoak diren sistema optiko eta detekzio sistemarekin zuzenketak egin ziren. Emaitza hauek CL duten liposomen aurrean eman litezkeen GABARAPL2 proteinaren aldaketa konformazionalak aztertzeke erabili ziren.

2.9.2 NBDren Fluoreszentzia Oinarritutako Espektroskopia

Proteina-lipido elkarrekintzak ikertzeko, aminoazido aromatikoaren fluoreszentzia intrinsekoa erabili ordez zunda estrintsekoak erabili daitezke. Azken hauetan lipidoa edota proteina zunda-fluoreszente bati lotzen dira. Komertzialki eskuragarri dagoen zunda kopurua oso handia da. Hauetako askok, adib. NBD (ingelesez *7-nitrobenz-2-oxa-1,3-diazol-4-yl*), uraren presentziarekiko oso sentikorrak dira, beste batzuk ordea, fluoreszeina bezala, pHaren edo ingurune elektrostatiakoaren aldaketan aurrean dira sentikorrak. Zunda-fluoreszente bat aukeratzeko orduan, zenbait faktore eduki behar dira kontuan:

- i. Zundak tamaina erlatibo txikia eduki beharko luke, izan ere, zunda oso handiek proteinaren egitura edo funtzioa aldatzeko arriskua daukate.
- ii. Zunda, ezin zen aldatu gabeko polipeptido natiboaren konformazioarekiko era independetean bigeruzan berez txertatzea eragiten duen besteko hidrofobikoa izan beharko. Adibidez, *acrylodan* edo *coumarin* bezalako zunda oso hidrofobikoak, beraien burua bigeruz apolarren muinean txertatzeko, itsatsiak dauden polipeptido katearen konformazioan eragingo lukete.
- iii. Markaketaren ostean, lotu ez diren zundak erraz ezabatu beharko lirateke, izan ere, era espezifikoan edo kobalentez lotuta ez dauden zunda-fluoreszenteak, espektroaren seinalearekin interferitu eta bere interpretazio egokia egitea eragotziko dute.

Azaldutakoa kontuan hartuz, IANBD, tiolarekiko espezifiko den NBDren iodoacetamida deribatu bat, tesi lan honetan erabilitako zundetako bat izan zen (ik. **9. Protokoloa**). NBD ingurune urtsu batetik (hau da, proteina zitosoliko baten gainazala) ingurune hidrofobiko batera (hau da, mintzaren edo proteina baten barnealde apolarra) mugitzen denean, bere emisioaren intentsitatea handitu eta bere igorpen maximoaren uhin luzera urdinerantz lekualdatzen da (**2.13 Irudia**). Gainera, proteina NBD motibo bakar batekin eta proteinaren posizio bakar batean markatuta dagoenez, metodo honek ere, proteinak mintzari lotuta dagoenean proteinaren gune espezifikoetan gertatzen diren egitura aldaketak aztertzeko aukera ematen digu. Proteinen markaketa bakoitzaren baldintza zehatzak dagokion kapituluaren Materialak eta Metodoak atalean aurkituko dira.

9. Protokoloa. NBDren Fluoreszentzian Oinarritutako Espektroskopia

1. Mutagenesi guleratua, proteinan dauden zisteina hondar endogeno guztiak kentzeko eta sortu berri den Cys-hondarrik gabeko proteinaren intereseko posizioetan Cys hondar berri bat txertatzeko erabiltzen da.

2. Markaketa prozedura:

- Proteinak 2 edo 3 mg/mL-tara kontzentratzen dira eta 1mM TCEPrekin inkubatzen dira 30 minutuz giro tenperaturan.
- Lagina, markaketa indargetzailean (10 mM HEPES, pH 7.3, 100 mM KCl, 1 mM EDTA) orekatutako PD-10 zutabe batera (GE Healthcare) gaineratuz TCEP ezabatzen da.
- Ondoren, proteina soluzioari dimetilsulfoxidoan (DMSO) disolbatutako IANBDren (Molecular Probes, Eugene, OR, EEBC) bolumen txiki bat (15 µL-tik 25 µL-ra) gaineratzen zaio, 1:10 proteina:zunda ratioa lortzeko (NBD stock soluzioa = 30 mM). Hau, irabiaketa geldo batekin eta lagina argitik babestuz gauean zehar 4°Ctan inkubatzen da.
- Lagina 10 minutuz 14000 x g-tan zentrifugatzen da eta markaketa indargetzailean orekatutako PD-10 zutabe batera gaineratzen da, zunda lotuta daukan proteina erreakzionatu ez duen zunda asketik banantzeko.
- Markaketa gradua zehazteko ondorengo ekuazioa erabiltzen da:

$$\% \text{ Markaketa gradua: } [(Abs_{\text{NBD}}/\epsilon_{\text{NBD}})/(Abs_{\text{prot}}/\epsilon_{\text{prot}})] \times 100$$

9. Ekuazioa. % Markaketa graduaren kalkulua.

non: Abs_{NBD} = NBDk 488 nm-tan duen absorbantzia.

Abs_{prot} = Proteinak 280 nm-tan duen absorbantzia.

ϵ_{NBD} = NBDren iraugipen molarraren koefizientea (eM).

ϵ_{prot} = Proteinaren iraugipen molarraren koefizientea (eM).

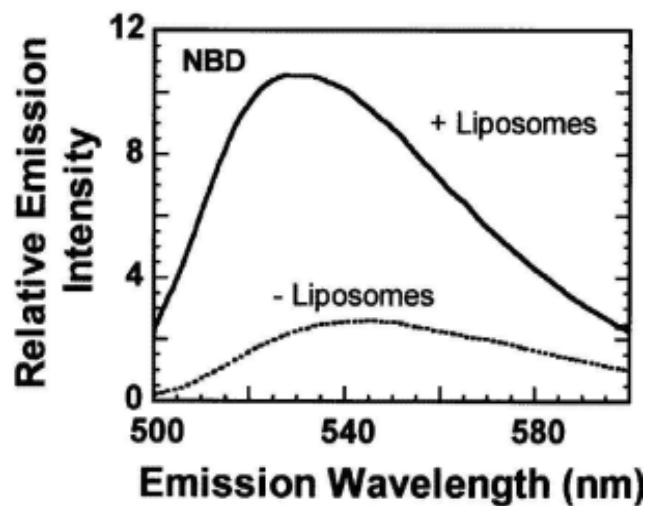
(Protokoloak hurrengo orrialdean jarraitzen du)

3. NBDren fluoreszentiaren neurketak:

- Neurketak egin aurretik laginaren osagaiak (proteina edo proteina gehi liposomak) 10 minutuz 37°Ctan inkubatzen dira.
- NBD espektroak, 1 cm-ko pasu optikoa duen kubeta erabiliz eta irabiaketa konstantean, QuantaMaster espektrofluorimetroan (Photon Technology International) erregistratu ziren, ondorengo baldintzen menpean:

$\lambda_{\text{ex}} = 465 \text{ nm}$ Banda zabalera = 5 nm
 $\lambda_{\text{em}} = 520 - 650 \text{ nm}$ Filtroa = 515 nm
 $T^\circ = 37^\circ\text{C}$ Abiadura = 1 nm/seg

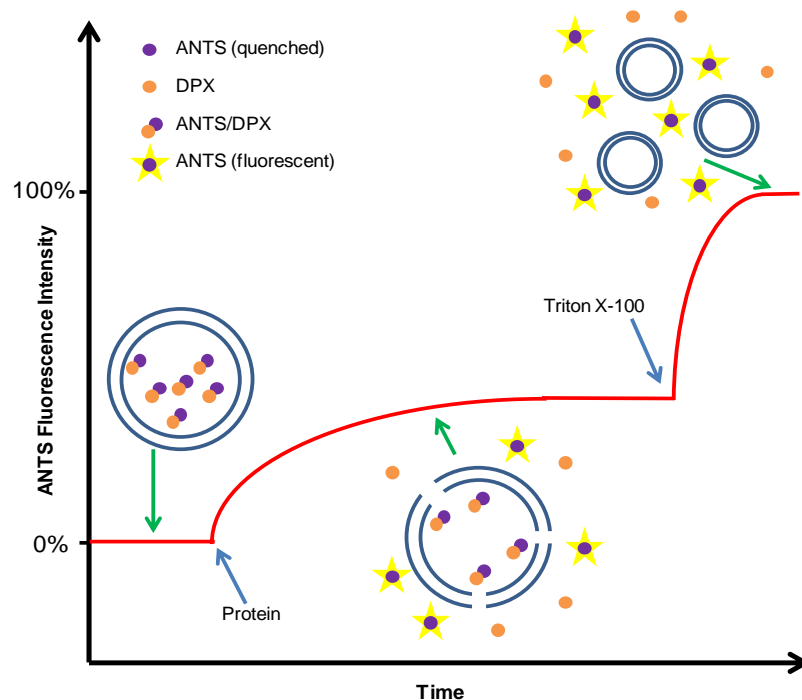
Kasu guztietan, proteinarik gabeko laginen intentsitateak proteinadun laginetan neurtutako intentsitateei kendu zitzairen.



2.13 Irudia. NBDren fluoreszentzia generatua. NBDrekin markatutako O perfringolisinaren NBD motiboaren fluoreszentzian ematen diren aldaketak, kolesterola duten liposometara lotu ostean (Heuck *eta* lank., 2000-etik hartua).

2.9.3 Besikulen Edukien Askapenaren Neurketak (Askapen Saioa)

Teknika honek molekula konkretuek besikulen bigeruz lipidikoa iragazkor bihurtzeko daukaten ahalmena neurtzen du. Molekula fluoreszenteak enkapsulatuak dituzten besikulen prestaketan oinarritzen den metodo sinplea da. Molekula espezifikoekin egindako inkubazioaren ostean, iragazkortze prozesua fluoreszentsian emandako aldaketan bidez jarraitzen da. Tesi lan honetan, zeramida eta dihidrozeramida besikulak iragazkor bihurtzeko daukaten ahalmena ikertu da, horretarako, ondo ezagutzen diren pisu molekular baxuko ANTS molekula fluoreszentea eta bere igorpena moteltzen duen DPXa erabili dira.



2.14 Irudia. ANTS/DPX askapen saioaren ikuspegi eskematikoa. Iragazkortasuna handitzea eragiten duen osagaia (kasu honetan proteina) mintzean txertatu ondoren, mintzaren ezegonkortzeagatik eratu daitezkeen mintzeko akatsek, besikuletan enkapsulatuta dauden ANTS eta DPX zunden askapena eragingo dute. ANTSaren fluoreszentsiaren igoera bat ikusten da. Besikulen erabateko solubilizazioa lortzeko eta horrela %100eko askapenaren balioa lortzeko (hau da, ANTSaren fluoreszentsia maximoa) normalean Triton X-100 gaineratzen da.

Metodo honen oinarria **2.14 Irudian** azaltzen da. ANTS eta DPX besikuletan enkapsulatuz, bata bestearengandik hurbil egoteak DPXa ANTSrekin elkarrengaitzat ahalbidetzen du, ANTSaren fluoreszentsia motelduz. Bi molekulak besikuletatik kanporatzen direnean, kanpo medioan diluitzen dira eta beraien arteko elkarrekintza asko zailtzen da. Honen ondorioz, ANTSren fluoreszentsia igotzen da. Beraz, ANTSren

fluoreszentzia neurtuz, besikulen bigeruzaren iragazkortzea aztertu daiteke. Metodo hau, 1985ean deskribatu zen lehen aldiz (Ellens *eta lank.*, 1985) eta besikulen arteko fusioa neurtzeko erabili zen. Kasu honetan, bi molekulak besikula populazio ezberdinetan enkapsulatzen ziren eta beraien arteko fusioaren ondoriozko ANTS-DPX elkarrekintzak ANTSaren fluoreszentzia moteltzen zuen (ik. Goñi *eta lank.*, 2003 eta Nieva *eta lank.*, 1989). **10. Protokoloan**, saioaren xehetasun guztiak deskribatzen dira.

10. Protokoloa. Besikulen Edukien Askapenaren Neurketak

1. 100 nm-ko LUVak aurretik azaldu den moduan (**3. Protokoloa**) prestatzen dira, ANTS eta DPX daukan ondorengo indargetzailea erabiliz: 20 mM ANTS, 70 mM DPX, 40 mM NaCl, 10 mM HEPES, 10 mM CaCl₂, 2 mM MgCl₂ (pH 7).
2. Besikulen barnean erabateko indargabetzea (*quenching*) lortzeko DPX/ANTS ratio handia erabiltzen da. Molekula fluoreszenteen argiaren ondoriozko zuriketa (*bleaching*) saihesteko, laginak aluminiozko paperrez estaltzen dira.
3. Besikuletan barneratu ez diren ANTS eta DPX molekulak baztertzeko, besikulen esekidura Sephadex G-25 zutabetik igaroarazten da. Aurretik, zutabea indargetzaile isoosmotiko batekin orekatzen da: 150 mM NaCl, 10 mM HEPES, 10 mM CaCl₂, 2 mM MgCl₂ (pH 7).
4. Lipido kontzentrazioa aurretik azaldu den bezala kalkulatu da (**6. Protokoloa**).
5. Iragazkortzea eragiten duen osagaia, 0.3 mM besikuletara gaineratzen da 1 mL-ko beirazko hodi batean irabiaketan eta QuantaMaster espektrofluorimetro (Photon Technology International) bat erabiliz, askapena ANTS fluoreszentiaren igoera gisa neurtzen da. Neurketak ondorengo baldintzen menpean egiten dira:

$$\lambda_{\text{ex}} = 355 \text{ nm}$$

$$\lambda_{\text{em}} = 520 \text{ nm}$$

(Protokoloak hurrengo orrialdean jarraitzen du)

Interferentzia filtroa = 450 nm

$T^{\circ} = 37^{\circ}\text{C}$

%0ko askapena = Besikulen hasierako fluoreszentzia.

%100ko askapena = 1 mM Triton X-100 bidezko besikulen erabateko solubilizazioaren osteko fluoreszentzia.

Askapenaren ehunekoa kalkulatzeko ondorengo ekuazioa erabiltzen da:

$$\% \text{ Askapena} = [(F - F_0)/(F_{100} - F_0)] \times 100$$

10. Ekuazioa. % Askapenaren kalkulua.

non: F = Oreako fluoreszentzia

F_0 = Hasierako fluoreszentzia

F_{100} = Askapen maximoa (Triton X-100 gaineratu osteko fluoreszentzia)

2.10 Ugaztunen Kultibo Zelularrak

2.10.1 Kultibo Zelularren Hasiera eta Mantenua

Tesi lan honetan erabili diren zelula lerroak **2.4 Taulan** aurkezten dira. Kultibo zelularren stock-ak kontuz izoztu ziren %10 DMSO eta %40 fetuko behi-seruma (FBS, ingelesetik *fetal bovine serum*) osagarri moduan zituen hazkuntza medioan, eta N_2 likidoan eta -80°C tan gorde ziren. Zelula lerroak berreskuratzeko, 37°C tan azkar urtu eta hazkuntza medio orokorraren 10 mL-tan diluitu ziren gauean zeharreko indarberritzerako. Hurrengo egunean, medioa aldatu zen DMSOaren aztarnak guztiz kentzeko. Ezer esaten ez bada, neurona kortikalak 4 putzutako plaketan hazi ziren eta gainontzeko zelula lerroak 10 cm-ko diametroko kultibo-plaketan, 37°C tan hezetutako inkubatzailer batean %5 CO_2 -arekin.

2.10.2 Transfekzio Iragankorra

Zelulen transfekzio iragankorra Lipofectamine[®]2000 transfekzio errektiboa (Invitrogen, Carlsbad, CA, EEBB) erabiliz burutu zen, fabrikatzailearen argibideak

jarraituz. Hitz gutxitan esanda, intereseko DNA zegokion opti-MEM[®] bolumenean diluitu zen, beste 1.5 mL-ko hodi esteril batean Lipofectamine[®]2000-a opti-MEM[®]-en diluitu zen eta biak 5 minutuz inkubatu ziren. Lipofectamine[®]2000/opti-MEM[®] kantitatea transfekzio kopuruen arabera egokitu zen. Bitartean, transfektatuak izango ziren zelula kopuru egokia nahi zen kontzentrazioan prestatu zen hazkuntza medio arruntean antibiotikorik gabe. Ondoren, Lipofectamine[®]2000-DNA konplexuak, tantaka zeluletara gaineratu ziren. Zelulak, 24 eta 48 ordu bitartean, 37°Ctan eta %5 CO₂tan hazi ziren tratamenduak egin aurretik.

Ugaztun zelulen transfekzio iragankorra eta lentibirusen bidezko transdukzioa burutzeko erabilitako plasmidoak Jon Lane (Bristoleko Unibertsitatea, Bristol, EB) eta Anne Simonsen (Institute of Basic Medical Sciences, Oslo, Norvegia) doktoreek adeitasunez eman zizkiguten (ik. **2.5 Taula**).

Zelula lerroa	Iturria	Kultibo medioa
U87MG	Giza glioma zelula lerroa (ATCC [®]) (G. Velasco doktorearekin egindako kolaborazioa, Madrilgo Konplutense Unibertsitatea)	DMEM (+ 10% FBS eta 5 µg/mL penizilina/estreptomizina)
mCherry-GFP-LC3B RPE1	Zelula lerro egonkorra	DMEM-High Glucose (+ 10% FBS)
mCherry-GFP-GABARAPL1 RPE1	Zelula lerro egonkorra	DMEM-High Glucose (+ 10% FBS)
GFP-ATG5 RPE1	Zelula lerro egonkorra	DMEM-High Glucose (+ 10% FBS)
iPSC-tik eratorritako giza neurona kortikalak	iPSC-tik eratorritako neurona kortikalak (P. Stathakos-ek eta P. A. Nistor doktoreak emanak, Bristol, EB)	N2B27, zeinak 1:1 Neurobasal eta glutamax-dun DMEM/F-12 dituen (amaierako glutamina kontzentrazioa 1mM) (+ 1:100 B27, 1:200 N2, 1:200 aminoazido ez esentzialak, 75 µM 2-merkaptetoetanola, 100 unitate/mL penizilina, 100 µg/mL estreptomizina)

2.4 Taula. Tesi lan honetan erabilitako zelula lerroak. Zelula lerro egonkorak J. Lane doktoreak (Bristoleko Unibertsitatea, Bristol, EB) eman zizkigun. B27, N2, aminoazido ez esentzialak eta 2-merkaptetoetanola LifeTechnologies-ek (Carlsbad, CA, EEBB) hornituak izan ziren. Penizilina eta estreptomizina Sigma Aldrich-ek hornitu zituen. ATCC[®], ingelesetik *American Type Culture Collection* (Rockville, MD, EEBB).

2.10.3 Lentibirusen bidezko Transdukzioa

Transfekzio iragankorraren ordezeko teknika bat, GIBean oinarritutako lentibirusen bektoreak erabiliz intereseko geneak zelulen genomatan era egonkorrean txertatzean datza. 1-GIBean oinarritutako eretrovirusak, lentibirusaren adierazpen bektorean genetikoki kodetutako edozein proteinaren adierazpen iraunkorra lortzeko, ugaztunen zelula lerroen transdukzioa ahalbidetzen du. Tesi lan honetan bi sistema lentibiral desberdin erabili dira (ik. **2.5 Taula**):

Fusio Genea	Bektorea	Metodoa
GABARAPL2-GFP	pDestEGFP	Transfekzio iragankorra
GABARAP-GFP	pDestEGFP	Transfekzio iragankorra
MTS-CFP	peCFP-N1	Transfekzio iragankorra (proiektu honetan, G. Mullally-k emandako MTS-peGFP plasmidotik azpiklonatua, Bristol, EB)
Mito-DsRed	pDsRed2	Transfekzio iragankorra
Mito-CFP	peCFP-N1	Lentibirusen bidezko transdukzioa (pLVX-Puro bektore lentibirala eta Lenti-X TM HT paketatze sistema)
mCherry-GFP-LC3B	pmCherry-C1	Lentibirusen bidezko transdukzioa (pLVX-Puro bektore lentibirala eta Lenti-X TM HT paketatze sistema)
GFP-LC3B	peGFP-C1	Lentibirusen bidezko transdukzioa (pxlg3-gfp sistema lentibirala)
GFP-GABARAPL1	peGFP-C1	Lentibirusen bidezko transdukzioa (pxlg3-gfp sistema lentibirala)

2.5 Taula. Plasmidoen zerrenda. Kontuan hartu beharrekoa: zelula lerro egonkorak modu ez zuzenean lortu ziren, aurretik pxlg3-gfp edo pLVX-Puro bektore lentibiralen plasmidoetan azpiklonatutako fusio-geneak erabiliz lentibirusen bidezko transdukziorako.

2.10.3.1 pLVX-Puro Bektore Lentibirala eta Lenti-XTM HT Paketatze Sistema

pLVX-Puro adierazpen bektore lentibiralak, LentiXTM HT paketatze sistemarekin (Clontech Laboratories, Mountain View, CA, EEBB) batera, komertzialki eskuragarria den zelula primarioen transdukziorako segurtasun eta gaitasun handiko sistema bat osatzen dute.

Lentivirusak, pLVX-Puro konstruktoekin eta LentiX™ HTX paketatzenahasketarekin (birusen paketatze proteinak kodetzen dituzten 5 plasmidoen nahasketa) %80-90 konfluente ziren HEK293T zelulak ko-transfektatuz sortu ziren, 10 cm-ko diametroko kultibo-plaketan, fabrikatzailearen argibideak jarraituz. Lentivirusak lortzeko, zelulen medio guztia 48 ordu ostean jaso eta 0.45 µm-tako zelulosa azetatozko filtro bat erabiliz filtratu zen. Filtratutako birusen suspentsioa kriohodietan alikuotatu eta -80°Ctan gorde zen.

pLVX-Puro bektorearekin lortutako adierazpen maila baxuak zirela eta, ondorengo esperimentuak burutzeko pxlg3-gfp sistema hautatu zen.

2.10.3.2 pxlg3-gfp Sistema Lentibirala

pxlg3-gfp bektorea, aldatutako pHR'SIN-SEW bektore bat da (Demaison *eta lank.*, 2002), zeinak WPRE eskualdea faltan duen (Danson *eta lank.*, 2007). pHR'SIN-SEW bektorea, bere buruaren inaktibazioa eragiten duen (SIN, ingelesetik *self-inactivating*) bektore batetik garatutako GIBean oinarritutako bektore lentibiral bat da [zeinetan 3' LTRaren U3 eskualdea kendu den (Zufferey *eta lank.*, 1998)], non CMW promotorea SFFVU3LTR (ingelesetik *U3 part of the spleen focus forming virus strain P long terminal repeat*) sekuentziagatik ordezkatu zen (Demaison *eta lank.*, 2002). Demaison *eta lankideek* bektore hau sortu zuten *in vitro* zelula progenitore hematopoietikokoak espezifikoki transduzitzeko (Demaison *eta lank.*, 2002). Giza neurona kortikaletan, pxlg3-gfp bektore batean GFP-LC3B edo GFP-GABARAPL1 zeramaten lentibirusekin (J. Lane doktoreak emandakoak) adierazpen maila altuak lortu ziren.

Ugaztunen zeluletan intereseko lentibirusekin egindako transdukzio egonkorra lortzeko erabili ziren bi sistemen kasuan, zelula ituak irudikapen-plaketan hazi ziren transdukzioaren egunean %50etik %70erako konfluentzia izateko. Desizoztutako lentivirusak tantaka zelula ituetan gaineratu ziren 1:4 (v/v) birusen esekidura:medioa ratioan. Transduzitutako zelulak 24 orduz inkubatuak izan ziren eta medio kutsatua hazkuntza medio berriaz ordezkatu zen. Zelulen irudikapena egin aurretik, zelulak gutxienez 72 orduz inkubatu ziren 37°Ctan eta %5 CO₂-rekin.

2.11 Fluoreszentziako Mikroskopia (FM)

Proteina espezifikoaren zelulan zeharreko kokapena markatzeko eta irudikatzeko (immunotindatuak edo isats fluoreszenteak erabiliz, hurrenez hurren), GUVetan lipido domeinuak aztertzeko edo proteinen GUVekiko lotura irudikatzeko, proteina- edo lipido-fluoreszenteak eta antigorputzei lotutako fluoroforoak erabili ziren. Horretarako, laginak hautatutako zunda-fluoreszentearen kitzikapen uhin luzeran argiztatu ziren eta fluoreszentzia igorpenaren seinalea mikroskopia erabiliz behatu zen.

Kitzikapen zein igorpen seinaleak, mikroskopioetan filtro espezifikoak erabiliz hautatu ziren. Lan honetan bi mikroskopio mota erabili izan dira: eremu zabaleko mikroskopioa (ingelesez *wide field*) eta mikroskopio konfokala. Eremu zabaleko mikroskopioak irudien eskurapen azkarra eta sentikortasun handia ahalbidetzen du, baina lagin mehetara hobeto doitzen da (adib. zelula atxikituak), eta denboran zeharreko mikroskopia egiteko eta zelula fixatuen irudikapenarako erabili zen. Hala ere, mikroskopia konfokalak fokatze gunetik kanpo dagoen argia baztertzen du eta zelula sekzioen irudikapena ahalbidetzen du, baita lagin meheetan ere, eta zelulen eta GUVen xehetasun espezifikoak irudikatzeko hobeto egokitzen zen, beraien forma zirkularra dela eta.

2.11.1 Zelula Fixatuen Irudikapena (Immunofluoreszentzia)

2.11.1.1 Zelulen Fixaketa eta Iragazkortzea

Detektatu behar den antigenoaren, markatuta eta bere horretan mantendua izan behar den organuluaren, eta baita erabiliko diren antigorputzen arabera ere, zelulak disolbatzaile organikoak (adib. metanola) edo elkargurutzatze errektiboak (adib. paraformaldehidoa, PFA) erabiliz fixatu daitezke. Disolbatzaile organikoek, lipidoak erauzi eta zelulak deshidratatzen dituzte, honek proteinak jalkitzea eragiten duelarik. Zelulen arkitekturaren kolapsoa eragin dezakete baina antigenizitatea ondo mantentzen dute. Disolbatzaile organikoak zitoeskeletoaren eta mintzera itsatsitako antigenoentzako ezin hobeak dira. Elkargurutzatzea eragiten duten errektiboek, zenbait aminoazidoren albo taldeen artean metileno zubiak eratzen dituzte. Zelularen arkitektura hobeto mantentzen duten arren, antigenizitatea murriztu dezakete, eta disolbatzaile organikoek ez bezala, aurretik iragazkor bihurtu diren zelulak soilik fixatzen dituzte.

Immunotindaketarako, zelulak euskarri solido bati atxikituak egotea beharrezkoa da. U87MG zelulak, 12 mm-ko beirazko estalduretan (Menzel-Gläser, Braunschweig, Alemania) hazi ziren. Estaldurak, PBSrekin behin garbitu eta %4 PFArekin (Sigma-Aldrich) fixatu ziren, 20 minutuz eta giro tenperaturan, ostean, berriz garbitu ziren. RPE1 (ingelesetik *hTERT-immortalized human retinal pigment epithelial 1*) zelulak 13 mm-ko beirazko estalduretan (Menzel-Gläser) hazi ziren. Estaldurak PBSrekin behin garbitu eta metanol oso hotzean (Thermo Fisher Scientific) 5 minutuz (HSP60aren immunotindaketarako) edo %2 PFArekin (Sigma-Aldrich) 15 minutuz, giro tenperaturan fixatu ziren, eta ostean garbitu egin ziren. Garbiketa guztiak PBSrekin 5 minutuz egindako 3 garbiketa izan ziren.

PFArekin fixatutako zelulak, 5 minutuz %0.1 edo %0.5 Triton X-100 (Sigma-Aldrich) duen PBSarekin iragazkor bihurtu ziren eta berriro ere garbitu ziren kasu honetan 1 M glizinarekin tanta bat gaineratuz, aldehido talde askeak blokeatu eta antigorputzen lotura inespezifikoak saihesteko. Aurretik aipatu den bezala, metanolaren bidezko fixaketan lipidoak erauzten ziren eta beraz ez zen iragazkortze pausurik behar.

2.11.1.2 Immunotindaketa eta Irudikapena

U87MG zelulak, %0.1 BSA (Sigma-Aldrich) duen PBSan diluitutako antigorputz primario egokiekin (LC3ren kasuan antigorputzak proteinaren B isoforma ezagutzen du) estalduretan bi orduz inkubatu ziren. Gero, estaldurak indargetzaile berdinarekin 3 aldiz garbitu ziren, garbiketa bakoitza gutxienez 5 minutukoa zelarik. RPE1 zelulak, PBSan (Sigma-Aldrich) diluitutako antigorputz primario egokiekin (LC3ren kasuan antigorputzak proteinaren B isoforma ezagutzen du) estalduretan 20 minutuz inkubatu ziren. Gero estaldurak indargetzaile berdinarekin 3 aldiz garbitu ziren, garbiketa bakoitza gutxienez 5 minutukoa zelarik.

Fluoroforoetara lotutako antigorputz sekundarioak (antigorputzen ezaugarri eta baldintzen deskribapen zehatz baterako ikusi Materialak eta Metodoak dagokion kapituluan) estalduretan iluntasunean eta giro tenperaturan, 20 edo 90 minutuz inkubatu ziren eta ostean PBSrekin beste 3 aldiz garbitu ziren. DAPI (4',6-diamidino-2-fenilindola) bidezko nukleoaren tindaketarako, estaldurak 0.1 µg/mL DAPIrekin (harizpi bikoitzeko DNARA lotuta dagoenean ~460 nm-ko igorpen uhin luzera duen nukleoaren tindatzailea) (Roche) 5-10 minutuz inkubatu ziren eta azkenik PBSrekin 2 garbiketa egin ziren. Estaldurak bi aldiz destilatutako urarekin (ddH₂O, ingelesetik *double distilled*

water) laburki garbitu ostean, Mowiol medioa (Calbiochem, Darmstadt, Alemania) edo DABCO gehigarria duen Mowiol medioarekin (25 mg/mL; 4 μ L 13 mm diametroko estaldura bakoitzeko) beirazko portetan buruz behera jarri ziren.

Fixatutako zelulen irudiak hurrengo mikroskopiaok erabiliz eskuratu ziren:

- i. Eremu zabaleko alderantzikatutako Olympus IX-71 mikroskopia (Olympus UK Ltd., Essex, EB) 60x Uplan Flourite olio objektibo batekin (0.65–1.25 NA), CoolSNAP HQ2 CCD kamera (Photometrics, Tucson, AZ, EEBC) batekin, MetaMorph *software*-aren (Molecular Devices, Sunnyvale, CA, EEBC) bidez erabilia eta bi argi iturriekin: argi transmititurako eta epifluoreszentziako irudikapenak egiteko xenonezko lanpara bat (LBSL-1256) eta merkuriozko lanpara bat (Olympus U-RFL-T) argiztapen handiagoa beharrezkoa zenean.
- ii. Leica TCS SP2 mikroskopia konfokala (Leica Microsystems CMS GmbH, Wetzlar, Alemania).

2.11.2 Zelula Bizien Irudikapena

Zelula bizien irudikapenarako, zelulak 35 mm-ko beirazko oinarria duten plaketan (MatTek Corp., Ashland, MA, EEBC) erein ziren, irudikapena egiterako orduan %60-tik %70-erako konfluentzia izateko.

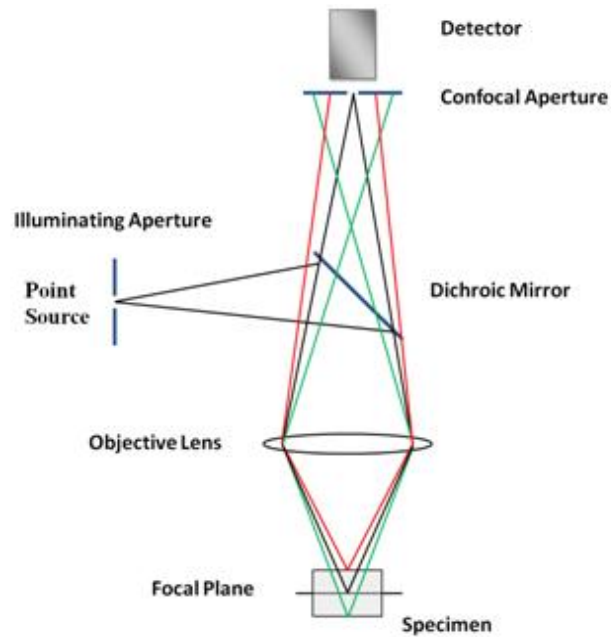
RPE1 zelulen mitokondrioen tindaketarako, gutxienez tratamenduak egin baino 24 ordu lehenago, MTS-CFP edo MitodsRed-rekin zelula atxikituen transfekzio iragankorra egin zen. Neurona kortikalen kasuan, mitokondrioen tindaketarako zelulak aurretik epeldutako 50 nM MitoTracker[®]Red CMXRos (Molecular Probes, Life Technologies) zuen medioarekin 15 minutuz inkubatu ziren. Ondoren, tindaketa medioa xurgatu zen, PBSrekin 3 garbiketa egin ziren eta hazkuntza medio berria gaineratu zen. MitoTracker[®]Red CMX-ROS-ek, mitokondrio aktiboak tindatzen ditu eta bere pilaketa mitokondrioetan, mitokondrioaren $\Delta\Psi$ -ren menpekoa da. Dena den, depolarizazioarekin tindaketa ez da guztiz galtzen eta zelulen fixaketaren ostean mantenduko litzateke (Poot *eta* *lank.*, 1996). LC3B eta GABARAPL1 proteinen tindaketarako, neuronan zegokien lentibirusen bidezko transdukzioa gutxienez saiakuntza burutu baino 72 ordu lehenago egin zen.

Zelula bizien irudikapena eremu zabaleko alderantzikatutako Olympus IX-71 mikroskopia erabiliz egin zen, zeinak bai transmitutako argirako zein epifluoreszentziarako xenonezko lanpara bat duen (LBLS-1256). Irudiak (fase-kontrastea eta fluoreszentzia) CoolSNAP HQ2 CCD kamera (Photometrics) bat erabiliz eskuratu ziren. Irudi hauek, MetaMorph *software*-aren kontrolpean, finkatutako denbora tarteetan eta zenbait z posizioetan hartu ziren. Irudikapen guztiak 37°Ctan eta %5eko CO₂arekin burutu ziren.

2.11.3 Fluoreszentziako Mikroskopia Konfokala

70. hamarkadaren amaieran zeharreko mikroskopia konfokalaren garapena, mikroskopia optikoaren aurrerapen nagusietako bat izan zen. Fluoreszentziako mikroskopia konfokalak hain zuzen, laginaren fokatutako plano mehe batetik datorren fluoreszentzia aztertzea ahalbidetzen du, eta honek hiru-dimentsioko (3D) irudiak eraikitzeke aukera ematen du. Fokatutako planoetatik kanpo datorren fluoreszentzia baztertzeke, eta epifluoreszentziako mikroskopia arrunta ez bezala, irudikapen konfokala lortzen da detektagailutik hurbil zulo txiki (ingelesez *pinhole*) bat jarritz. Honela, fokatutako planoaren fluoreszentzia soilik iritsiko da detektagailura. **2.15 Irudiak** fluoreszentziako mikroskopia konfokal baten osagai nagusien irudikapen eskematikoa erakusten du. Funtsean, kitzikapen argi gisa laser bat erabiltzen da, zeina objektibo batetik zehar laginaren puntu txiki batera bideratuko den argia hautatuko duen ispilu dikroiko batean fokatzen den. Hala ere, hau ez da nahikoa aztertzen ari den osagaiaren 3D irudi bat eraikitzeke. Helburu honetarako, mikroskopia ispilu bereziak behar dira laser sortak x-y planoan eskaneatzeko, horrela laser ekorketa mikroskopia konfokala bezala ezagutzen den mikroskopia konfokal berezia sortzen da. Modu honetan, laginaren z mugimenduak era zehatzean kontrolatuz, 3 dimentsioko irudiak eratu daitezke.

Tesi lan honetan, laser ekorketa mikroskopia konfokalaren bidez, GUVen lipido domeinuak aztertu edo proteinen GUVekiko lotura behatu da, Nikon D-ECLIPSE C1 mikroskopia konfokala (Nikon Inc., Melville, NY, EEBA) erabiliz. Bestalde, zelula fixatuetan immunotindatutako zenbait proteinen kokapena aztertzeke Leica TCS SP2 mikroskopia konfokala (Leica Microsystems CMS GmbH) erabili zen.



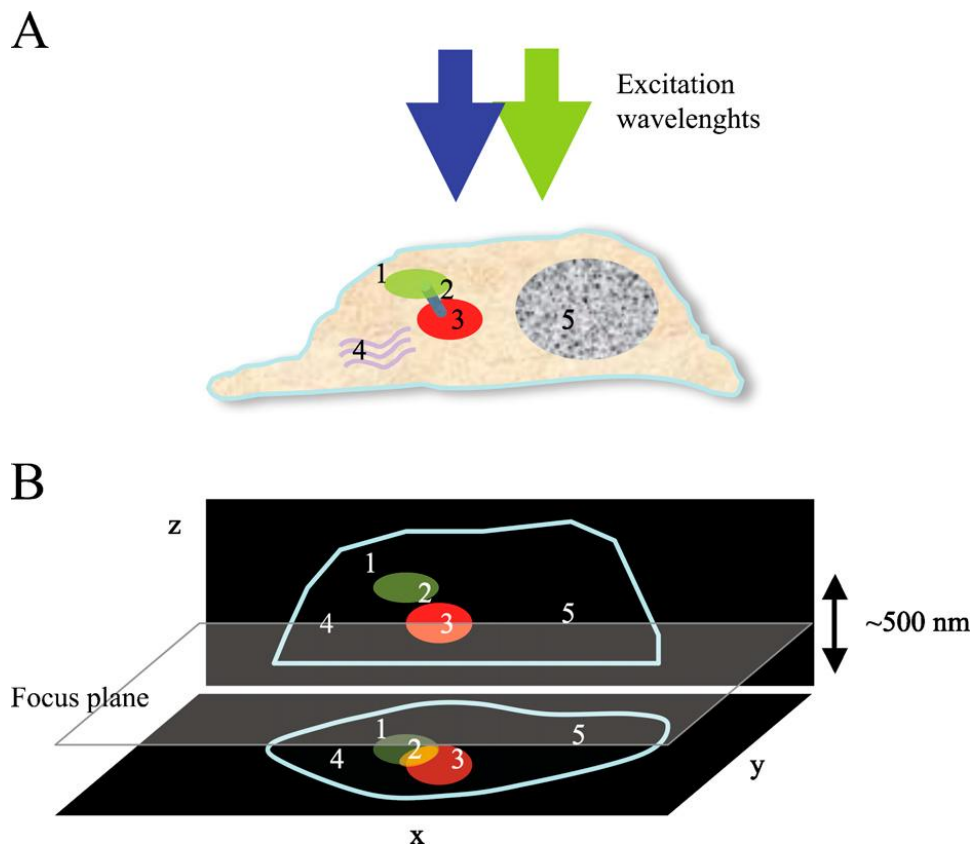
2.15 Irudia. Fluoreszentziako mikroskopia konfokal baten oinarrizko osagaiak.

2.12 Argizko Mikroskopia eta Mikroskopia Elektroniko Korrelatiboa (CLEM)

Azken urteotan, bi mikroskopia tekniken indarrak bateratzen dituen CLEM (ingelesezik *correlative light and electron microscopy*) teknikaren inguruko interesa asko handitu da. Hau ez da soilik argitalpen zientifikoan ugaritzean oinarritzen, baita CLEM bidezko ikerketara zuzenduak dauden produktu komertzial berrien agerpenean ere. CLEM teknikan lagin baten, ahal izanez gero eskualde bera, lehenengo argizko mikroskopiaren (LM, ingelesezik *light microscopy*) bidez eta gero mikroskopia elektronikoarekin (EM, ingelesezik *electron microscopy*) behatzen da (2.16 Irudia). Normalean, CLEM ikerketetan, beste LM metodo batzuen aldean, nahiago da FM erabiltzea, izan ere, eskuragarri dauden zunda-fluoreszente eta tindatzaile espezifiko ugariak erabiliz, zenbait proteina eta zelulako egiturek daukaten kokapenaren inguruko informazioa lor daiteke. Normalean, lehendabizi laginaren FM bidezko behaketa egiten da, eta ondoren, lagina mikroskopia elektronikora eramaten da.

FMa, intereseko eskualde bat (ROI, ingelesezik *region of interest*) edo lagin konplexu eta handietan ematen diren ezohizko gertaerak kokatzeko balio dezakeen laginaren ekorketa bat egiteko erabil daiteke, fluoreszente markatutako proteinak edota egiturak dauden posizioetan oinarrituz. Ondoren, aukeratutako ROI hauek, bereizmen handiko eta informazio ultraestruturala ematen duen transmisiozko

mikroskopia elektronikoan (TEM, ingelesetik *transmission electron microscopy*) behatzen dira. CLEM zelula bizien irudikapena (FM) EMrekin lotzeko erabiltzen denean (van Rijnsoever *eta lank.*, 2008; Spiegelhalter *eta lank.*, 2014), laginen FM irudikapen dinamikoak beharrezko informazioa eskaintzen du zelularen gertaera konplexuak dimentsio guztietan ulertzeko, baita denboraren inguruko informazioa ere (berrikustapen gaurkotuago batentzat ik. Karreman *eta lank.*, 2016). Gero, aukeratutako momentu konkretu batean lagina fixatu egiten da eta FM bidezko behaketa dinamiko hauek, EM irudikapenaren bidez lortzen den arkitektura zelularren inguruko informazio garrantzitsuarekin konbinatzen dira.



2.16 Irudia. CLEM teknikaren abantailak. (A) Zelula baten 5 ezaugarri adierazten dira (1–5). Gezi urdin eta berdeek LM irudikapenerako erabiltako kitzikapen uhin luzera ezberdinak adierazten dituzte. Fluoreszentzian oinarritutako irudikapenak soilik markatuta dauden osagaien (2 eta 3) identifikazioa ahalbidetzen duen bitartean, EMk osagai guztiak ikusgarri diren (1-5) erreferentziako esparrua eskaintzen du. Fluoreszentziako mikroskopia optikoaren (FLM, ingelesetik *fluorescence light microscopy*) bereizmen axial mugatua dela eta, 200 nm baino gertuago dauden osagaiak, x-y proiektzioan puntu bakarrean batzen dira (B). CLEMek bi teknikak konbinatzen ditu zelula osagaien edo zelulan gertatzen ari diren gertaeren inguruko informazioa eta bereizmen handiena lortzeko (Cortese *eta lank.*, 2009-etik hartua).

Biologikoki garrantzitsuak diren auziak argitzeko erabilgarria izan daitekeen arren, CLEM erabileraren hedapena hainbat oztopok mugatzen dute, horien artean aurkitzen dira: laginak prestatzeko protokolo optimizatuak eta CLEM teknikarekin bateragarriak diren markatzaileak ez egotea, emaitzak eskuratzeko prozesu geldoa, FM-EM korrelazioaren zehaztasun falta, eta protokolo esperimentalen konplexutasuna. Gaur egun gero eta ikerketa lan gehiago daude protokoloak optimizatzea (Kolotuev *eta lank.*, 2010; Karreman *eta lank.*, 2014; Peddie *eta* Collinson, 2014; Russell *eta lank.*, 2016), markaketa hobetzea (Grabebauer *eta lank.*, 2005; Shu *eta lank.*, 2011), eta CLEM bidezko ikerketekin lotutako teknologien garapena (Verkade, 2008; Koning *eta lank.*, 2013) helburutzat dutenak.

CLEM esperimentu guztiak Wolfson Bioimaging Facility-an (Bristoleko Unibertsitatea, Bristol, EB) eskuragarri dauden errektiboak eta ekipoa erabiliz burutu ziren.

2.12.1 Zelulen Fixaketa Kimikoa eta Epon Erretxinan Eginiko Barneraketa

Zelulak beirazko lauki-saredun oinarria duten 35 mm-ko plaketan (MatTek Corp.) hazi ziren eta FM bidezko irudikapena (zelula bizien irudikapena) egin ostean TEM egiteko, fixaketa kimikoa eta Epon erretxinan eginiko barneraketaren bidez prozesatu ziren.

11. Protokoloa. Zelulen Fixaketa Kimikoa eta Epon Erretxinan Eginiko Barneraketa

1. Hazkuntza medioan esekiduran dauden zelulak, %3 glutaraldehidoaren bolumen berdinarekin 15-30 minutuz fixatzen dira.
2. Glutaraldehidoarekin fixatutako zelulak bi aldiz garbitzen dira kakodilato indargetzailearekin (0.1 M Na kakodilato).
3. Ondoren, mintzen eta zitoplasmaren arteko kontrastea sortzeko, lipidoen tindatzailea den osmio tetroxidoa (OsO₄, Electron Microscopy Science, Hatfield, PA, EEBC) erabiltzen da. Laginak ordu batez ilunpetan %1.5 potasio ferrizianida, %1 OsO₄ eta 0.1 M kakodilatoarekin inkubatzen dira.

(Protokoloak hurrengo orrialdean jarraitzen du)

4. Gero zelulak 3 aldiz 0.1 M kakodilatoarekin eta beste 3 aldiz urarekin garbitzen dira.
5. Epon erretxinan barneraketa egiteko, lehendabizi laginak errotore batean deshidratatzen dira, hiru aldiz 5 minutu %70 etanolarekin, hiru aldiz 5 minutu %90 etanolarekin eta bi aldiz 5 minutu %100 etanolarekin.
6. Gero, Epon erretxina (3 g Epon, 2 g DDSA, 1.25 g MNA, 0.1625 mL BDMA, dena TAAB Laboratories Equipment Ltd., Berks, EB-ekoa) eta etanola 1:1 bolumen ratioan errotorean gauean zehar inkubatzen dira.
7. Laginak Epon erretxina purura pasatzen dira ondorengo 24 orduetan egindako bi aldaketen bidez. Epon erretxina polimerizatzen uzten da 48 orduz 60°Ctan.

Fixaketa kimikoa hainbat artefaktuekin lotuta dago (van Weering *eta lank.*, 2010-ean berrikusia), adibidez, mintzak kolapsatzea deshidratazioaren ondorioz eta zitoeskeletoaren ezegonkortzea, zeinak zenbaitetan organuluaren identifikazioa eragozten duen. Ikerketa lan honetan zelulen fixaketarako eta erretxinaren eginiko barneraketarako erabili den protokoloa zehaztua deskribatzen da (**11. Protokoloa**).

2.12.2 Erretxinaren Barneratutako Laginen Mozketa eta Irudikapena

2.12.2.1 Blokearen Prestaketa eta Mozketa

Erretxina blokeetatik euskarriak kentzeko, lehenengo euskarri bakoitzari gehiegizko erretxina kendu zitzaion kurrika bat erabiliz. Blokeak N₂ likidotan 10 segundoz eta beste 10 segundoz ur berotan (80°C) barneratu ziren izotz-kristalak sortu arte eta euskarriak kurrika bat erabiliz kontuz kendu ziren, intereseko zelulak blokearen goiko partean utziz.

Blokeak moztu ziren piramide-formako alde bat eratzeko lauki-sareko aurkitze koordenatuak (ingelesez *finder grid coordinates*) erabiliz, eta ondoren blokearen alde hori diamantezko labain bat (3.0 mm eta 45°C Diatome Ultra Diamond knife, Agar Scientific, Stansted, EB) erabiliz, 70 nm-ko elkarren segidako sekzioetan moztu zen. Sekzioak 3-4 sekziozko zintetan pintzak erabiliz 100 edota 300 laukietako sarea duten kobrezko lauki-sareen (*grids*) alde opakotan (3.05 mm, Agar Scientific) hartu ziren.

2.12.2.2 Tindaketa eta Mikroskopia Elektronikoaren (EM) Bidezko Irudikapena

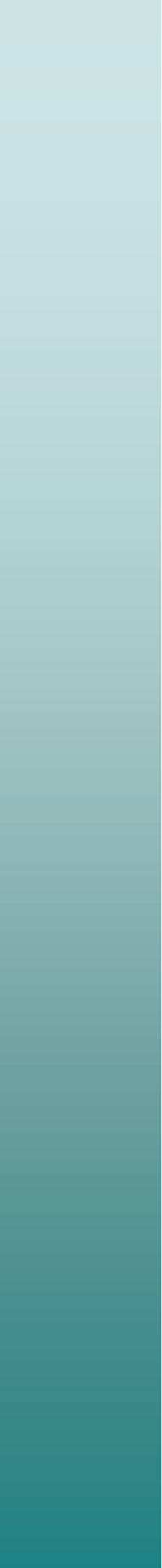
Kontrastea handitzeko eta TEM bidezko behaketa hobetzeko asmoz, sekzio meheak kontraste-tindaketaren bidez tindatu ziren. Soluzioak erabiliak izan aurretik 0.22 μm -ko filtro baten bidez filtratu ziren. *Nescofilm* izeneko filmean lauki-sareak soluzio bakoitzaren tanten gainean jarri ziren, sekzioak (alde opakoa) likidoarekin kontaktuan jarritz.

Lehendabizi, sekzioak %3 uranil azetako urtsuarekin 10 minutuz iluntasunean negatiboki tindatu ziren, ostean 3 aldiz, 5 minutu aldi bakoitza, aurretik irakindako eta hoztutako ur distilatuarekin garbitu ziren. Uranil azetatoa tindatzaile negatibo gisa erabiltzen da; dentsitate elektronikoa handitzen du atomo tindatzaile astunak jalkituz, eta horrela irudiaren kontrastea handitzen du. Lagin biologikoen elektroien-ekorketa ezaugarriak areagotzeko asmoz, berun zitratoa erabili zen. Honek, Epon erretxinaren sekzioetan mintz zitoplasmatikoen, erribosomen eta material nuklearraren intentsitate handiko tindaketa eragiten du (Reynolds, 1963). Berun zitrato soluzioa, 189 mM berun nitratoaren 1 mL, 282 mM sodio zitrato tindatzailearen 1 mL-rekin nahastatuz prestatu zen, ondoren 1M NaOH-ren 0.38 mL nahasturara gaineratu ziren. Lauki-sareak, berun zitrato tindatzailearekin 10 minutuz inkubatu ziren iluntasunean, gero 3 aldiz (5 min) garbitu ziren aurretik irakindako eta hoztutako ur distilatuarekin eta, gutxienez 45 minutuz, lehortzen utzi ziren giro tenperaturan.

Zelulak, TEM bidez behatu ziren Tecnai 12-FEI 120 kV BioTwin Spirit transmiziozko mikroskopia elektronikoa (FEI Europe, Eindhoven, Holanda) erabiliz eta irudiak FEI Eagle 4 k x 4 k CCD kamerarekin eskuratu ziren. Aurretik zelula bizien irudikapenean behatutako intereseko eskualde bakoitzaren irudikapena irudian zelula osoa ikusgarria izatea ahalbidetzen zuen magnifikazioan egin zen eta magnifikazioa gorde zen. Zelula guztian zehar beste 4 edo 5 irudi magnifikazio handiago batekin hartu ziren organuluen identifikazioa errazteko.

Chapter 3

Individual human Atg8 orthologs respond differently to cardiolipin in membranes: Specific properties of LC3B, GABARAPL2 and GABARAP



CHAPTER 3:

Individual human Atg8 orthologs respond differently to cardiolipin in membranes: Specific properties of LC3B, GABARAPL2 and GABARAP

3.1 Introduction

Macroautophagy, ‘autophagy’ in the context of this work, is an intracellular degradation pathway conserved in all eukaryotes (Mizushima & Komatsu, 2015; Yang & Klionsky, 2010). It involves the formation of a double-membrane structure, the phagophore, and its subsequent maturation to form an AP that fuses with lysosomes where the autophagosomal components are degraded (Shibutani & Yoshimori, 2014). This process does not only provide nutrients under amino-acid limiting conditions (nonselective autophagy) but works as well as a quality control system by selectively removing misfolded aggregate-prone proteins and damaged or superfluous organelles (selective autophagy) (Reggiori *et al.*, 2012). Thus far several cargo-specific autophagy processes have been reported, including the specific removal of mitochondria known as mitophagy (Ashrafi & Schwarz, 2013; Stolz *et al.*, 2014; Youle & Narendra, 2011). Beyond quality control, mitophagy is required for steady-state turnover of mitochondria (Tal *et al.*, 2007), for the adjustment of mitochondrion numbers to changing metabolic requirements (Kiššova *et al.*, 2004) and during specialized developmental stages in mammalian cells (Schweers *et al.*, 2007). Several non-mutually exclusive mechanisms have been reported including changes in the nutrient conditions of yeast media (Kanki & Klionsky, 2008), damage- or drug-induced loss of mitochondrial membrane potential

(Wang *et al.*, 2012), decreased ATP production (Chistiakov *et al.*, 2014) or alteration of mitochondrial Ca^{2+} homeostasis (Cherra *et al.*, 2013). This cellular process is thought to delay aging (García-Prat *et al.*, 2016) and its dysregulation has been linked to multiple human pathological states including neurodegeneration (Nixon, 2013; Aufschneider *et al.*, 2016), myopathies (Kubli & Gustafsson, 2012) and cancer (Lu *et al.*, 2013).

Several molecular components involved in mitophagy have been identified both in yeast (Kanki *et al.*, 2015) and higher eukaryotes (Reggiori *et al.*, 2012; Youle & Narendra, 2011), including members of the Atg8 protein family (mammalian LC3 and GABARAP subfamilies). However one outstanding question is how damaged or superfluous mitochondria are primarily recognized by the autophagic machinery (Randow & Youle, 2014). Specific mitophagy receptors, by binding on one side to mitochondria and on the other end to the above autophagosome-specific proteins, physically link mitochondria to the growing phagophores. Lazarou and coworkers using knockout models of the 5 mitophagy receptors, namely TAX1BP1, OPTN, NBR1, p62, and NDP52, have found that LC3B is specifically recruited by OPTN and NDP52 during mitophagy and LC3C by TAX1BP1. Remarkably they have observed that GABARAP family proteins are not recruited to mitochondria, suggesting that they are not actively involved in mitophagy (Lazarou *et al.*, 2015). By contrast, other groups have found that GABARAPL1 colocalizes with depolarized mitochondria during basal, starvation, and apoptotic conditions (Novak *et al.*, 2010; Betin & Lane, 2009; Boyer-Guittaut *et al.*, 2014). In addition, CL externalization to the OMM has been shown to function as a signal for LC3 that mediates both mitochondrial recognition and AP formation, ultimately leading to targeted removal of damaged mitochondria (Chu *et al.*, 2013).

On the basis of these observations, we have attempted to gain further insight into the molecular basis of the interaction of LC3B with CL (Antón *et al.*, 2016). Here we show that LC3B interacts preferentially with CL over other di-anionic lipids such as PtdIns4P and that the LC3B-CL interaction relies on both electrostatic forces and CL-specific changes in membrane properties. We also provide evidence indicating that the LC3B C terminus remains exposed to the hydrophilic environment after protein binding to CL-enriched membranes. Finally, we extended our analysis to the LC3 paralogs GABARAPL2 and GABARAP, and we found that GABARAP, but not GABARAPL2, interacts with CL *in vitro*. Altogether, our study indicates that (i) LC3B-cardiolipin interaction is modulated by particular membrane properties that could allow the specific recognition of impaired mitochondria and (ii) the various human orthologs of yeast Atg8 may play specific roles in different autophagic processes.

3.2 Materials and Methods

3.2.1 Materials

PC from egg yolk was purchased from Lipid Products (Redhill, UK). Chol, bovine heart CL [90% TLCL (CL 18:2), 5% TOCL, 5% unknown], TOCL (CL 18:1), TMCL (CL 14:0), CL*, PtdIns4P, PtdIns[4,5]P₂, PtdIns[3,4,5]P₃, brain PS, liver PtdIns, egg PG, egg PA, Rho-PE, DOPE and eCer were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Anti-LC3 monoclonal antibody, anti-GABARAP monoclonal antibody and anti-GABARAPL2 polyclonal antibody were from MBL International (Medical & Biological laboratories Co. Ltd., Nagoya, Japan).

3.2.2 DNA Constructs and Site-Directed Mutagenesis

Plasmids for expression of several Atg8 family proteins (LC3B, GABARAPL2 and GABARAP) and LC3B lacking the C-terminal Gly (LC3B^{GA}) were kindly provided by Dr. I. Tanida. Note that each Atg8 ortholog is a truncated form ending in the reactive C-terminal Gly such that no ATG4-mediated preprocessing is necessary. These plasmids were used as templates for site-directed mutagenesis using QuikChange SDM kit (Stratagene, San Diego, CA, USA) according to the manufacturer's instructions. The following mutants were made using this SDM kit: a double mutant for Arg to Ala at positions 10 and 11 of LC3B (LC3B^{R10,11A}) and at positions 14 and 15 of GABARAP (GABARAP^{R14,15A}); a single mutant for Lys to Ala at position 47 of GABARAP (GABARAP^{K47A}); a double mutant for Lys to Ala at positions 46 and 47 of GABARAP (GABARAP^{K46,47A}); a triple mutant for Lys to Ala at positions 46, 47 and 48 of GABARAP (GABARAP^{K46,47,48A}); and single Cys point mutants of LC3B (LC3B^{Q15C} and LC3B^{S101C}) and GABARAP (GABARAP^{S16C} and GABARAP^{S88C}). All primers designed to introduce site-directed mutations were synthesized and purified by Sigma-Aldrich (See **Table 2.1**). The polymerase chain reaction-amplification products were evaluated by agarose gel electrophoresis and the parental methylated and hemimethylated DNA was digested by *DpnI* endonuclease (New England Biolabs). After inactivation of *DpnI* (80°C for 20 min), the digested polymerase chain reaction product was transformed into DH5- α *E. coli* chemo-competent cells and inoculated on LB (Sigma-Aldrich) plates containing 100 mg/ml ampicillin (Sigma-Aldrich). A total of 5 colonies were selected and their plasmids were isolated by mini-prep (GeneJET Plasmid Miniprep Kit, Thermo Fisher Scientific) and used for DNA sequencing (Secugen S.L.).

3.2.3 Recombinant Protein Expression and Purification

All proteins were purified from soluble fractions of bacterial extracts obtained in the absence of detergents, and were >90% pure as evaluated by Coomassie Blue-stained SDS-PAGE. *E.coli* BL21(DE3) cells were transformed with appropriate plasmids and cells were grown to OD₆₀₀ of 0.8 and induced with 0.5 mM IPTG (Sigma-Aldrich) for 4 h at 20°C in the case of GABARAP, GABARAP^{R14,15A}, GABARAP^{K47A}, GABARAP^{K46,47A}, GABARAP^{K46,47,48A}, GABARAP^{S16C}, GABARAP^{S88C} and 3 h at 37°C for GABARAPL2 and LC3B, LC3B^{GA}, LC3B^{R10,11A}, LC3B^{Q15C}, LC3B^{S101C}. Following centrifugation at 4,500 x g for 15 min, the pellet was resuspended and sonicated in breaking buffer [10 mM PBS, 20 mM Tris-HCl, pH 7, 150 mM NaCl, supplemented with freshly prepared 1 mg/ml lysozyme (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), bacterial protease inhibitors (Roche) and 2.5 µg/ml DNase (Sigma-Aldrich)]. After removal of cellular debris by centrifugation at 30,000 x g for 30 min at 4°C, the sample supernatant fraction was incubated with 1 ml glutathione Sepharose 4B (GE Healthcare) for 3 h at 4°C to bind GST-tagged proteins. Then PreScission Protease (GE Healthcare) was added at 100 units/ml in a 2-bed volume of PreScission Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) freshly prepared with 1 mM DTT and cleavage was performed for 4 h at 4°C. Cleaved protein was eluted and concentrated. Proteins were stored in 20% glycerol at -80°C.

3.2.4 Alexa Fluor 488 Protein Labeling

Purified proteins were first concentrated to 4-5 mg/ml (protein concentration should be at least 2 mg/ml for optimal results) and dialyzed against 0.1 M sodium bicarbonate (pH 8.2), 150 mM NaCl buffer to remove any amine-containing substances that would interfere with the conjugation reaction. Then 10 µL of the reactive dye solution [10 mg/mL of the Alexa Fluor[®] 488 dye (Molecular Probes) dissolved in DMSO (Sigma-Aldrich)] was slowly added to the protein solution. The reaction was incubated for 2 h at 37°C with continuous stirring. A Sephadex G-25 chromatography column (GE Healthcare) was used to separate the conjugate from unreacted labeling reagent with PreScission buffer used as the eluent. The degree of labeling was determined measuring the absorbance of the protein-dye conjugate at 280 nm and at 488 nm for the dye. Labeled samples were subjected to SDS-PAGE and visualized using a VersaDoc MP 4000 Imaging System (Bio-Rad).

3.2.5 IANBD Cysteine-substituted Protein Labeling

In a typical labeling reaction, cysteine-substituted proteins were first

concentrated to 2-3 mg/ml and incubated with 1 mM TCEP (Sigma-Aldrich) for 30 min at room temperature. Then proteins were dialyzed against the labeling buffer [10 mM HEPES (Sigma-Aldrich), pH 7.3, 100 mM KCl, 1 mM EDTA] to remove TCEP. IANBD (Molecular Probes) dissolved in DMSO was then added in a small volume (15 to 25 μ L) to the protein solution to give a final protein:probe molar ratio of 1:10 (NBD stock solution concentration was 30 mM). The mixture was incubated overnight at 4°C with gentle stirring. Next the mixture was passed again through a PD-10 column (GE Healthcare) equilibrated with labeling buffer to separate the conjugate from unreacted labeling reagent. The stoichiometry of the NBD-protein conjugation reaction was calculated as the molar ratio of conjugated NBD and labeled proteins. The molar concentrations of the conjugated NBD and protein were determined from the absorbance at 488 nm (for NBD) and 280 nm (for proteins) using the molar extinction coefficients (ϵ M) of 26500, 5960, 18450 and 11920 $\text{M}^{-1}\text{cm}^{-1}$ for NBD, LC3B, GABARAPL2 and GABARAP respectively.

3.2.6 Liposome Preparation

The appropriate lipids were mixed in organic solution [chloroform:methanol (2:1 v/v)] and the solvent was evaporated to dryness under a N_2 stream. Then the sample was kept under vacuum for 2 h to remove solvent traces and the lipids were swollen in PreScission buffer. Large unilamellar vesicles were prepared from swollen lipids, subjected to 10 freeze/thaw cycles, and then extruded [LIPEX Liposome Extrusion System (Transferra Nanosciences)] using 0.1 μ m pore size Nuclepore filters (Whatman, Maidstone, UK) as described by Mayer and coworkers (Mayer *et al.*, 1986). SUVs were obtained by sonicating MLVs with a probe tip sonicator (MSE Soniprep 150, MSE) for 20 min (10 sec on, 10 sec off) on ice. Vesicle size was checked by quasi-elastic light scattering using a Malvern Zeta-Sizer 4 spectrometer (Malvern Instruments). LUVs had an average diameter of 100 nm and SUVs average diameter was 60 nm. Lipid concentration was determined by phosphate analysis.

3.2.7 Monolayer Surface Pressure Measurements

Surface pressure experiments were carried out in a multi-well Delta Pi-4 Langmuir balance (Kibron Inc., Helsinki, Finland) under constant stirring. Protein-induced changes in surface pressure at the air-water interface and protein-lipid monolayer interactions were studied at either 25°C or 37°C. Monolayers were formed by spreading a small amount of the lipid mixtures in chloroform:methanol (2:1 v/v) solution on top of assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 0.2

mM DTT) until the desired initial surface pressure was reached. Proteins were injected with a micropipette through a hole connected to the subphase.

3.2.8 Protein-Lipid Overlay Assay

Stock solutions of different lipids were solubilized in 2:1:0.8 MeOH:CHCl₃:H₂O, spotted onto Hybond C nitrocellulose (GE Healthcare) and allowed to dry. To detect LC3B by immunoblotting, the nitrocellulose was first blocked with 5% fat-free milk in PBS for 1 h and further incubated for 1 h with LC3B in PreScission buffer at 37°C. The nitrocellulose was washed 4 times, 10 min each, with PBS, and soaked in 5% fat-free milk in PBS with an anti-LC3 monoclonal antibody at 1:1000 dilution overnight at 4°C. The nitrocellulose was washed 4 times with PBS and soaked in 5% fat free milk in PBS with HRP-conjugated anti-mouse antibody (Santa Cruz Biotechnology) at a 1:5000 dilution for 1 h at room temperature. After washing with PBS 4 x 10 min at room temperature, the protein was detected by chemiluminescence. Protein bands were digitalized and integrated densities were measured using Quantity One software (Bio-Rad).

3.2.9 Sucrose Gradient Centrifugation of Liposomes

Recombinant proteins as indicated (10 µM) were incubated with 3 mM liposomes (containing 0.05 mol percent Rho-PE for detection) for 1 h at 37°C in 200 µl PreScission buffer. 125 µl protein/lipid mix was diluted to 300 µl in PreScission buffer containing 2.4 M sucrose (Sigma-Aldrich). Then the reaction mix was transferred to a centrifuge tube. The 1.4 M sucrose layer was overlaid with 400 µl PreScission buffer containing 0.8 M sucrose and 300 µl PreScission buffer containing 0.5 M sucrose. Sucrose step gradients were centrifuged in a TLA-120.2 rotor (Beckman Coulter) at 356,160 x g for 3 h at 4°C. Four 250-µl fractions were pipetted, starting from the bottom. The top fraction (T), containing liposomes as indicated by the rhodamine fluorescence, and the bottom fraction (B) containing the unbound protein were analyzed by SDS-PAGE and western blotting.

3.2.10 Fluorescence Spectroscopy

Fluorescence measurements were performed in a QuantaMaster spectrofluorometer (Photon Technology International) in a thermostatically controlled 1-cm path length cuvette with constant stirring at 37°C. For Trp measurements, excitation wavelength was 295 nm, the band-pass was 5 nm, and emission spectra of Trp were measured between 310 nm and 450 nm at a rate of 1 nm/sec. For NBD fluorescence

measurements, the excitation wavelength was 465 nm, the band-pass was typically 5 nm, and emission spectra of NBD were measured between 520 nm and 650 nm at a rate of 1 nm/sec. A 515 nm cut-off filter was placed between the sample and the emission monochromator to avoid scattering interference. In all cases, background intensities from samples lacking protein were subtracted from the intensities measured in protein containing samples.

3.2.11 Far-UV CD Measurements

Far-UV CD spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd.) equipped with a JASCO PTC-423S temperature control unit using 1-mm path length cell. Data were collected every 0.2 nm at 50 nm/min from 260 to 200 nm with a bandwidth of 2 nm, and results were averaged from 20 scans. The contribution of buffer to the measured ellipticity was subtracted for each protein as the blank.

3.2.12 GUV Preparation

GUVs were prepared using the electroformation method developed by Angelova & Tsoneva, (1999). For direct visualization under the microscope a homemade chamber was used (Fidorra *et al.*, 2006). Transferred GUVs were formed in a PRETGUV 4 chamber supplied by Industrias Técnicas ITC. Stock solutions of lipids (2 mM total lipid containing 0.5 mol% Rho-PE) were prepared in chloroform:methanol (2:1, v/v), 4 μ l of the lipid stocks were added onto the surface of Pt electrodes and solvent traces were removed by evacuating the chamber under high vacuum for at least 2 h. Next, the Pt electrodes were covered with 400 μ l of a 300 mM sucrose solution. The Pt electrodes were connected to a generator (TG330 function generator, Thurlby Thandar Instruments) under AC field conditions (10 Hz, 1 VRMS for 2 h, followed by 2.5 Hz, 1 VRMS, 1 h 30 min).

3.2.13 Confocal Fluorescence Microscopy

After GUV formation, the vesicle suspension (in 300 mM sucrose) was transferred to microscopy chambers pretreated with BSA (2 mg/ml) and containing an equiosmolar buffer solution of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1 mM DTT, pH 7.5. Due to the different density of the two solutions, the vesicles sedimented at the bottom of the chamber, and this facilitated observation under the microscope. Finally, LC3B- or GABARAP-Alexa 488 (10 μ M) were added to study protein binding to GUVs after incubation at 37°C. The chamber was placed on an inverted confocal

fluorescence microscope (Nikon D-ECLIPSE C1, Nikon). The excitation wavelengths were 488 nm for protein-Alexa488 and 561 nm for Rho-PE; and the emission was collected using 515 ± 15 nm and 593 ± 20 nm band-pass filters, respectively. Imaging was performed at room temperature. Image treatment was performed using the EZ-C1 3.20 software (Nikon).

3.2.14 Statistical Analyses

Statistical analyses were performed by ANOVA with a *post hoc* analysis by the Student-Neuman-Keuls test.

3.3 Results

3.3.1 Human Orthologs of Yeast Atg8

Atg8 family proteins are essential factors in the execution phase of autophagy (Mizushima *et al.*, 2011). Whereas the yeast *Saccharomyces cerevisiae* contains a single ATG8 gene, in humans the ATG8 family contains 4 LC3 subfamily genes (LC3A, LC3B, LC3B2 and LC3C) and 3 GABARAP subfamily paralog genes (GABARAP, GABARAPL1 and GABARAPL2); another human paralog of this subfamily, GABARAPL3, is currently considered to be a pseudogene, and no data at present link it with autophagy.

A

```

>Atg8_YEAST                MKSTFKSEYPFEKRKAESERIADRFRKRIPVICEKAEK-SDIPEIDKRKYLV 51
>MAP1LC3A_HUMAN           MPSDRPFKRRSFADRCKEVQQIRDQHPSKIPVIIERYKGEKQLPVLDKTKFLV 54
>MAP1LC3B_HUMAN           MPSEKTFKRRTFEQRVEDVRLIREQHPTKIPVIIERYKGEKQLPVLDKTKFLV 54
>MAP1LC3C_HUMAN           MPPPQKIPSVRPFKRRSLAIRQEEVAGIRAKFPNKIPVVVERYPRETFLPPLDKTKFLV 60
>GABARAP_HUMAN            MKFVYKEEHPFEKRRSEGEKIRKKYPDRVPVIVEKAPK-ARIGDLDKKKYLV 51
>GABARAPL1_HUMAN          MKFYQYEDHPFEYRKKKEGKIRKKYPDRVPVIVEKAPK-ARVPDLDKRKYLV 51
>GABARAPL2_HUMAN          MKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSQ-SQIVDIDKRKYLV 51
>GABARAPL3_HUMAN          MKFYQYEVHPFEYRKKKEGKIRKKYPDRVPLIVEKAPK-ARVPDLDRRKYLV 51
>Ubiquitin_HUMAN          MQIFVKT-----LTGKTITL 25
  
```

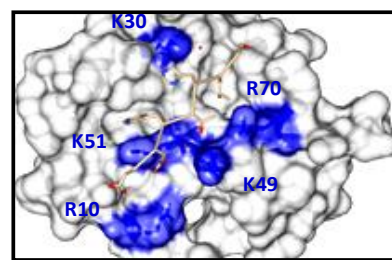
```

>Atg8_YEAST                PADL--TVGQFVYVIRKRIMLPPEKAIFIFVNDT-LPPTAALMSAIYQEHKDKDGFYLV 108
>MAP1LC3A_HUMAN           PDHV--NMSELVKIIRRRLQLNPTQAFLLVNQHSMVSVSTPIADIYEQEKDEDGFYLMV 112
>MAP1LC3B_HUMAN           PDHV--NMSELIKIIRRRLQLNANQAFLLVNGHSMVSVSTPISEVYESEKDEDGFYLMV 112
>MAP1LC3C_HUMAN           PQEL--TMTQFLSIIRSRMVLRATEAFYLLVNNKSLVMSATMAEYRDKDEDGFVYMT 118
>GABARAP_HUMAN            PSDL--TVGQFYFLIRKRIHLRAEDALFFFVNNV-IPPTSATMGQLYQEHHEEDFFLYIA 108
>GABARAPL1_HUMAN          PSDL--TVGQFYFLIRKRIHLRPEDALFFFVNNV-IPPTSATMGQLYEDNHEEDYFLYVA 108
>GABARAPL2_HUMAN          PSDI--TVAQFMWIIRKIQLPSEKAIFLFVDKT-VPQSSLTMGQLYEKEKDEDGFYVA 108
>GABARAPL3_HUMAN          PSDL--TDGQFYLLIRKRIHLRPEDALFFFVNNV-IPPTSATMGQLYEDSHEEDDFLYVA 108
>Ubiquitin_HUMAN          EVEPSDTIENVKAKIQDKEGIPPDQQLIFAGK-QLEDGRT----LSDYNIQKESTLHLV 70
  
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>Atg8_YEAST                YSGENTFGR----- 117
>MAP1LC3A_HUMAN           YASQETFGF----- 121
>MAP1LC3B_HUMAN           YASQETFGMKLSV----- 125
>MAP1LC3C_HUMAN           YASQETFGCLESAAPRDGSSLEDRPCNPL 147
>GABARAP_HUMAN            YSDESVYGL----- 117
>GABARAPL1_HUMAN          YSDESVYGK----- 117
>GABARAPL2_HUMAN          YSGENTFGF----- 117
>GABARAPL3_HUMAN          YSNESVYGK----- 117
>Ubiquitin_HUMAN          LRLRGG  ↑ 76
  
```

B



LC3B-SQSTM1

Figure 3.1. Human Atg8 family members. (A) Sequence alignment (Clustal Omega) of yeast Atg8, Atg8 human orthologs and ubiquitin. Highlighted in grey are the highly conserved basic amino acids, which have the potential to bind negatively charged amino acids located at the C- and N-terminal regions of the conserved LIR (consensus: W/Y/FxxL/I/V) present in the different cargo receptors during selective autophagy. (B) View of LC3B bound to the SQSTM1 LIR motif (PDB: 2ZJD). Highlighted in blue are the positively charged residues in LC3B that stabilize binding to SQSTM1 LIR motif. Picture was created by UCSF Chimera.

Originally implicated in intracellular trafficking processes all Atg8 orthologs are found within autophagosomes through binding to PE via a conserved C-terminal glycine (see **Figure 3.1A**, black arrow). Each of the 3 families is very well conserved across its entire length and all are very similar to each other in most of their positions (**Figure 3.1A**). However, the amino-terminal region differs between the various mammalian Atg8 proteins (the N terminus of LC3 subfamily members is strongly basic, whereas in the GABARAP subfamilies this region is acidic) (see **Figure 3.10**). It has been proposed that differences between the various human Atg8 proteins in this N-terminal region might reflect their distinct functions. Moreover, in spite of being ubiquitously expressed some subfamily members are expressed at increased levels in certain tissues.

All Atg8 orthologs share a strong structural similarity containing 2 amino-terminal α -helices (N-terminal arm) in addition to their C-terminal ubiquitin core (ubiquitin-like domain). The ubiquitin core contains a hydrophobic feature that is conserved among family members and is suggested to have a role in protein-protein interactions (**Figure 3.1B**), namely binding to the conjugation machinery proteins.

In this study, LC3B, GABARAPL2, GABARAP and their mutant forms were expressed in their GST-tagged form in *E. coli* BL21 (DE3) and purified using glutathione Sepharose beads. All Atg8 human orthologs were expressed and purified with the C-terminal Gly exposed (**Figure 3.2**).

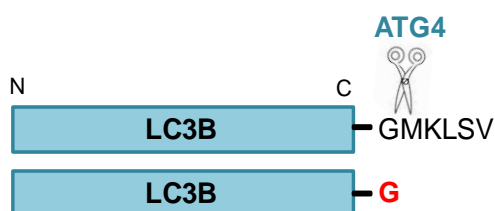


Figure 3.2. Schematic representation of the expressed Atg8 orthologs. LC3B wild type and LC3B with the C-terminal Gly exposed are shown. All human orthologs were expressed with the C-terminal Gly exposed such that no ATG4-mediated cleavage would be necessary.

Next, recombinant ATG protein purity was evaluated and the secondary structure and correct folding were assessed using CD. As expected from previously reported data of these proteins, LC3B, GABARAPL2 and GABARAP gave rise to CD spectra with minima near 205 nm compatible with a mix of α -helix and intrinsically disordered structures (**Figures 3.3 and 3.4**). Circular dichroism was also used to check for any conformational changes induced by protein mutagenesis or labeling.

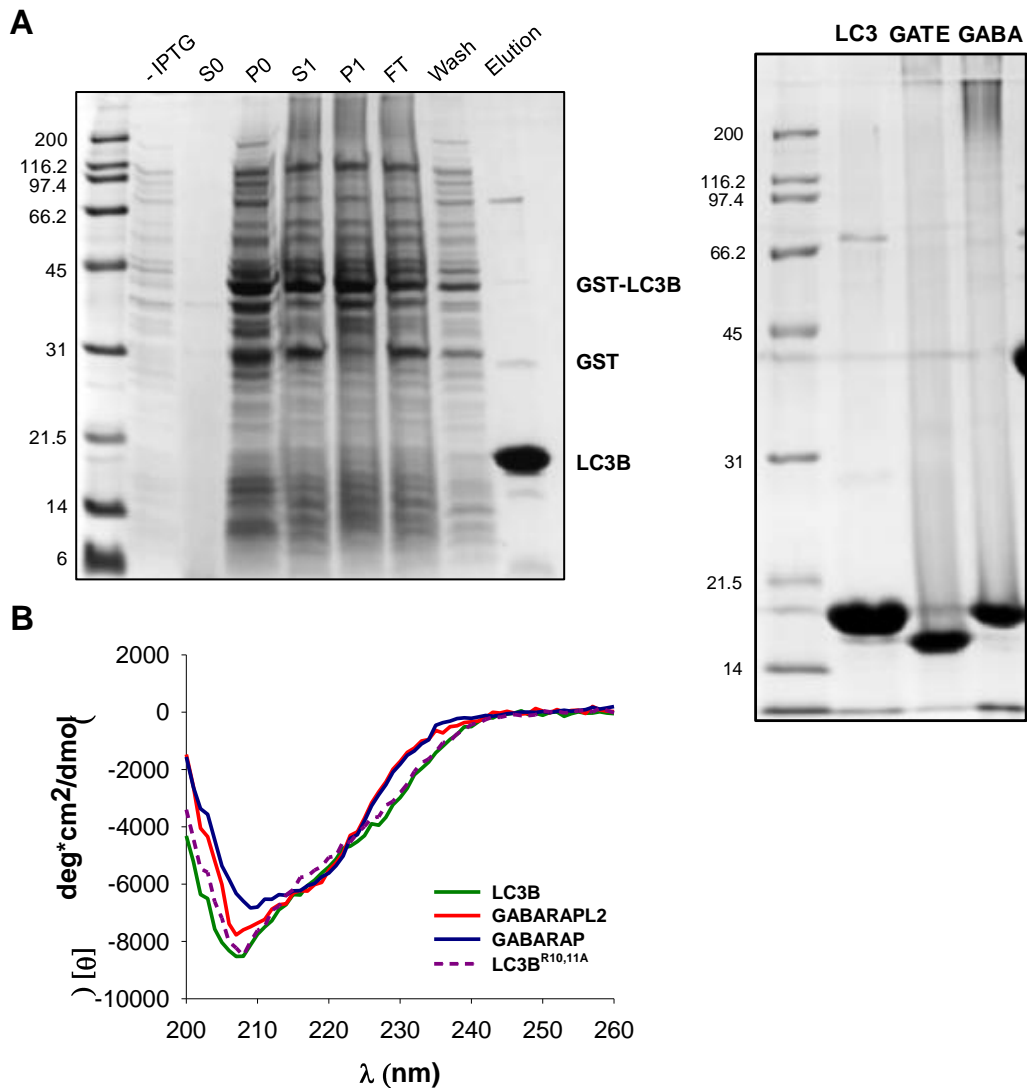


Figure 3.3. Expression and purification of recombinant LC3B, GABARAPL2/GATE-16 and GABARAP. (A) Coomassie Blue-stained gel showing purification steps. LC3B-expressing *E.coli* cells were lysed and soluble and insoluble fractions were separated by centrifugation. S0/P0 and S1/P1 correspond to the soluble and insoluble fractions before and after tip sonication, respectively. S1 was incubated with glutathione Sepharose beads for 3 h at 4°C to allow binding of LC3B to the beads (left-hand panel). After SEC, all proteins were >90% pure as evaluated by Coomassie-stained SDS-PAGE (right-hand panel). Molecular masses are shown in kDa on the left-hand side. (B) CD analysis of Atg8 orthologs and LC3B^{R10,11A} mutant in 50 mM Na₂HPO₄, 20 mM KCl, pH 7.0.

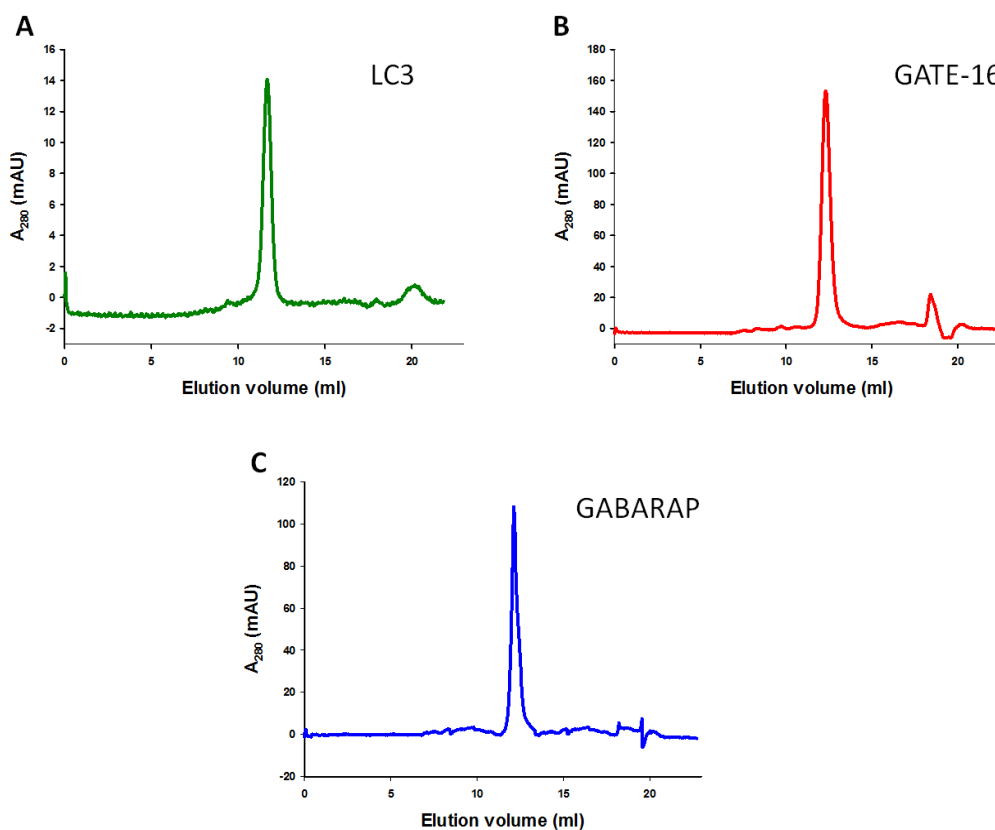


Figure 3.4. Elution profiles of human LC3B, GATE-16/GABARAPL2 and GABARAP (A-C). After elution from glutathione beads, ATG proteins were subjected to size exclusion chromatography on a Superdex 75 10/300 GL column.

3.3.2 LC3B Interaction with CL-containing Membranes

CL, the signature lipid of mitochondria, is a unique glycerol-based phospholipid comprising two phosphate moieties and four acyl groups of which linoleic acid is predominant (Osman *et al.*, 2011). CL is essential not only for normal mitochondrial function (Maguire *et al.*, 2016; Ren *et al.*, 2014) but it also plays a prominent role in disease-related processes such as apoptosis (Gonzalvez & Gottlieb, 2007; Maguire *et al.*, 2016) or mitophagy (Chu *et al.*, 2013; Hsu & Shi *et al.*, 2016; Maguire *et al.*, 2016; Antón *et al.*, 2016). In fact, although CL is usually considered to be a specific component of the inner mitochondrial membrane, the intramitochondrial location of CL is highly dynamic and CL externalization to the outer mitochondrial membrane has recently been shown to act as a signal for LC3, resulting in LC3 translocation to the OMM and targeted removal of damaged mitochondria via autophagy (Chu *et al.*, 2013). Based on these observations, we decided to define in more detail the molecular mechanisms underlying LC3-CL interaction (Antón *et al.*, 2016).

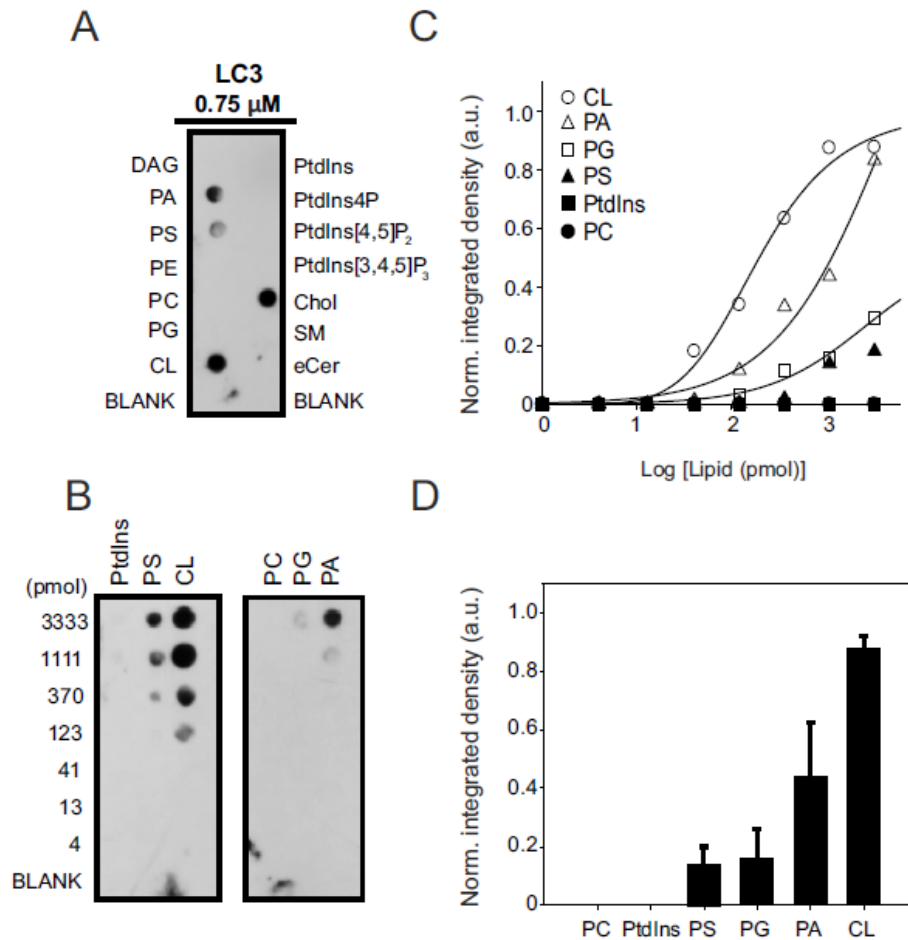


Figure 3.5. LC3B binds CL directly and preferentially over other anionic lipids. LC3B immunoreactivity is shown from a representative protein:lipid dot-blot experiment with some of the most common glycerolipids and sphingolipids present in mammalian cells (1 nmol of each lipid) (A) and with increasing amounts of some of those lipids (B). (C) Integrated LC3B immunoreactivity was measured at each spot and normalized for each lipid, and the data were fitted with a sigmoidal equation of the type $f = (y_0 + a \cdot x^b) / (c + x^b)$, from which an apparent Hill coefficient (b) of 5.3 ± 1.2 for CL binding could be estimated ($R^2 = 0.991$). (D) Quantitative assessment of bound LC3B (0.75 μM) with 1.11 nmol of each lipid. LC3B immunoreactivity was measured at each spot by immunoblotting using anti-LC3 antibody and densitometry. Data shown as mean \pm SEM ($n = 3$).

First we performed a PLO assay in which recombinant LC3B was incubated with a nitrocellulose membrane containing some of the most common glycerolipids and sphingolipids present in mammalian cells (Figure 3.5A). Among the lipid species examined, LC3B bound most strongly CL and Chol and less strongly the anionic lipids PS and PA. Interestingly, LC3B binding was not detectable for PtdIns4P, which has the same net negative charge as CL, nor with the polyanionic lipids PtdIns[4,5]P₂ or PtdIns[3,4,5]P₃, with even higher net negative charges. Dose-response experiments indicated that LC3B bound CL with 5-fold higher apparent affinity than PS or PG, and 2-fold higher affinity than PA (Figure 3.5B,D). These results indicate that LC3B

recognizes different anionic phospholipid species and displays a preference for CL. The dose-response data for LC3B-CL binding (**Figure 3.5C**) can be fitted to a sigmoidal curve, as detailed in the figure legend, showing a high positive cooperativity for the binding, apparent Hill coefficient $n = 5.3$. This might mean that binding of LC3B to one of the phosphate groups of CL would make easier the binding of the second phosphoryl moiety.

Next, we analyzed the interaction of LC3B with lipid monolayers using a Langmuir balance. Injection of LC3B into the aqueous phase led to a rapid, dose-dependent increase of lateral pressure (π) at the air-water interface (**Figure 3.6A, left-hand panel**). An increase in π is an indication that the protein is adsorbing onto the air-water interface, i.e. that it has surface-active properties. The increase in surface pressure appears to reach a plateau value of ~ 15 mN/m at $1.5 \mu\text{M}$ LC3B.

Lipid monolayers composed of either PC:DOPE (80:20 mol ratio), PC:DOPE:CL (55:20:25 mol ratio), PC:DOPE:PS (55:20:25 mol ratio), or PC:DOPE:PtdIns4P (55:20:25 mol ratio) were prepared with constant surface area at an initial pressure of 19 mN/m, i.e. above the surface pressure measured for the pure protein (~ 15 mN/m). Recombinant LC3B was added to the subphase, and the resulting increase in monolayer surface pressure was monitored in real-time (**Figure 3.6A, right-hand panel**). As shown in **Figure 3.6B**, the increase in surface pressure observed with PC:DOPE:CL monolayers under near-equilibrium conditions was higher than the value observed with electrically-neutral PC:DOPE monolayers, indicating that LC3B inserted more favorably into negatively charged monolayers.

For a quantitative measure of the LC3B ability to penetrate into lipid monolayers, critical surface pressure values were determined. In these experiments, the $\Delta\pi$ upon LC3B addition was measured as a function of the π_0 . The data were fitted to a straight line, whose x-intercept corresponded to the monolayer π_c . The results in **Figure 3.6B** show that LC3B insertion was highest (or easiest) when anionic lipids were present in the monolayer. The LC3B^{GA} mutant was also examined, lacking the C-terminal Gly of LC3B, to test whether differences observed in LC3B also appeared in its inactive mutant form. LC3B^{GA} was found to be surface-active and it also exhibited a higher insertion into PC:DOPE:CL monolayers.

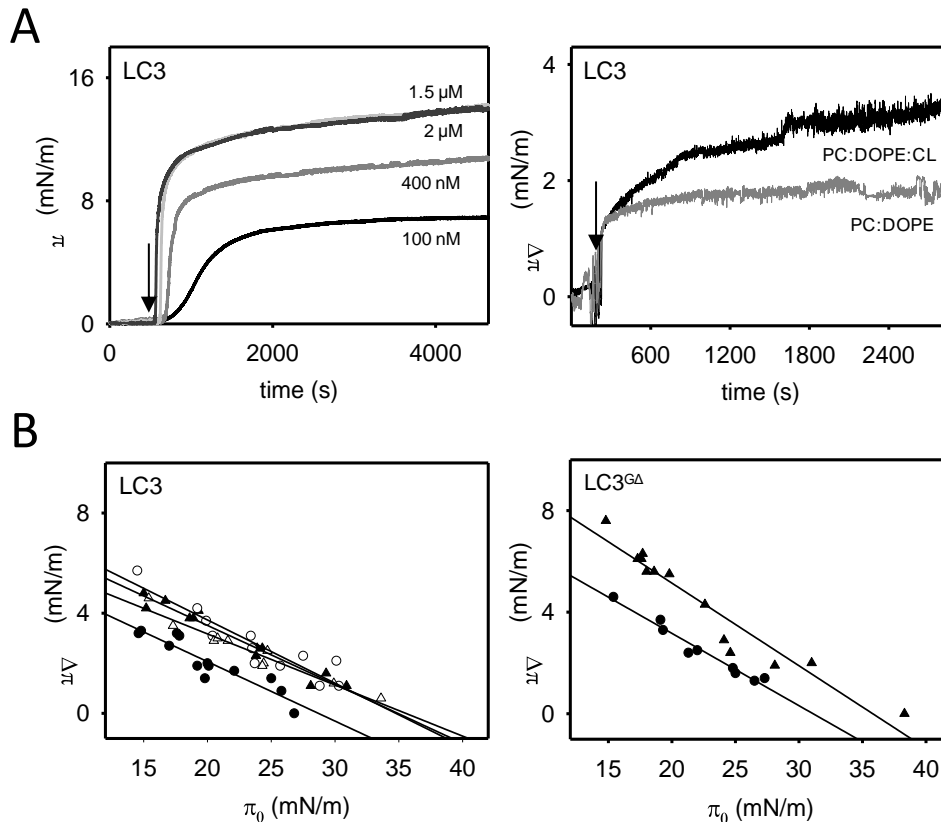


Figure 3.6. LC3B and LC3B^{GA} insertion into lipid monolayers. (A) Representative time courses of LC3B adsorption at the air-water interface (left-hand panel) and representative time courses of increase in lateral pressure after LC3B (1.5 μ M) insertion into PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (55:20:25 mol ratio) monolayers (right-hand panel) at 25°C. (B) Maximum increase in lateral pressure after LC3B or LC3B^{GA} insertion into lipid monolayers at 25°C. Lipids were: [●] PC:DOPE (80:20 mol ratio), [▲] PC:DOPE:CL, [○] PC:DOPE:PS and [Δ] PC:DOPE:PtdIns4 (55:20:25 mol ratio). Data reported as a function of initial lateral pressure π_0 . Very similar results were obtained at 37°C (data not shown).

Protein	Lipid Composition	π_c
LC3B	PC:DOPE (80:20)	28.7 mN/m (± 0.40)
	PC:DOPE:CL (55:20:25)	35.0 mN/m (± 0.30)
	PC:DOPE:PtdIns4P (55:20:25)	35.6 mN/m (± 0.29)
	PC:DOPE:PS (55:20:25)	34.7 mN/m (± 0.53)
LC3B ^{GA}	PC:DOPE (80:20)	31.0 mN/m (± 0.23)
	PC:DOPE:CL (55:20:25)	35.8 mN/m (± 0.64)

Table 3.1. Critical pressures (π_c) for LC3B and LC3B^{GA} insertion into lipid monolayers. Data calculated from the straight lines in Figure 3.6B. The tendency line-associated standard error is given for each π_c .

The critical surface pressure is a measure of the penetration capacity of a protein entering a monolayer: if this pressure exceeds 30 to 32 mN/m, then the protein is considered to be capable of membrane insertion (see **Table 3.1**). This is based on the estimation of ~30 mN/m as the average physiological surface pressure in cell membranes (Marsh, 1996).

Thus, our data indicate that LC3B inserts at least part of its mass into the monolayer and that negatively-charged groups favor the insertion. This is compatible with the idea of CL stabilizing initial electrostatic interactions leading to a partial insertion of LC3B into the bilayer (Chu *et al.*, 2013).

To test whether LC3B-CL interaction would occur in a bilayer membrane vesicle in which lipids maintain a certain bilayer curvature and could form domains, we performed liposomal float-up assays. In these experiments, pure LC3B was incubated with liposomes of a defined curvature and lipid composition and protein association with liposomes was assessed by the protein ability to float with the vesicles after equilibrium sucrose gradient centrifugation. Equivalent amounts of the top (T) and bottom (B) fractions of the gradient, corresponding respectively to proteoliposomes and to unbound protein, were then subjected to SDS-PAGE and western blot analysis. The immunoblot response of LC3B was quantified at different protein concentrations by densitometry and the data were linearly fitted by the least-squares method (**Figure 3.7A**). The LC3B maximal binding response was observed with CL-containing LUVs, while it did not bind PC:DOPE vesicles. Interestingly, LC3B did not bind either PtdIns4P-containing liposomes, although those vesicles had the same net negative charge as the CL-containing ones (**Figure 3.8A**). Furthermore, considering that LC3B exhibited strong binding affinity for Chol in PLO assays (**Figure 3.5A**), we also analyzed the binding of LC3B to model membranes containing both CL and Chol. However cholesterol inclusion in CL-enriched membranes did not lead to a higher binding response of the protein (**Figure 3.8A**). Moreover, no LC3B binding was observed with LUVs or SUVs composed of PC:DOPE:Chol (50:20:30) (data not shown). Thus the presence of Chol in those lipid compositions did not significantly affect LC3B binding to liposomes. We also analyzed the protein dose-dependence of the interaction by incubating liposomes with LC3B at 3 different protein concentrations. As shown in **Figure 3.8B**, little or no increase in LC3B binding to CL-containing vesicles was detected above 10 μ M protein. Finally, **Figure 3.8C** shows the dose-dependent binding of LC3B to CL-containing membranes; half-maximal binding occurred at 1.65 ± 0.2 mM total lipid. This suggests that the observed association of LC3B with PC:DOPE:CL vesicles is specific. Indeed, no protein binding was observed with

PC:DOPE vesicles even at the highest lipid concentration. These results suggest that LC3B is not an integral membrane protein but that it binds membranes through an interaction with acidic phospholipids, in agreement with the hypothesis proposed above that LC3B inserts only part of its mass into the membrane and that negatively-charged groups favor its insertion.

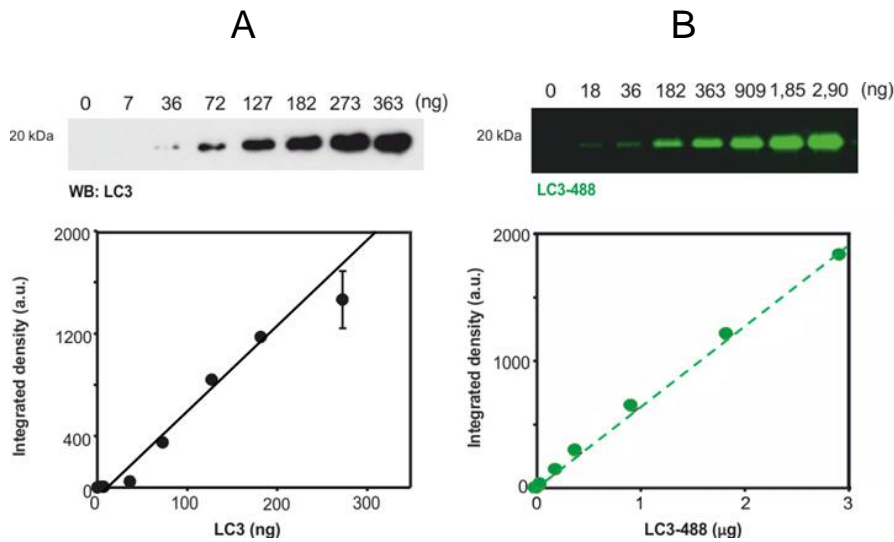


Figure 3.7. Least-squares linear fit to the experimental data. (A) Plot of immunoblot response of LC3B quantified by densitometry versus protein concentration. Line represents least-squares linear fit to the experimental data ($R^2 = 0.972$). (B) Plot of Alexa 488 fluorescence of LC3B quantified by densitometry versus protein concentration. Dashed line represents least-squares linear fit to the experimental data ($R^2 = 0.997$).

Moreover we have investigated the possible effect of changes in environmental pH on LC3B binding to model CL-containing membranes. CL is supposed to have only one negative charge below pH 7.5 (Kates *et al.*, 1993). In that case the electrostatic repulsion between CL phosphate groups might be reduced which together lowers the effective size of the headgroup and the smaller size of the polar headgroup should enhance the propensity of CL to form inverted nonlamellar lipid phases (Lewis & McElhaney, 2009). Regarding the pH effect on LC3B, the protein has a theoretical pI of 8.89 thus a net positive charge under our experimental conditions, with the charge becoming more positive as pH is lowered. As predicted, *in vitro* interaction of LC3B with CL-containing bilayers appeared to be highest at lower pH (**Figure 3.8D**).

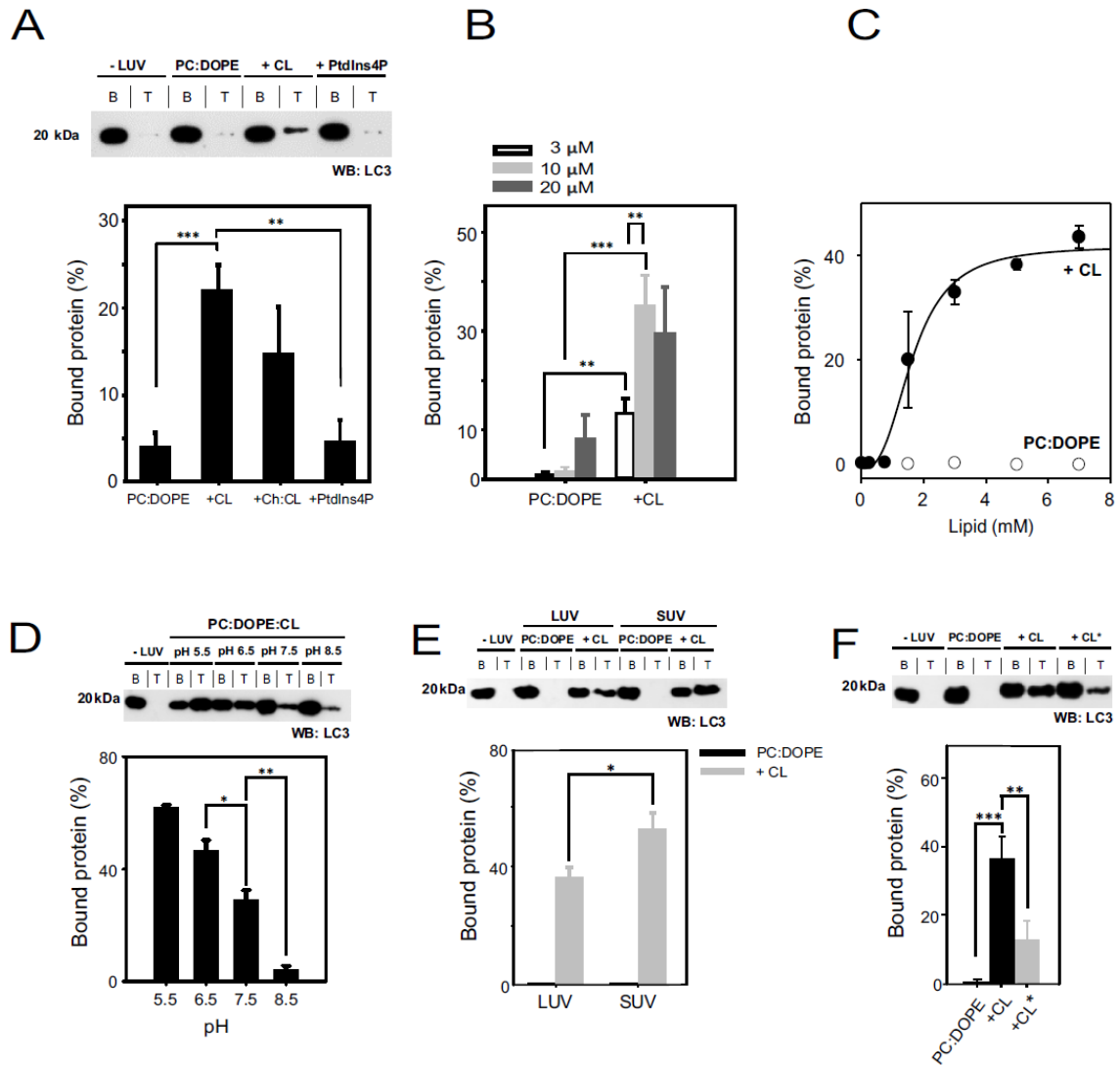


Figure 3.8. LC3B interacts preferentially with CL-containing liposomes. The liposome-bound protein fraction was analyzed after flotation in a sucrose density gradient by SDS-PAGE/immunoblot analysis and quantified by densitometric integration of the dots. **(A)** 10 μ M LC3B was incubated with 3 mM LUVs composed of either PC:DOPE (80:20 mol ratio), PC:DOPE:CL (50:20:30 mol ratio), PC:DOPE:Chol:CL (30:14:33:23 mol ratio) or PC:DOPE:PtdIns4P (50:20:30 mol ratio). Molecular mass is shown in kDa on the left-hand side. Data shown as mean \pm SEM (n = 3); **P = 0.001 to 0.01, ***P < 0.001. **(B)** LC3B dose-dependence analysis using 3, 10 and 20 μ M protein and 3 mM liposomes. Data shown as mean \pm SD (n = 3); **P = 0.001 to 0.01, ***P < 0.001. **(C)** Protein:lipid ratio effect on LC3B binding to PC:DOPE or PC:DOPE:CL vesicles. The continuous line represents the best fit of the data, assuming an EC₅₀ of 1.65 \pm 0.2 mM for binding to PC:DOPE:CL vesicles. Data shown as mean \pm SEM from at least 3 independent experiments. **(D)** Effect of pH on LC3B (10 μ M) binding to PC:DOPE:CL large vesicles (3 mM). Data shown as mean \pm SEM (n = 3); *P = 0.01 to 0.05, **P = 0.001 to 0.01. **(E)** 10 μ M LC3B was incubated with 3 mM LUV or SUV to analyze vesicle size effect on the interaction. Data shown as mean \pm SD (n = 3); *P = 0.01 to 0.05. **(F)** Comparison between heart bovine (CL) and *E. coli* (CL*) cardiolipins in the interaction. 10 μ M LC3B was incubated with 3 mM LUV of either composition. Data shown as mean \pm SD (n = 3); **P = 0.001 to 0.01, ***P < 0.001.

Membrane curvature is increasingly being reported to play an important role during autophagy (Fan *et al.*, 2011; Rostislavleva *et al.*, 2015; Antonny, 2011; Horenkamp *et al.*, 2015). Mitophagy is preceded by mitochondrial fission, which divides elongated mitochondria into smaller pieces of manageable size for encapsulation, and lipidation of LC3 has been recently proposed to occur preferentially on small, highly curved membranes *in vivo* and *in vitro* (Nath *et al.*, 2014). To address the effect of vesicle radius on LC3 membrane binding, we examined the interaction of LC3B with PC:DOPE and PC:DOPE:CL vesicles of 3 different sizes: SUV (~60 nm), LUV (~100 nm) and GUV (5-100 μ m). As shown in **Figures 3.8E and 3.13B**, we observed almost no binding of LC3B to PC:DOPE vesicles independently of their size. However LC3B bound CL-enriched SUVs better than LUVs and did not bind CL-containing GUVs. These results support the notion that interaction of LC3B with CL is promoted in the context of a curved membrane.

Apart from containing net negative charges CL is also characterized by a high degree of unsaturation of its acyl chains. This confers a high degree of flexibility to the membrane and the latter may in turn promote LC3 association. To evaluate this particular issue we analyzed LC3B binding to liposomes containing CL from *E.coli* which presents relatively short and fully-saturated or mono-unsaturated acyl chains. As shown in **Figure 3.8F**, LC3B bound significantly less the more rigid vesicles containing bacterial CL.

Additionally to the float-up assays, we used NBD-conjugated monocysteine LC3B mutants (LC3B^{Q15C} and LC3B^{S101C}) for studying LC3B interaction with CL-containing membranes. To enhance the efficiency of covalent modification with NBD, Cys substitutions were made at sites in LC3B with SASA values greater than 45% (**Table 3.2**). Since the protein was labeled in each case with a unique NBD moiety, this approach allowed us to characterize the association to the membrane of specific sites of the protein. We first examined the spectral properties of the NBD-labeled LC3B^{Q15C} mutant in the absence (black line) and presence (green line) of PC:DOPE, PC:DOPE:CL and PC:DOPE:PtdIns liposomes (**Figure 3.9A**). In the absence of liposomes, LC3B^{Q15C-NBD} displayed NBD $\lambda_{em(max)}$ values around 550 nm indicating a predominantly solvent-exposed NBD environment. No changes in the NBD spectrum were observed upon incubation with SUVs composed of PC:DOPE or LUVs composed of PC:DOPE:PtdIns, in agreement with the low affinity of the protein observed for liposomes that did not contain CL (**Figure 3.8A**). However incubation with CL-containing liposomes produced a substantial increase in LC3B^{Q15C-NBD} fluorescence intensity and blue-shifts in $\lambda_{em(max)}$ values, the change observed in the presence of CL-

enriched SUVs being larger than with LUVs (**Figure 3.9A**). As shown in **Figure 3.9B**, virtually no change in the emission spectrum of LC3B^{S101C-NBD} was observed upon addition of large or small vesicles containing CL (red line), unlike the situation found with the LC3B^{Q15C} mutant. Based on these data, we propose a structural model in which residue C101, which is located within the C terminus of the protein, remains in a hydrophilic environment before and after LC3B binding to CL-containing liposomes, while residue C15, which is localized within the N terminus, moves from a hydrophilic to a hydrophobic environment upon LC3B membrane binding (**Figure 3.9C**).

Protein	Residue	Localization	Modification	SASA (%)
LC3B	Q15	N terminus	Mutation to Cys and NBD labeling	47.0
	S101	C terminus	Mutation to Cys and NBD labeling	71.9
GABARAP	S16	N terminus	Mutation to Cys and NBD labeling	58.0
	S88	C terminus	Mutation to Cys and NBD labeling	90.8
GABARAPL2	C15	N terminus	NBD labeling	40.4

Table 3.2. Characterization of NBD-labeled protein forms. SASA values were obtained from GETAREA for PDB: 1UGM (LC3), 1GNU (GABARAP) and 1EO6 (GABARAPL2).

To sum up, results obtained using 4 independent lipid-interaction assays (lipid dot-blot, lipid monolayer surface pressure measurements, liposome flotation and NBD fluorescence spectroscopic analysis) concur in suggesting that (i) LC3B interacts preferentially with CL over other anionic phospholipids, (ii) LC3B-CL interaction relies on both electrostatic and other CL-mediated changes in membrane properties, e.g. bilayer fluidity and (iii) LC3B C terminus remains exposed to the hydrophilic environment after protein binding to CL-enriched membranes.

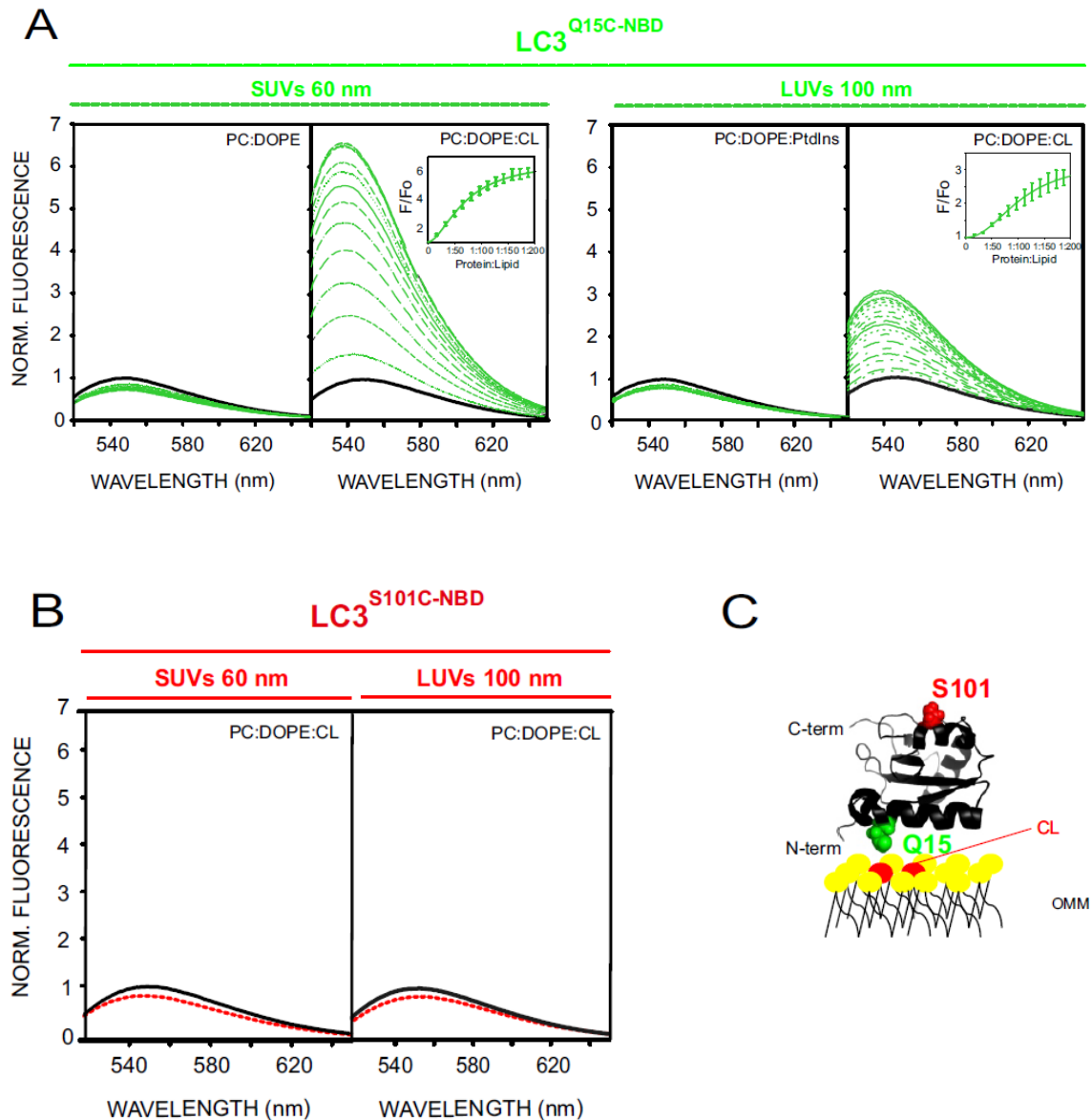


Figure 3.9. LC3B C terminus remains exposed to the hydrophilic environment after protein binding to CL-enriched membranes. Representative NBD fluorescence emission spectra of (A) LC3B^{Q15C-NBD} (1 μM) in the absence or presence of increasing amounts of liposomes containing either PC:DOPE (80:20 mol ratio), PC:DOPE:CL or PC:DOPE:PtdIns (50:20:30 mol ratio); and (B) LC3B^{S101C-NBD} (1 μM) in the absence or presence of PC:DOPE:CL liposomes. In each case, fluorescence was normalized to the peak intensity of the protein spectrum in the absence of liposomes. (C) Structural model generated with PyMol depicting the 2 LC3B residues that were individually mutated to cysteine obtaining single-cysteine LC3B mutants. The environmentally sensitive fluorophore NBD was used to label each of these single cysteine residues. PDB: 1UGM.

3.3.3 GABARAPL2 and GABARAP Interaction with CL-containing Membranes

To determine whether mechanisms underlying LC3-CL interaction are protein-specific or else they are the same for all human orthologs, we analyzed GABARAPL2 and GABARAP interaction with CL-enriched membranes (Antón *et al.*, 2016).

First, estimations of the electrostatic surface potential of Atg8 orthologs in solution revealed that the predicted CL-interacting region was markedly less basic for GABARAPL2 and GABARAP than for LC3B (**Figure 3.10**).

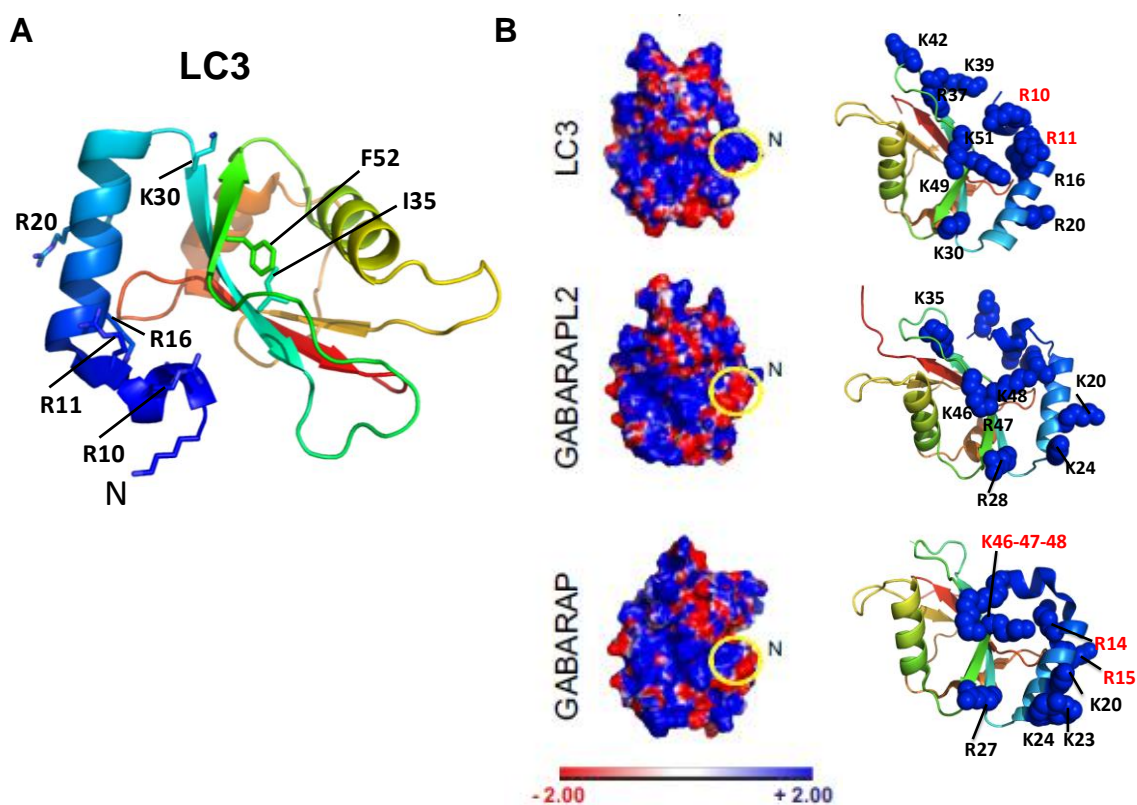


Figure 3.10. Location of the predicted cardiolipin-binding site in Atg8 orthologs. (A) Crystal structure of LC3, rainbow colored (blue N terminus to red C terminus) generated with PyMOL. The side chains of the N-terminal positively charged amino acids (Arg10, Arg11, Arg16, Lys30 and Lys51) are shown. These residues are concentrated on a basic patch that is predicted to interact with cardiolipin (Chu *et al.*, 2013). The side chains of Phe52 and Ile35 are also shown. These amino acids are located in the LC3 central globular domain and might be involved in ceramide-binding (Sentelle *et al.*, 2012). (B) Electrostatic surface diagram of each LC3 paralog in solution. Electrostatic potential surface was calculated using the Poisson-Boltzmann equation and displayed with PyMOL. The predicted cardiolipin-binding site is highlighted in yellow, and the color code is indicated below (left-hand panel). Right-hand panel shows locations of positively charged residues in two ribbon representation of Atg8 ortholog three-dimensional structures. Residues in LC3 and GABARAP that were mutated to alanine are highlighted in red. PDB: 1UGM (LC3), 1EO6 (GABARAPL2), 1GNU (GABARAP).

To test whether these structural features affected the lipid-binding profile of each LC3 paralog we first decided to explore the ability of GABARAP and GABARAPL2 to interact and insert into lipid monolayers using a Langmuir balance. In both cases an increase in π at the air-water interface similar to that of LC3B was observed after protein injection into the aqueous phase (**Figure 3.11A, left-hand panel**), also reaching a plateau value of ~ 15 mN/m at $1.5 \mu\text{M}$ protein concentration. Moreover, monolayer insertion of GABARAPL2 and GABARAP was also facilitated by the presence of CL or PtdIns4P [**Figure 3.11A (right-hand panel) and B**]. PS however did not distinctly modify the insertion of these 2 homologs, resulting in a change in lateral pressure equivalent to that observed with PC:DOPE (See **Table 3.3**).

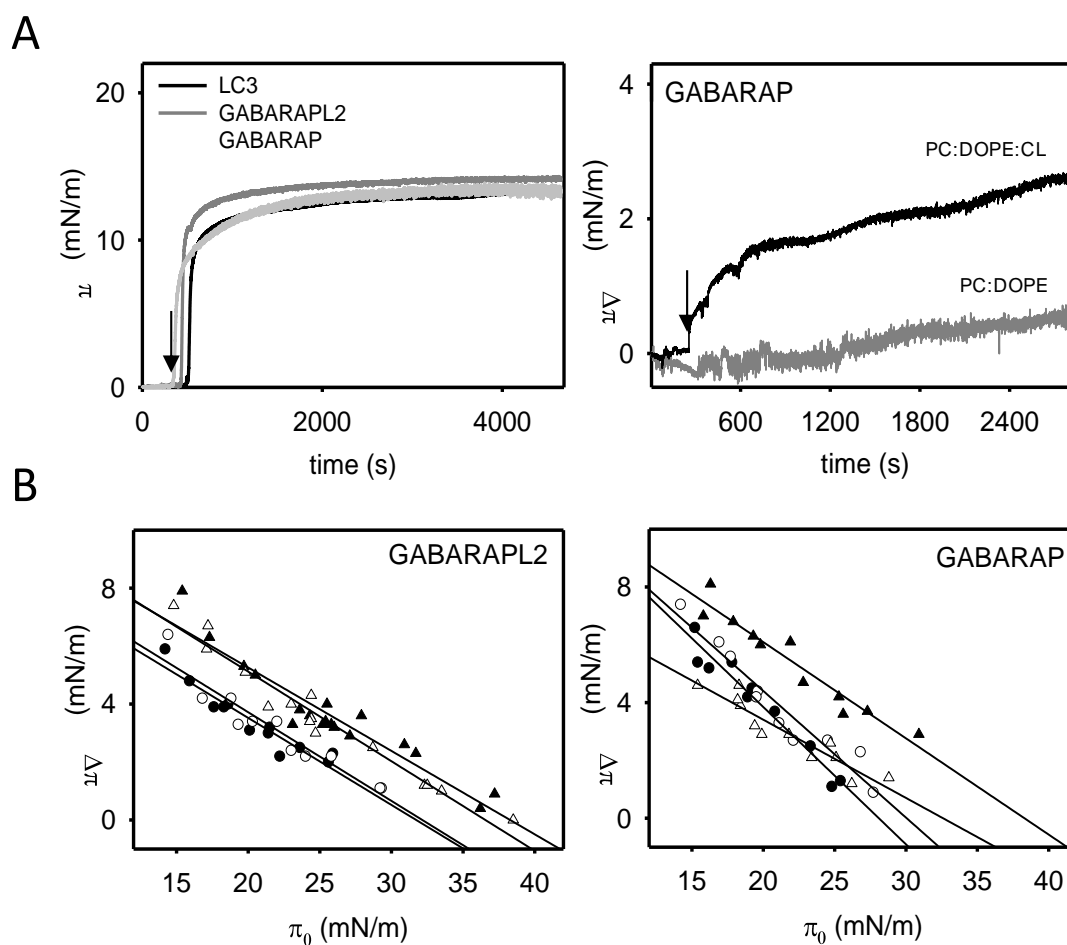


Figure 3.11. GABARAPL2 and GABARAP insertion into lipid monolayers. (A) Representative time courses of adsorption of LC3B, GABARAPL2 and GABARAP ($1.5 \mu\text{M}$) at the air-water interface (left-hand panel) and representative time courses of increase in lateral pressure after GABARAP ($1.5 \mu\text{M}$) insertion into PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (55:20:25 mol ratio) monolayers (right-hand panel) at 25°C. (B) Maximum increase in lateral pressure after GABARAPL2 and GABARAP insertion into lipid monolayers at 25°C. Lipids were: [●] PC:DOPE (80:20 mol ratio), [▲] PC:DOPE:CL, [○] PC:DOPE:PS and [Δ] PC:DOPE:PtdIns4P (55:20:25 mol ratio). Data reported as a function of initial lateral pressure π_0 . Very similar results were obtained at 37°C (data not shown).

Protein	Lipid Composition	π_c
GABARAPL2	PC:DOPE (80:20)	31.8 mN/m (± 0.39)
	PC:DOPE:CL (55:20:25)	38.2 mN/m (± 0.57)
	PC:DOPE:PtdIns4P (55:20:25)	36.6 mN/m (± 0.48)
	PC:DOPE:PS (55:20:25)	32.1 mN/m (± 0.49)
GABARAP	PC:DOPE (80:20)	28.1 mN/m (± 0.43)
	PC:DOPE:CL (55:20:25)	38.3 mN/m (± 0.48)
	PC:DOPE:PtdIns4P (55:20:25)	32.5 mN/m (± 0.42)
	PC:DOPE:PS (55:20:25)	30.0 mN/m (± 0.55)

Table 3.3. Critical pressures (π_c) for GABARAPL2 and GABARAP insertion into lipid monolayers. Data calculated from the straight lines in **Figure 3.11B**. The tendency line-associated standard error is given for each π_c .

Furthermore, we assessed the binding of GABARAPL2 and GABARAP to PC:DOPE and PC:DOPE:CL vesicles by liposomal float-up assays. As depicted in **Figure 3.12**, GABARAP displayed a significantly lesser CL-binding response compared to that of LC3B and even less GABARAPL2 was observed to bind CL-containing liposomes.

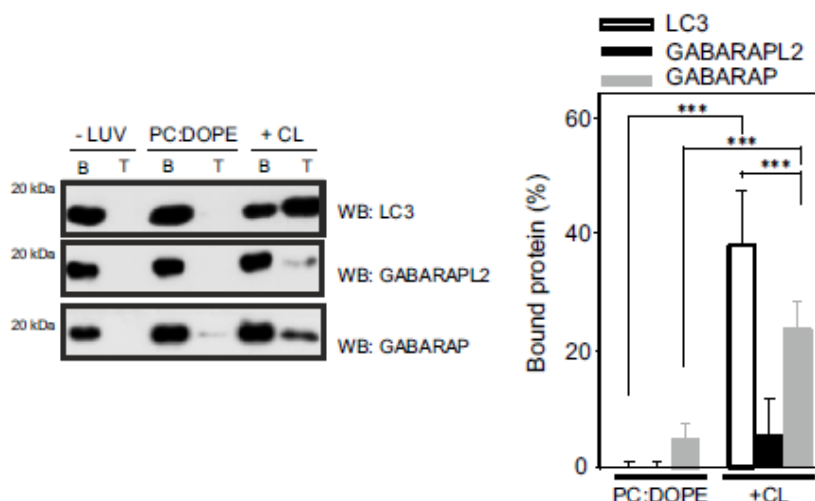


Figure 3.12. Different CL-interacting properties among human Atg8 orthologs. Each ortholog (10 μ M) was incubated with 3 mM LUVs composed of PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (50:20:30 mol ratio) followed by flotation of the liposomes by gradient centrifugation. Bound protein fraction was quantified by SDS-PAGE/immunoblot analysis using anti-LC3, GABARAPL2 and GABARAP antibodies. Molecular masses are shown in kDa on the left-hand side. Data shown as mean \pm SD ($n \geq 3$); *** $P < 0.001$.

Additionally, as mentioned above, LC3B bound significantly less the more rigid vesicles containing bacterial CL (**Figure 3.8F**) and this is in agreement with a decrease in LC3B, but not GABARAP, membrane association with a higher saturation degree of CL fatty acyl chains (**Figure 3.13A**).

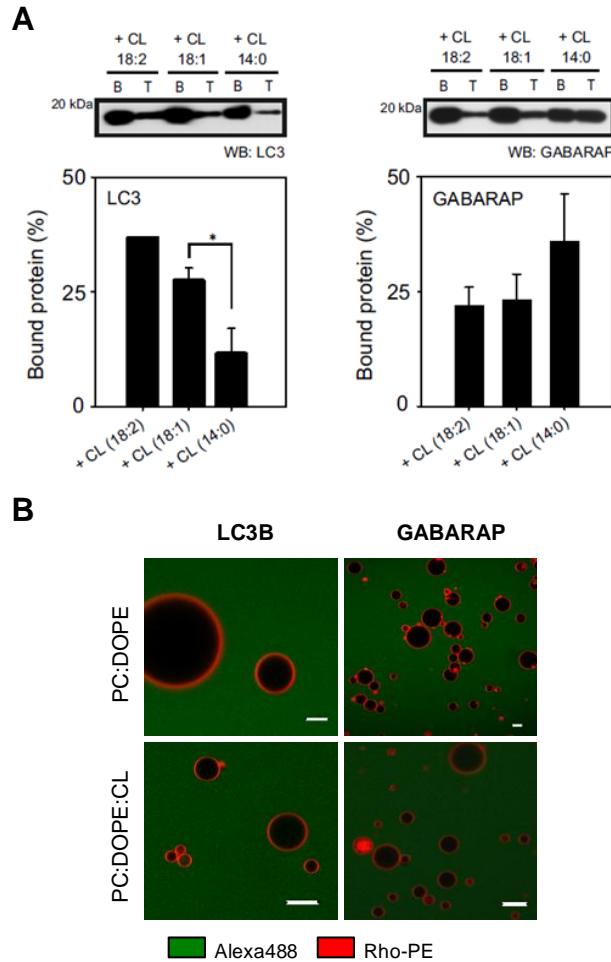


Figure 3.13. LC3B and GABARAP interaction with CL-enriched vesicles. (A) A higher degree of CL acyl chain unsaturation may facilitate LC3B, but not GABARAP membrane association. LC3B or GABARAP (10 μ M) were incubated with 3 mM LUVs composed of PC:DOPE:CL (CL 18:2), PC:DOPE:TOCL (CL 18:1) or PC:DOPE:TMCL (CL 14:0) (50:20:30 mol ratio) followed by flotation. Bound protein fraction was quantified by SDS-PAGE/immunoblot analysis using anti-LC3 or anti-GABARAP antibodies. Molecular masses are shown in kDa on the left-hand side. Data shown as mean \pm SEM (n = 3); *P = 0.01-0.05. (B) LC3B and GABARAP do not interact with GUVs composed of PC:DOPE:CL (50:20:30 mol ratio). Membrane and protein labeled with Rho-PE and Alexa 488 respectively. Lipid composition is given at the left-hand side. Bar: 10 μ m.

We also compared the effect of liposomes containing CL on GABARAPL2 and GABARAP labeled at equivalent positions with NBD. To this aim, GABARAP^{S16C} and GABARAP^{S88C} mutants and GABARAPL2, on its endogenous C15 cysteine, were specifically labeled with NBD. To enhance the efficiency of covalent modification with NBD, Cys substitutions were made at sites in GABARAP with SASA values greater

than 45% (**Table 3.2**). For each monocysteine mutant, a structural model is shown in **Figure 3.14B** indicating the location of the Cys-substituted amino acid which was labeled with NBD. A representative NBD spectrum for each mutant in the absence (black line) or presence (green line) of PC:DOPE and PC:DOPE:CL is also shown. In solution, the $\lambda_{em(max)}$ values of NBD attached to monocysteine GABARAPL2 or GABARAP mutants were similar to those of LC3B mutants. The magnitude of NBD spectral changes induced by the presence of CL-enriched liposomes was somewhat smaller for GABARAP^{S16C} than for LC3B^{Q15C} (**Figure 3.14A**) and even smaller for GABARAPL2^{C15}, in correlation with the electrostatic surface potential of each protein and the flotation experiments shown above (**Figures 3.10B and 3.12**). Moreover, no increase in the emission spectrum of GABARAP^{S88C-NBD} was observed upon its incubation with CL-containing large vesicles (red line) (**Figure 3.14A**). On the basis of the previously observed NBD spectral changes upon moving from solution to CL-containing liposomes, both LC3B and GABARAP would interact with CL-enriched membranes while exposing their C terminus to the hydrophilic environment (**Figures 3.9C and 3.14B**).

To further confirm the observed lack of effect of cardiolipin on GABARAPL2, we examined the protein tertiary structure by Trp fluorescence spectra analysis. GABARAPL2 is the only human ortholog studied in this work to contain Trp residues, namely two Trp residues in helix 1 and helix 3 (**Figure 3.15A**). In solution, the average Trp fluorescence emission of GABARAPL2 displayed a maximum value (Trp $\lambda_{em(max)}$) of 349 nm. The addition of both PC:DOPE and PC:DOPE:CL liposomes gave rise to a small increase in Trp fluorescence intensity (~1.2-fold increase) but it was not accompanied by a blue shift in Trp $\lambda_{em(max)}$ (**Figure 3.15B**). The observations from NBD and Trp fluorescence, as evident from the float-up assays (**Figure 3.12**), indicate that CL-containing membranes do not cause significant conformational rearrangements in GABARAPL2.

Taken together, these results strongly suggest that differences shown in the electrostatic surface potential of LC3 family proteins may lead to lipid-binding specificities among them.

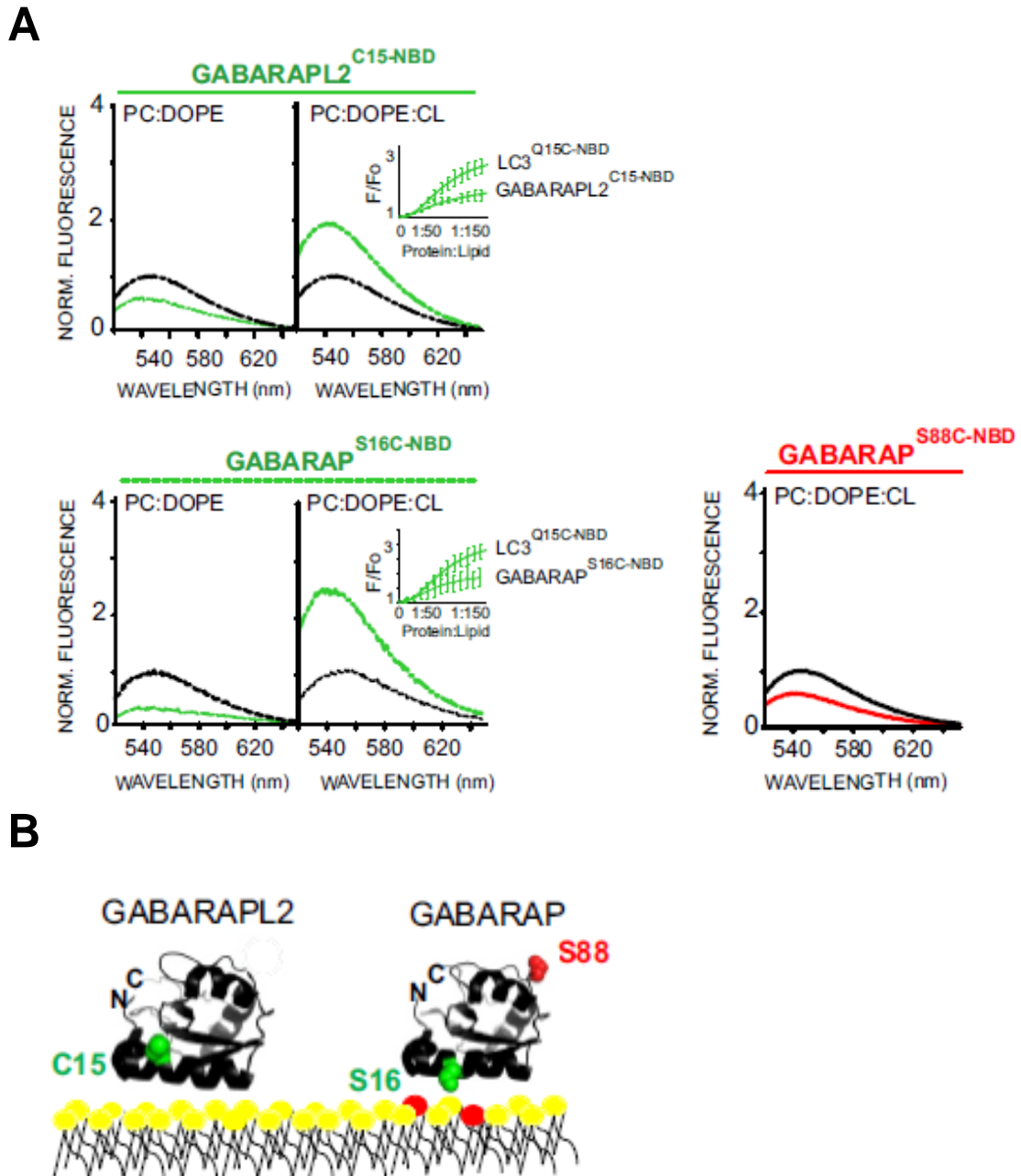


Figure 3.14. Effect of CL-containing liposomes on NBD-labeled GABARAPL2 and GABARAP. (A) Representative NBD fluorescence emission spectra of GABARAPL2^{C15-NBD} and GABARAP^{S16C-NBD} (1 μ M) in the absence or presence of increasing amounts of liposomes containing PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (50:20:30 mol ratio); and GABARAP^{S88C-NBD} (1 μ M) in the absence or presence of PC:DOPE:CL liposomes. In each case, fluorescence was normalized to the peak intensity of the protein spectrum in the absence of liposomes. (B) A structural model generated with PyMol is also shown depicting the 2 GABARAP residues that were individually mutated to cysteine obtaining single-cysteine GABARAP mutants and the endogenous cysteine of GABARAPL2. The environmentally sensitive fluorophore NBD was used to label each of these single cysteine residues. PDB: 1EO6 (GABARAPL2), 1GNU (GABARAP). CL is colored in red.

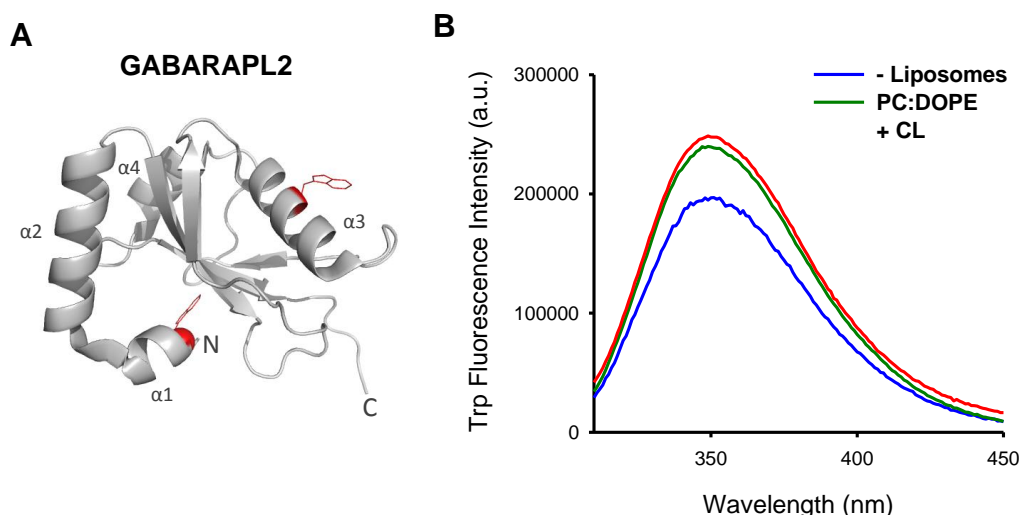


Figure 3.15. Tryptophan fluorescence analysis of GABARAPL2 conformation. (A) Structural model of GABARAPL2 generated with PyMol displaying the side chains of Trp (W) residues that served as spectroscopic probes of protein conformational changes in the presence of liposomes. Red color highlights the location of W3 in helix 1, and of W62 in helix 3. PDB: 1EO6. (B) Representative Trp fluorescence spectra of GABARAPL2 (2 μ M) incubated for 1 h at 37°C in the absence or presence of SUVs (0.6 mM) composed of PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (50:20:30 mol ratio). In all cases, 2 μ M protein was excited at 295 nm and the fluorescence spectra were collected from 310 nm to 450 nm. Lines represent the average corrected emission spectra from three replicate experiments.

In their study, Kagan and colleagues (Chu *et al.*, 2013) have determined that cardiolipin recognition for mitophagy depended on N-terminal amino acids (R10 and R11) of LC3B (see **Figure 3.10**). To further validate our liposome-binding assay, we mutated those residues to alanine in LC3B and evaluated the LC3B^{R10,11A} capacity to bind CL-enriched liposomes (**Figure 3.16A**). Substitution of those residues did not produce structural changes in the protein as tested by secondary structure examination of both WT and mutant LC3B forms using far-UV circular dichroism spectroscopy (**Figure 3.3B**). Mutating those 2 residues made the anti-LC3 antibody unable to recognize LC3B in western blots (data not shown). To circumvent this we decided to label both LC3B and LC3B^{R10,11A} with Alexa Fluor 488. The Alexa Fluor 488 fluorescence of LC3B-A488 was quantified at different protein concentrations by densitometry and the data were linearly fitted by the least-squares method (**Figure 3.7B**). Consistent with a critical role of these 2 basic amino acids in the interaction of LC3 with CL (see **Figure 3.10**), the RR to AA mutation clearly decreased LC3B^{R10,11A} affinity for CL (**Figures 3.16B and C**).

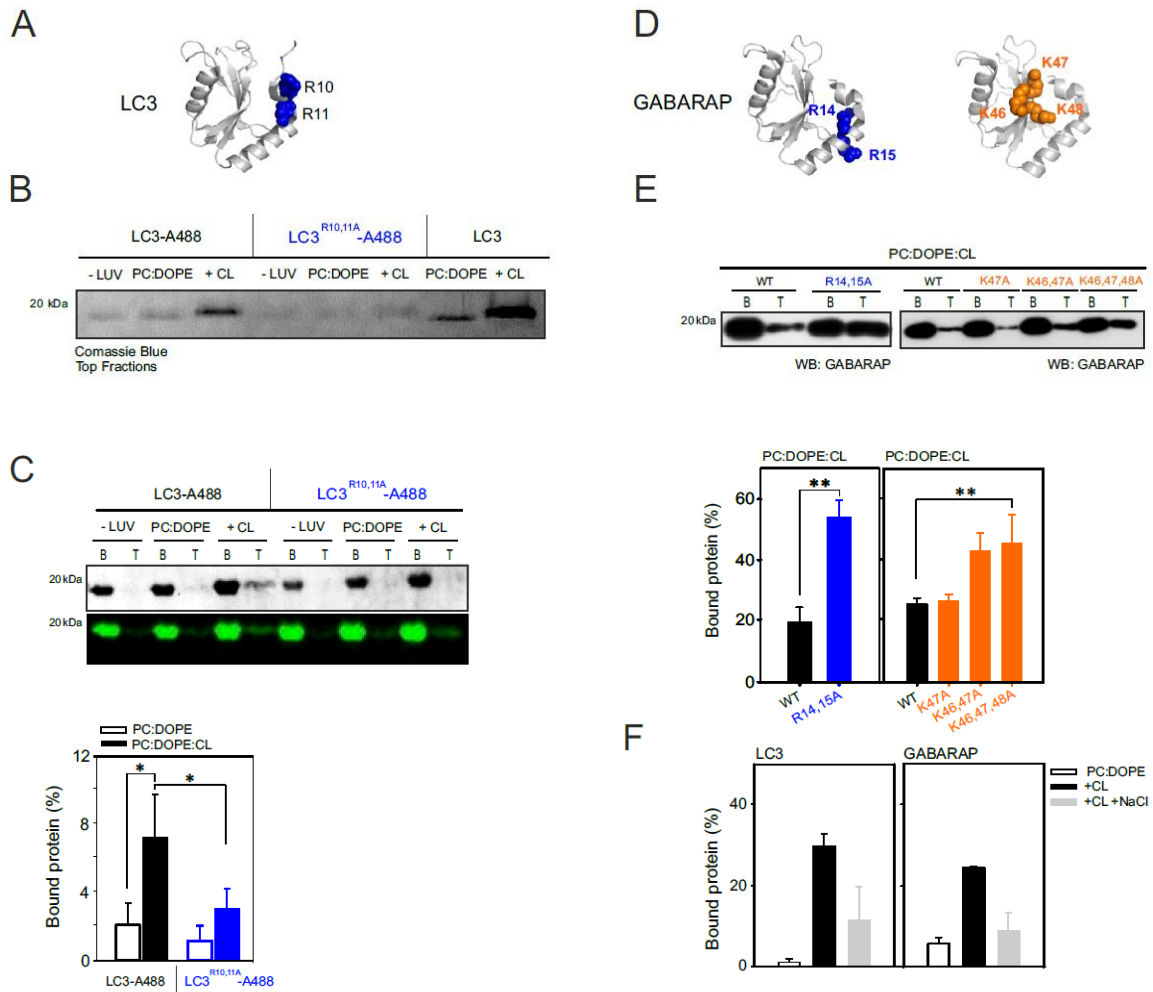


Figure 3.16. N-terminal RR to AA mutation reduces LC3B, but not GABARAP, ability to interact with CL. (A) Crystal structure of LC3 displaying the N-terminal residues (R10 and R11, colored in blue) that were mutated to alanine. PDB: 1UGM. (B) Control experiment comparing liposome-binding of unlabelled LC3B with labelled LC3B and LC3B^{R10,11A} under the same experimental conditions. (C) 10 μ M LC3B- or LC3B^{R10,11A}-A488 were incubated with 3 mM LUV composed of PC:DOPE (80:20 mol ratio) or PC:DOPE:CL (50:20:30 mol ratio) followed by flotation in a sucrose density gradient. Liposome-bound protein fraction was quantified by SDS-PAGE and Alexa Fluor 488 fluorescence. Molecular masses are shown in kDa on the left-hand side. Data shown as mean \pm SD (n = 3); *P = 0.01 to 0.05. (D) Crystal structures of GABARAP displaying the N-terminal residues (R14 and R15, colored in blue and K46, K47 and K48, colored in orange) that were mutated to alanine. PDB: 1GNU. (E) 10 μ M GABARAP, GABARAP^{R14,15A}, GABARAP^{K47A}, GABARAP^{K46,47A} or GABARAP^{K46,47,48A} were incubated with 3 mM LUV composed of PC:DOPE:CL (50:20:30 mol ratio) followed by flotation in a sucrose density gradient. Liposome-bound protein fraction was quantified by SDS-PAGE and immunoblot analysis. Molecular mass is shown in kDa on the left-hand side. Data shown as mean \pm SD (n = 3); **P = 0.001 to 0.01. (F) LC3B or GABARAP (10 μ M) binding to liposomes (3 mM) composed of PC:DOPE or PC:DOPE:CL with/without addition of 300 mM NaCl. Data shown as mean \pm SEM (n = 3).

In order to test whether these N-terminal residues are also involved in GABARAP interaction with CL-containing membranes, R14 and R15 GABARAP amino acids equivalent to the ones in LC3B, and K46, K47, K48 amino acids, that are also

within the proposed interaction patch (see **Figure 3.10B**), were mutated to alanines (**Figure 3.16D**). Note that K46, K47 and K48 are highly conserved basic amino acids (see **Figure 3.1A**) that have been shown to interact with amino acids present in the conserved LIR of the different cargo receptors during selective autophagy (Stolz *et al.*, 2014). Notably, all GABARAP mutants were still capable of recognizing CL, some of them with an even higher affinity than WT GABARAP (**Figure 3.16E**). Taking into account that substitution of the positively charged residues R10 and R11 had a negative effect on LC3B but not on GABARAP binding to CL, the contribution of electrical charge to each protein-CL interaction was further analyzed. To this aim, the interaction of LC3B and GABARAP with CL-enriched liposomes was examined in the presence of 300 mM NaCl (**Figure 3.16F**). We found that increasing the ionic strength of the medium both LC3B and GABARAP binding to CL-containing liposomes decreased, suggesting that in both cases the interaction depends at least in part on an electrostatic component. Given that R14, R15, K46, K47 and K48 residues are not essential for GABARAP binding to CL, other N-terminal residues may be involved in the protein interaction with CL.

3.4 Discussion

The expansion of the Atg8 family proteins in higher eukaryotes has been proposed to facilitate differential cargo handling during selective autophagy (Schreiber & Peter, 2014; Stolz *et al.*, 2014). The components of the ULK1/ULK2 complex, for instance, bind preferentially GABARAP-like over LC3-like proteins (Alemu *et al.*, 2012; Joachim *et al.*, 2015) whereas the receptor protein FYCO1 displays a strong preference for LC3 (Pankiv *et al.*, 2010). Structural studies have highlighted the importance of specific features of individual members in determining these preferential bindings. Importantly while most ubiquitin-directed autophagy receptors (SQSTM1, NBR1, and OPTN) interact with both GABARAP and LC3 subfamily proteins (**Figure 3.1**) (Sugawara *et al.*, 2005; Kirkin *et al.*, 2009a; Wild *et al.*, 2011), specific binding of CALCOCO2 to LC3C (and not the other LC3 isoforms) is crucial for innate immunity during xenophagy (von Muhlinen *et al.*, 2012). Again structural analysis revealed that the selectivity of CALCOCO2 for LC3C is conferred by a noncanonical LIR in CALCOCO2, in which the lack of an aromatic residue is balanced by specific interactions within the N-terminal region of LC3C. In addition to its role in microbial clearance, LC3C has been related to a unique anticarcinogenic activity (Mikhaylova *et al.*, 2012) whereas LC3B promotes tumor growth. While it is very likely that this

opposite effect is related to differential recruitment of critical autophagy targets, the identity of these cargos and the structural basis for these specific interactions are still elusive.

Selective elimination of mitochondria by mitophagy regulates not only the steady-state mitochondrial number required for changing metabolic demands, but also maintains cell quality control by removing damaged organelles. Although mitochondria can be engulfed nonselectively along with other cytosolic contents during bulk autophagy, different lines of evidence indicate that mitophagy can be mechanistically differentiated from other forms of autophagy, and mitophagy-specific mechanisms are now beginning to emerge (Randow & Youle, 2014).

Yeast genetic screening identified Atg11 as an important mitophagy regulator. By binding to Atg32 at the OMM and to Atg8 at the growing phagophore, Atg11 recruits the canonical autophagic machinery to the mitochondria and primes mitochondria for degradation (Okamoto *et al.*, 2009; Sakakibara *et al.*, 2015). In mammals, the ubiquitin-binding adaptor SQSTM1 is thought to act in the same way as Atg11 (Geisler *et al.*, 2010) and during erythrocyte differentiation mitochondrial resident BNIP3L functions as a mitophagy receptor at least in part by binding to LC3 homologs (Novak *et al.*, 2010).

Alternatively, upon mitochondrial membrane depolarization, PINK1 accumulates at the OMM and recruits the E3 ubiquitin-protein ligase PARK2 from the cytosol specifically to the damaged mitochondrion (Lazarou *et al.*, 2015). PARK2 then induces mitochondrial degradation through both ubiquitin-dependent and -independent pathways. The genes encoding PINK and PARK2 were found to be mutated in certain forms of autosomal recessive Parkinson disease (Valente *et al.*, 2004; Vives-Bauza *et al.*, 2010) and loss of either protein in *Drosophila* results in mitochondrial dysfunction (Narendra *et al.*, 2010a). Nevertheless, pink1- or park2-knockout mice appear largely normal and have only subtle phenotypes, suggesting that in the absence of PINK1 or PARK2 other factors can sustain effective mitophagy. In this line, it has been recently shown that exposed CL on the surface of the OMM combines with LC3 and gives rise to the recognition of injured mitochondria (Chu *et al.*, 2013). The process was not accompanied by membrane depolarization and was independent of PINK1 or PARK2. Interestingly, CL has also been reported to interact with other mitophagy-related proteins including DNM1L (Bustillo-Zabalbeitia *et al.*, 2014), human IRGM (Singh *et al.*, 2010) and Beclin 1 (Huang *et al.*, 2012), making CL an essential lipid in this process for both cargo reception and protein function regulation.

3.4.1 LC3B-CL Interaction

The aim of this study was to characterize the association of the autophagy protein LC3 with CL-containing membranes. In this work, we have described novel molecular mechanisms underlying this protein-lipid interaction (Antón *et al.*, 2016). Using several compositionally defined *in vitro* reconstituted systems we show that LC3B avidly interacts with CL, both when CL was spread onto nitrocellulose membranes in the context of a PLO assay and in CL-enriched model membranes (**Figures 3.5 to 3.9**). LC3B binds preferentially CL over other anionic lipids such as PtdIns4P, even though the latter also carries 2 negative charges and has been related to certain types of selective autophagy. Furthermore, we show that association of LC3B with CL predominantly relies on electrostatic interactions (**Figures 3.10 to 3.12 and 3.14**). These results are consistent with the notion that the interaction is mediated by basic patches at LC3 surface that stabilize the phosphate moieties of CL (Chu *et al.*, 2013). Our results with the LC3B^{R10,11A} mutant (**Figure 3.16**) strongly support the idea that some of the residues within those patches are essential for LC3-CL interaction, modulating the binding capacity of the protein (see **Figure 3.10**). Moreover, molecular docking analyses of LC3B upon binding to CL-containing membranes implicate N-terminal Arg10 and Arg11 in the initial interactions of LC3 with CL (Chu *et al.*, 2013). In the *in silico* model the C terminus of LC3, which becomes crosslinked to autophagosome membranes through a conserved glycine residue (see **Figure 3.1A**, black arrow) is predicted to remain exposed to the solvent. Here, using site-specific NBD labeling coupled to NBD fluorescence spectroscopic analysis, we provide experimental data supporting this model in which the N- and C-terminal regions would bind mitochondria and autophagosomal membranes respectively (**Figure 3.9**). This membrane-interacting surface of LC3B located at the N-terminal domain would approach the hydrophobic region of the bilayer and directly interact with CL in the lipid bilayer. This N-terminal interaction with CL may induce a conformational rearrangement in LC3 that would lead to an enhancement of LC3 autophagic activity via its C terminus binding the autophagosomal membrane. Moreover, our previous study has found that fusion induced by LC3 human orthologs is facilitated by the presence of CL in model membranes (Landajuéla *et al.*, 2016). Therefore, CL-mediated modulation of LC3B function would occur via changes in both membrane and protein properties. However, whether or not the CL effects on LC3B will affect the role of the LC3 conjugation system in autophagy needs to be clarified. Furthermore, the relative bilayer depth or surface exposure of LC3B potential membrane interacting sites could also be examined by the analysis of NBD fluorescence quenching. Indeed, the Atg8

orthologs membrane insertion degree still remains poorly characterized. Membrane insertion could be distinguished from peripheral association with the membrane by quenching of (i) intrinsic fluorescence by brominated lipids, and (ii) NBD fluorescence by doxyl/nitroxide moieties located at different depths in the bilayer.

Additionally, LC3B interaction with CL-containing membranes would be facilitated in an acidic environment (**Figure 3.8D**), which is in agreement with LC3 interacting with CL in the OMM of damaged mitochondria. Indeed inhibition of the mitochondrial ATP synthase could induce a higher proton concentration in the intermembrane space that would finally cause a local cytosolic pH decrease. When the mitochondrial respiratory chain is blocked lactic acid fermentation could also be activated as an alternative for the cell to obtain energy, and lactic produced in that process would tend to acidify the intracellular environment, thus to facilitate LC3-CL interaction. However the relationship between pH, CL net charge and LC3-CL binding may not be straightforward. Changing the environmental pH would change the protonation state of CL, but also the protein net charge, and CL packaging in the membrane (Khalifat *et al.*, 2011; Lemmin *et al.*, 2013). Since at lower pH CL would have a smaller negative charge, less binding of LC3B to CL would be expected, but at the same time LC3 charge would become more positive, and this would facilitate LC3 electrostatic interaction with CL. Moreover we must take into account that this interaction relies only partially on electrostatic forces, thus CL negative charge is just one of the several membrane properties that facilitate the interaction. Instituting an artificial pH gradient in CL-containing giant unilamellar vesicles (pH 4 to 5 outside) generates profound invaginations, potentially enriched in CL that would mimic mitochondrial cristae (Khalifat *et al.*, 2008). If these are indeed CL-enriched microdomains, they would facilitate the local binding of LC3.

LC3B preferential binding to smaller and more curved CL-containing liposomes may suggest that vesicle curvature is another factor increasing LC3 affinity for CL (**Figure 3.8E**). Indeed, LC3B does not interact with GUVs (**Figure 3.13B**) suggesting that LC3B interaction with membranes requires a vesicle curvature (GUV curvature is almost zero). This could be a mechanism of recognition of previously processed mitochondria, which would have been converted into pieces of manageable size for their engulfment by autophagosomes. Studies in different mammalian cells have shown that after DNM1L-mediated fission, depolarized mitochondrial products are much less likely to fuse and are eventually autophagocytosed (Twig *et al.*, 2008). Moreover, inhibition of DNM1L prevents both mitochondrial fragmentation and mitophagy, indicating that fission is related to efficiency of mitophagy. Therefore, mitochondrial

shape and size appear to be critical for LC3-CL interaction and targeted mitochondrial degradation. Note in this respect that the negative intrinsic curvature of the lipid CL is conceptually different from the geometrical curvature of a vesicle, in fact lipids with an intrinsic negative curvature can exist in vesicles, e.g. SUVs, with a high geometrical curvature (small radius) (Goñi, 2014).

CL isolated from heart cells displays not only a unique tandem head group arrangement but also a high degree of unsaturation in the acyl chains, linoleic (C18:2) being the most abundant fatty acid. This structural feature contributes to a high degree of flexibility within the membrane that may facilitate LC3 insertion and association. In support of this idea, LC3B binding to CL was significantly lower with vesicles enriched in bacterial CL that contains more saturated acyl chains leading to a more rigid membrane (**Figure 3.8F**). Thus bilayer rigidity would be another regulative mechanism in LC3B association with CL-enriched membranes. Indeed, lipid oxidation increases markedly mitochondrial membrane rigidity observed in aging animals (Chen & Yu, 1994). Hence free radical reaction-induced decrease in membrane fluidity would affect LC3 recognition of damaged mitochondria and this would be an aging-related process (García-Prat *et al.*, 2016).

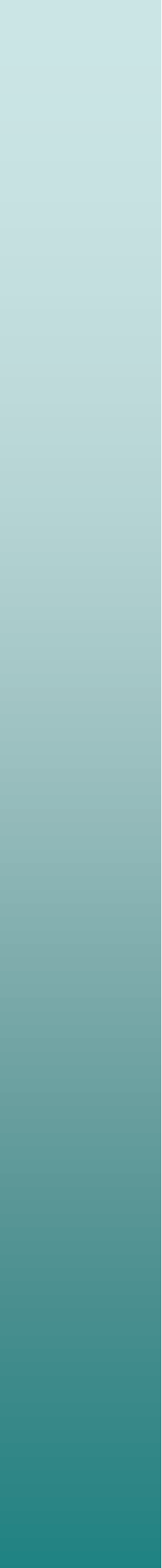
3.4.2 GABARAPL2 and GABARAP

Since most studies have been focused on LC3, the role of other human Atg8 orthologs in mitophagy is less well characterized. LC3 family proteins have different specificities guiding the selective recruitment of different LIR-containing proteins (Weidberg *et al.*, 2010).

CL-binding assays and electrostatic surface potentials shown in this study revealed that human Atg8 orthologs also differ in their lipid-binding affinities (**Figures 3.10 to 3.16**). In contrast to LC3B and GABARAP, GABARAPL2 hardly binds CL-containing membranes (**Figures 3.12 to 3.15**). Therefore, LC3B, GABARAPL2 and GABARAP may play different roles during selective and nonselective autophagy, and there might be different mechanisms involved in the regulation of mitophagy pathways depending on the cellular and physiological context. The data presented in the next chapter may have important implications for the understanding of LC3B, GABARAP and GABARAPL2 localization in bulk autophagy and rotenone-dependent mitophagy *in vivo*.

Chapter 4

*Rotenone-dependent regulation of
Atg8-ortholog mitochondrial
recruitment during mitophagy*



CHAPTER 4:

Rotenone-dependent regulation of Atg8-ortholog mitochondrial recruitment during mitophagy

4.1 Introduction

Macroautophagy (referred to hereafter as autophagy) is an intracellular degradation pathway conserved in all eukaryotes (Klionsky, 2007; Mizushima & Komatsu, 2015). This process does not only provide nutrients to maintain vital cellular functions under amino acid limiting conditions, but also plays an important role in a wide range of physiological processes (Boya *et al.*, 2013). During macroautophagy, cytoplasmic material including entire organelles are sequestered by a double membrane-bound organelle called autophagosome. Eventually, autophagosome fuses with membranes of the endocytic and lysosomal pathways for cargo degradation and recycling (Shibutani & Yoshimori, 2014; Shen & Mizushima, 2014). One outstanding question is how the autophagosomal membrane is formed and elongated. The most widely accepted hypotheses propose the sequential recruitment of a series of regulatory proteins to a specialized site on the ER known as the autophagosome assembly site or “isolation membrane”, upon which adhesion and fusion of further membrane structures would occur (Ktistakis & Tooze, 2016; Mi *et al.*, 2015).

Mitophagy is the specific turnover of mitochondria via a selective autophagy pathway (Hamacher-Brady & Brady, 2016; Randow & Youle, 2014) and inhibition of this process is found to cause mitochondria accumulation and excessive exposure to ROS (Rouschop *et al.*, 2009). LC3/GABARAP family proteins play a key role in the recognition and engulfment of specific mitochondria through interaction with autophagy

adaptor proteins that bind both the mitochondria and LC3/GABARAP family proteins through their LIRs (Wild *et al.*, 2014). Certain lipids such as cardiolipin or ceramide can also act as cargo receptors for the autophagy machinery (Dany & Ogretmen, 2015; Maguire *et al.*, 2016). For instance, Chu and colleagues proposed that the OMM-localized CL acts as a mitochondrial receptor for LC3 triggering mitophagy in cortical neurons and neuroblastoma cells treated with the electron transport chain complex I inhibitor rotenone (Chu *et al.*, 2013). However, much remains to be elucidated regarding the regulation and molecular mediators involved in cargo selection during selective autophagy pathways.

This area of research has attracted considerable attention recently as the mitophagy process is found to play an important role in multiple human pathological states including not only neurodegeneration (Nixon, 2013; Aufschnaiter *et al.*, 2016; Vidal *et al.*, 2014) but also cancer (Lu *et al.*, 2013) and myopathies (Kubli & Gustafsson, 2012). However, how the specific LC3/GABARAP subfamily protein members modulate the targeting and removal of damaged mitochondria in different cell-types and stress conditions remains largely unexplored. In this study, the previously described relevance of the LC3-CL interaction (Antón *et al.*, 2016; see Chapter 3), was further evaluated in several human cell lines, namely U87MG human glioblastoma cells, RPE1 cells and iPSC-derived human cortical neurons expressing LC3B, GABARAP, GABARAPL1, or GABARAPL2 proteins. Various imaging methods (light microscopy, confocal microscopy and CLEM) were used to gain further insight into the kinetics of mitophagy and the specific roles of different human Atg8 orthologs in selective targeting and removal of damaged mitochondria during PARK2-independent rotenone-induced mitophagy. Here CL translocation to the OMM induced by rotenone (Chu *et al.*, 2013), but not by other bulk autophagy-inducing signals, such as THC and AZD8055 treatments or nutrient starvation conditions, was found to induce LC3B translocation to mitochondria and its subsequent delivery to lysosomes in U87MG glioma cells and RPE1 cells. On the basis of these observations, CLEM was used next to locate and analyze the structure of mitophagic compartments within RPE1 cells using stage-specific fluorescently-tagged autophagosome markers. Ultrastructural analysis of LC3B recruitment to mitochondria via lipid attachments in different stages of the process upon rotenone treatment revealed mitochondria surrounded by large autophagosomal structures indicating the targeting and engulfment of mitochondria and fusion events between autophagosomes. By contrast, unlike the situation found with LC3B, neither GABARAPL2 nor GABARAP were found to be translocated to mitochondria upon rotenone treatment in U87MG cells. Moreover, GABARAPL1-labeled autophagosomes

were observed to be grouped in mitochondria-rich areas in rotenone-treated RPE1 cells but, contrary to that found for LC3B, GAPARAPL1 punctae were not observed in the vicinity of mitochondria in cortical neurons upon rotenone exposure.

Location of ATG5 puncta as a marker for isolation membranes or preautophagosomes was also evaluated upon induction of both nonselective and selective autophagy processes with the aim of determining whether LC3B lipidation is necessary for its recruitment to mitochondria. Induction of nonselective bulk autophagy was found to cause a significant increase in steady state puncta counts for GFP-ATG5 which was also significant for Parkin-dependent and -independent mitophagy induced by the mitochondrial uncoupler CCCP and rotenone, respectively. However, ATG5-positive preautophagosomes were not notably located to mitochondria under rotenone-induced mitophagy conditions in human RPE1 cells.

Together, the *ex vivo* studies presented in this chapter shed light on the specific regulation and function of the LC3/GABARAP family proteins in mitochondria recognition during mitophagy and provide evidence indicating that mechanisms for selective targeting and recruitment of individual family members may depend on environmental factors (e.g., metabolic or stress conditions) and reflect cell-specific functions.

4.2 Materials and Methods

4.2.1 Materials

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Leupeptin and THC were prepared in H₂O and in an alcohol base, and were used at a final concentration of 20 and 4 μ M, respectively. Rotenone, CCCP, and AZD8055 were prepared in DMSO and unless otherwise indicated were used at a final concentration of 1 or 5 μ M, 20 μ M, and 1 nM, respectively.

The following antibodies were used: anti-LAMP2 (1:100; BD Biosciences, San Jose, CA, USA), anti-LC3B (1:1000), and anti-TOMM20 (Santa Cruz Biotechnology) in experiments with U87MG cells; and anti-HSP60 (1:200), anti-ATPB (1:100), and anti-LC3B (1:400) for immunostaining of RPE1 cells.

4.2.2 Molecular Cloning

The construct containing the MTS, MTS-CFP, used for mitochondrial staining of RPE1 cells, was subcloned by insertion of MTS fragment into a peCFP-N1 vector, cut from the MTS-peGFP construct (from Ms G. Mulally) using EcoRI and BamHI. The ligation product was used to transform *E. coli* competent cells to select the colonies that contained the peCFP-N1 with the MTS inside and was further confirmed by electrophoresis (**Figure 4.7A**).

4.2.3 Cell Culture

U87MG cells were cultured in DMEM (Lonza, Basel, Switzerland) containing 10% FBS and penicillin/streptomycin (5 µg/mL) (Lonza). When required, cells were seeded at a density of 5000 to 10000 cells/cm² and transferred to medium containing 0.5% FBS, 18 h before performing the different treatments. For nutrient deprivation experiments, cells were incubated in EBSS medium (Lonza). RPE1 cells were grown in DMEM with 4.5 g/L glucose (high glucose) and 2 mM L-glutamine supplemented with 10% FBS (Gibco, Life Technologies), sodium pyruvate and HEPES. Cells were starved for 2 h in a pre-warmed serum-free media previously described by the group of N. Ktistakis (140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose and 20 mM HEPES at pH 7.4 supplemented with 1% (w/v) fresh BSA and filter sterilized) (Axe *et al.*, 2008). iPSC-derived human cortical neurons were cultured in N2B27 containing 1:1 Neurobasal (Life Technologies) and DMEM/F-12 with glutamax (Life Technologies) previously described in Nistor *et al.*, 2015. Neuronal cells were cultured either in 4-well plates (Thermo Fisher Scientific) or in live-cell imaging dishes (MatTek Corp.).

4.2.4 Transfection and Viral Transduction

Transfections of expression vectors GABARAPL2-GFP and GABARAP-GFP (from Dr. A. Simonsen) into U87MG cells and of plasmids MTS-CFP (sub-cloned from MTS-peGFP) and Mito-DsRed (from Dr. J. Lane) into RPE1 cells were carried out using the Lipofectamine®2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. iPSC-derived human cortical neurons were transduced with lentiviral vectors containing the sequences for LC3B and GABARAPL1 with N-terminal GFP tags (from Dr. J. Lane).

4.2.5 Fluorescence Microscopy

Standard protocols for immunofluorescence microscopy were used. Incubation with appropriate Alexa Fluor 488-, Alexa Fluor 594- or Alexa Fluor 647-conjugated

secondary antibodies (Invitrogen) was performed in the dark at room temperature for 20-90 min.

U87MG cells were observed in a Leica TCS SP2 confocal microscope (Leica Microsystems CMS GmbH). Wide-field fluorescence imaging of RPE1 cells was performed using an Olympus IX-71 inverted microscope (Olympus UK Ltd.) driven by MetaMorph software (Molecular Devices). For live-cell imaging, RPE1 cells and iPSC-derived human cortical neurons were seeded in 35 mm glass-bottomed live-cell imaging dishes (MatTek Corp.). In case of cortical neurons, mitochondrial staining was performed using MitoTracker[®] Red CMXRos (Molecular Probes, Life Technologies). All live-cell imaging was performed at 37°C and 5% CO₂.

4.2.6 Autophagic Flux Analysis by LC3B, GABARAPL1 or ATG5 Puncta Counting

Tandem fluorescent-tagged LC3B or GABARAPL1 and GFP-tagged ATG5 were used as a tool to analyze autophagic flux based on the principles described previously (Kimura *et al.*, 2007). Fixed RPE1 cells stably expressing mCherry-GFP-LC3B, mCherry-GFP-GABARAPL1 or GFP-ATG5 puncta were imaged using an Olympus IX-71 inverted microscope (Olympus UK Ltd.). An automated tandem LC3B-, tandem GABARAPL1- or GFP-ATG5-labelled autophagosome scoring was performed using the TopHat morphology filter in MetaMorph software, selecting for round objects of diameter 5 pixels (=1 µm) or less, on images taken at an optimal but constant exposure for a whole experiment. Individual items selected by the morphology filter were counted automatically by MetaMorph using fixed fluorescence thresholds, determined on images of the positive and negative controls for each experiment. Thresholds were set manually for each channel and experimental data set, and the MetaMorph “count cells” plugin was used to automatically count the number of puncta/field.

4.2.7 Correlative Light and Electron Microscopy (CLEM)

mCherry-GFP-LC3B stably expressing RPE1 cells plated in 35 mm glass-bottomed dishes with finder grids (MatTek Corp.) were first imaged by wide-field fluorescence and phase contrast microscopy (live-cell imaging) using an Olympus IX-71 inverted microscope (Olympus UK Ltd.) and then processed using chemical fixation and embedding in Epon resin for TEM. Resin blocks were trimmed and 70 nm serial sections were cut and stained. Images were obtained using a Tecnai-12-FEI 120kV BioTwin Spirit Transmission Electron Microscope (FEI Europe). Details of sample processing can be found in Section 2.12.

4.2.8 Statistical Analyses

Statistical analyses were performed by ANOVA with a *post hoc* analysis by the Student-Neuman-Keuls test.

4.3 Results

The conversion of the soluble form of LC3 (LC3-I) to the lipidated and autophagosome-associated form (LC3-II) is considered to be one of the hallmarks of autophagy. In intact cells, formation of LC3-II is associated with the appearance of LC3 puncta readily detected by conventional fluorescence microscopy. The mitochondrial complex I inhibitor rotenone and other promitophagic stimuli have been shown to increase the number of LC3 puncta and the level of colocalization with mitochondria. Interestingly, rotenone treatment also induced externalization of CL to the mitochondrial surface (Chu *et al.*, 2013).

4.3.1 CL Translocation to the OMM Leads to LC3B, but not GABARAP or GABARAPL2, Mitochondrial Localization in U87MG Glioblastoma Cells

U87MG cells were selected as a cellular model to investigate, in collaboration with Dr. G. Velasco, the activation of autophagy and mitophagy by different stimuli. In addition it is known that autophagy plays an important role in cancer cells and therefore the mechanism of regulation of mitochondria degradation studied in this cell line could also be relevant in the control of cancer cell survival. Cells were treated with rotenone for 4 h and colocalization of mitochondria with endogenous LC3B puncta was examined. In control experiments, nutrient starvation or THC were used as nonselective autophagy inducers. In agreement with Kagan and coworkers, rotenone treatment elicited robust colocalization of mitochondria with LC3B puncta, indicative of LC3B translocation to the organelle (**Figure 4.1A**). In parallel experiments, both THC and nutrient starvation caused potent LC3B puncta formation consistent with reports indicating that THC and nutrient deprivation induce autophagy-mediated death and protective autophagy, respectively (Salazar *et al.*, 2009; Weidberg *et al.*, 2011). Nevertheless, LC3B puncta formed under these treatment conditions did not exhibit mitochondrial colocalization unlike the situation observed with rotenone. Rotenone-induced mitophagy was further confirmed by colocalization of both mitochondria

(**Figure 4.1B**) and LC3B (**Figure 4.1C**) with lysosomes. Finally, consistent with THC and nutrient starvation not inducing mitophagy, LC3B puncta (**Figure 4.1C**) but not mitochondria (**Figure 4.1B**) exhibited lysosomal colocalization. Altogether, these results indicate that rotenone, but not other well-characterized autophagy inducers (THC, nutrient starvation) elicited mitophagy in U87MG human glioblastoma cells.

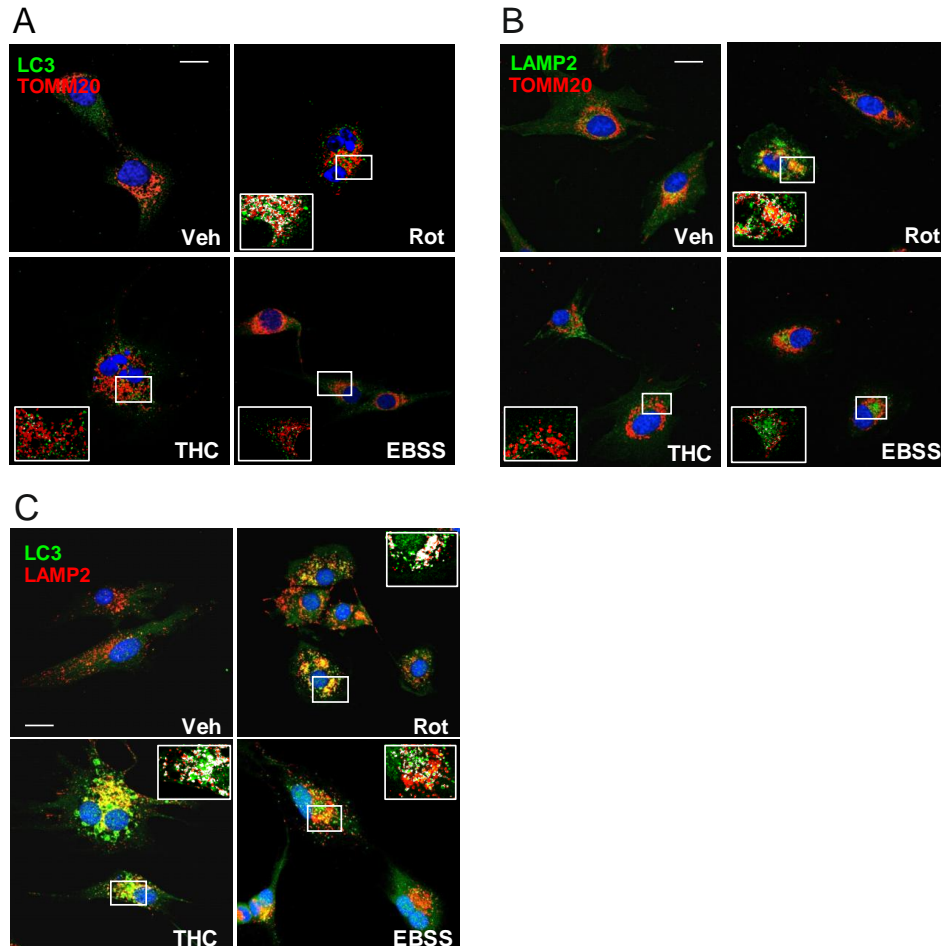


Figure 4.1. Rotenone, but not THC or nutrient starvation, elicits mitophagy in U87MG human glioblastoma cells. (A) Analysis of endogenous LC3B puncta colocalization with TOMM20-stained mitochondria in response to Rot (1 μ M; 4 h) or THC (4 μ M; 6 h) treatments, or to nutrient deprivation (EBSS) conditions in U87MG cells. (B) Rotenone, but not THC or EBSS, increased delivery of TOMM20-stained mitochondria to LAMP2-stained lysosomes in U87MG cells. (C) LC3B puncta colocalization with lysosomes (LAMP2) in response to Rot (1 μ M; 4 h) or THC (4 μ M; 6 h) treatments, or to nutrient deprivation (EBSS) conditions in U87MG cells. Insets from the same cells showing colocalization pixels in white are also shown. Bars: 20 μ m.

Next, in order to analyze the functional implications of the previously observed differences among human orthologs *in vitro* (see Chapter 3), the possible rotenone-induced GABARAPL2 and GABARAP translocation to mitochondria was examined in glioblastoma cells. To this aim, U87MG cells were transiently transfected with plasmids expressing GFP-tagged human GABARAPL2 or GABARAP, and subjected to either nutrient starvation, THC or rotenone treatments. As shown in **Figure 4.2A and B**, all

treatments elicited an increase in GABARAPL2- and GABARAP-GFP puncta within cells. However GABARAP- or GABARAPL2-labeled autophagosomes did not exhibit mitochondrial colocalization after rotenone treatment. Thus, LC3B translocation to mitochondria in response to externalized CL might play a specific role in this cell line, different from that of GABARAPL2 and GABARAP.

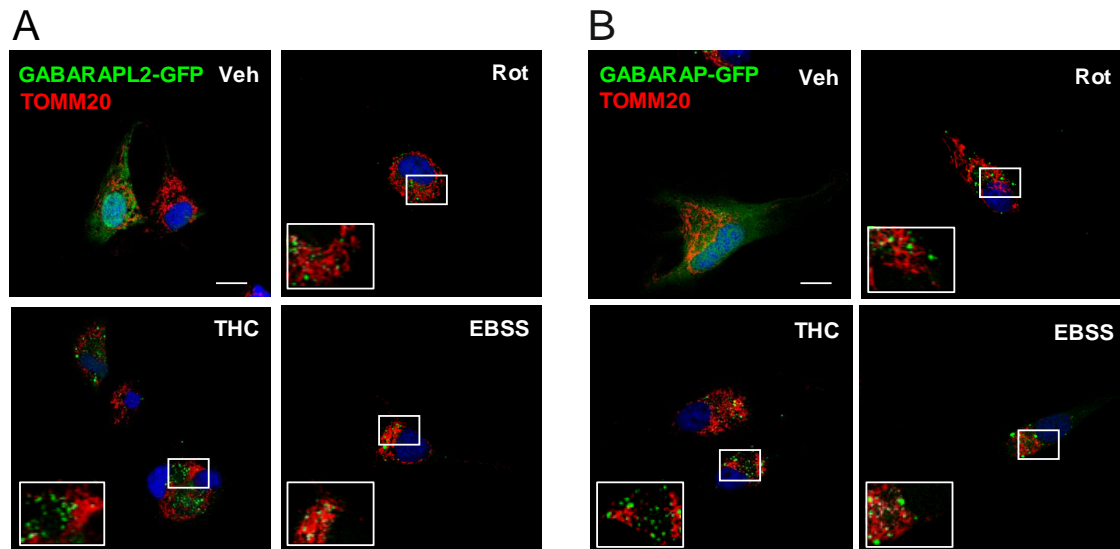


Figure 4.2. Neither GABARAPL2 nor GABARAP exhibit mitochondrial colocalization after rotenone treatment in U87MG cells. (A-B) Analysis of GABARAPL2-GFP or GABARAP-GFP puncta colocalization with TOMM20-stained mitochondria in response to rotenone (1 μ M; 4 h) or THC (4 μ M; 6 h) treatments, or to nutrient deprivation conditions in U87MG cells. Bars: 20 μ m.

4.3.2 LC3B, GABARAPL1 and ATG5 Mitochondrial Recruitment during Mitophagy in Human RPE1 Cells

Mitochondrial complex I inhibition and CL externalization to the outer mitochondrial membrane act as signals for LC3B in U87MG human glioblastoma cells triggering the recruitment of the protein to mitochondria and leading to the selective removal of damaged mitochondria via autophagy (**Figure 4.1**; Ant3n *et al.*, 2016). However, whether this mechanism and pathway are specific for certain cell types and/or for LC3B, remains a matter of investigation.

4.3.2.1 LC3B, GABARAPL1 and ATG5 Intracellular Localization during Mitophagy

As a first approach to investigate the possibility of the previously observed LC3B mitochondrial recruitment during rotenone-induced mitophagy (Chu *et al.*, 2013; Ant3n *et al.*, 2016) to be a cell type-dependent process, a stable RPE1 cell line expressing tandem-tagged mCherry-GFP-LC3 was employed to examine the mitochondrial

localization of the LC3B-labelled autophagosomes (**Figure 4.3A**). Autophagosome maturation can be tracked by using the mCherry-GFP-LC3 fusion construct as, upon lysosomal fusion, GFP is quenched in a pH-dependent manner whereas mCherry resists the acidic lysosomal environment (Kimura *et al.*, 2007). As a result, late autolysosomes fluoresce red only, but earlier autophagosomes are decorated both green and red, thus appearing as yellow puncta when green and red channels are overlaid. As an example, during steady autophagy induction the number of autophagosomes (yellow) and autolysosomes (red) are expected to increase concurrently, however if autophagic flux is inhibited, only the autophagosomes (yellow) will increase in number. In the context of this project this method was used to quantitatively analyze LC3B puncta recruitment to mitochondria during nonselective bulk autophagy or mitophagy.

The predominant cellular response to rotenone exposure depends largely on dose and exposure time, as well as on the prior condition of the cells (Fleming *et al.*, 2004; Maurya *et al.*, 2015; Meurers *et al.*, 2009; Zhu *et al.*, 2004). Indeed, in some cases low doses have only mild toxic effects, as indicated by a high cell survival rate (Frank *et al.*, 2012; Shaikh & Nicholson, 2009), but a prolonged exposure or higher concentrations of rotenone can induce excessive accumulation of ROS, inclusion body formation or apoptosis, e.g. in dopaminergic neurons and striatal neurons (Höglinger *et al.*, 2003; Lapointe *et al.*, 2004; Maurya *et al.*, 2015; Shaikh & Nicholson, 2009; Zhu *et al.*, 2004). Chu and coworkers have shown that rotenone, under sublethal or prelethal conditions (involving low doses), elicits mitophagy in primary rat cortical neurons and SH-SY5Y neuroblastoma cells. Moreover under these early, mitophagy-inducing treatment conditions no increased CL peroxidation products were detected (Chu *et al.*, 2013). Thus cell response to rotenone highly depends on experimental conditions and also varies with cell types.

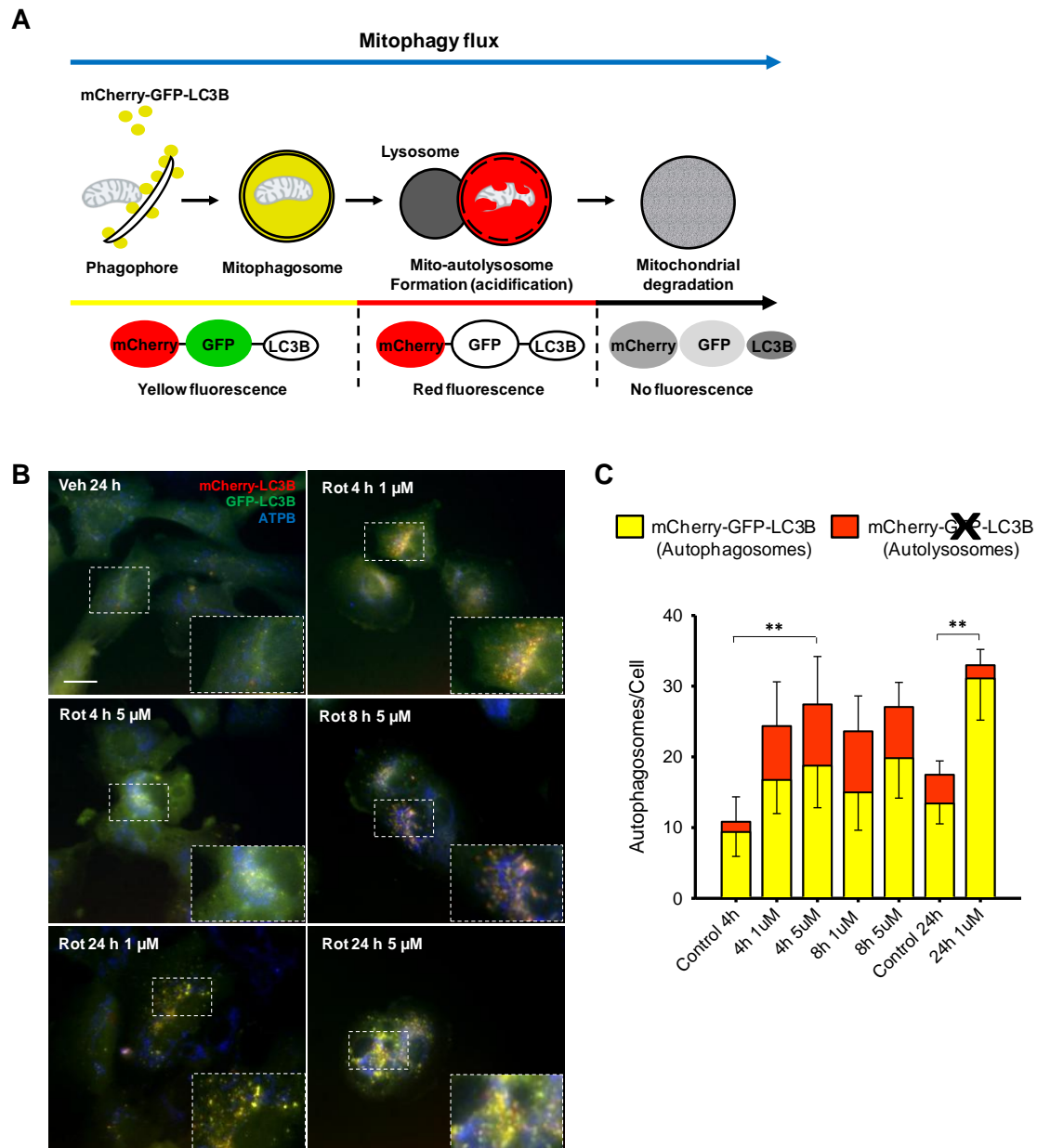


Figure 4.3. Rotenone shortest treatment and lowest dose for a mitochondrial colocalization of tandem LC3B in RPE1 cells. (A) Outline of mitophagy flux analysis using the mCherry-GFP-LC3B tandem tag. By merging green and red channels as shown, autophagosomes were identifiable as yellow puncta and autolysosomes as red puncta. (B) Colocalization analysis of mCherry-GFP-LC3B with ATPB-stained mitochondria in response to rotenone 1 or 5 μ M, 4 h; 5 μ M, 8 h and 1 or 5 μ M, 24 h treatments in RPE1 cells. (C) mCherry-GFP-LC3B puncta quantitation of >400 cells/condition across $n = 3$ experiments are shown. Yellow bars represent red/green autophagosomes; red bars represent red-only autolysosomes (control = vehicle; upward error bars are SD of the red-only puncta and downward error bars are SD for green/red puncta; ** $P = 0.001$ to 0.01). Bar: 20 μ m.

In this study rotenone treatment dose and duration were designed to minimize the exposure length of rotenone-treated cells and cell damage, with the goal of avoiding the relatively acute cell death induced by higher doses or prolonged exposures to the reagent, which would likely bias the analysis at a late stage in the process (Betarbet *et*

al., 2000; Höglinger *et al.*, 2003; Maurya *et al.*, 2015). To determine the minimum rotenone dose and exposure time sufficient to trigger LC3B puncta colocalization with mitochondria (mitophagy), an optimization experiment was first performed in mCherry-GFP-LC3B expressing RPE1 cells (**Figure 4.3**). Cell mitophagy response to varying concentrations of rotenone for 4, 8 and 24 h was measured, whereupon a statistically significant increment in autophagosome-bound LC3B localized to mitochondria was detected in rotenone-treated cells as compared to non-treated control cells (**Figure 4.3B, C**).

To confirm that induction of mitophagy would lead to LC3B localization in the outer mitochondrial membrane by a mechanism specific for this pathway, four different autophagy- or mitophagy-inducers were tested in the mCherry-GFP-LC3B cell line, namely nutrient deprivation, or AZD8055, CCCP or rotenone treatments. As previously shown for U87MG glioblastoma cells (**Figure 4.1**), amino acid/growth factor starvation is known to induce nonselective autophagy and LC3B dots formed under this treatment do not exhibit mitochondrial colocalization (Antón *et al.*, 2016; Hernández-Tiedra *et al.*, 2016). AZD8055 is an ATP-competitive mTOR kinase inhibitor that induces robust bulk autophagy (Chan *et al.*, 2012; Egan *et al.*, 2015; Hsieh *et al.*, 2012). For a positive mitophagy control, CCCP – a protonophore that causes an uncoupling of the respiratory chain and a loss of mitochondrial membrane potential by increasing permeability to protons (Betin *et al.*, 2012; Perry *et al.*, 2011) – was used. CCCP exposure is generally used to simulate mitochondrial dysfunction and induces PARK2-mediated mitophagy (Rüb *et al.*, 2016). Finally, low rotenone doses induce CL externalization to the OMM, LC3 translocation to mitochondria and mitophagy (Chu *et al.*, 2013). In neurons and SH-SY5Y cells, in contrast to the effects observed with strong depolarizing agents such as CCCP (Kondapalli *et al.*, 2012), rotenone causes no more than 15% decrease of mitochondrial membrane potential and PINK1/PARK2 translocation is not observed (Chu *et al.*, 2013; Chu *et al.*, 2014).

LC3B intracellular localization was examined and LC3B puncta numbers were measured during nonselective autophagy, using nutrient starvation or AZD8055 treatment, or during CCCP- or rotenone-induced selective mitophagy (**Figure 4.4**). Correspondingly, in RPE1 cells expressing mCherry-GFP-LC3B, AZD8055 administration or starvation conditions caused a dramatically increased LC3B puncta numbers (~4.7-fold) (**Figure 4.4B**) that were largely diffuse in the cytoplasm, but no translocation to mitochondria was observed (**Figure 4.4A**). CCCP also caused a strong cell response (4.5-fold LC3B puncta) and in this case LC3B predominantly localized in the surrounding mitochondria. Cells treated with 5 μ M rotenone do not give rise to a

particularly strong stress-induced autophagy response (**Figure 4.4B**), however they clearly induced LC3B translocation to mitochondria (**Figure 4.4A**).

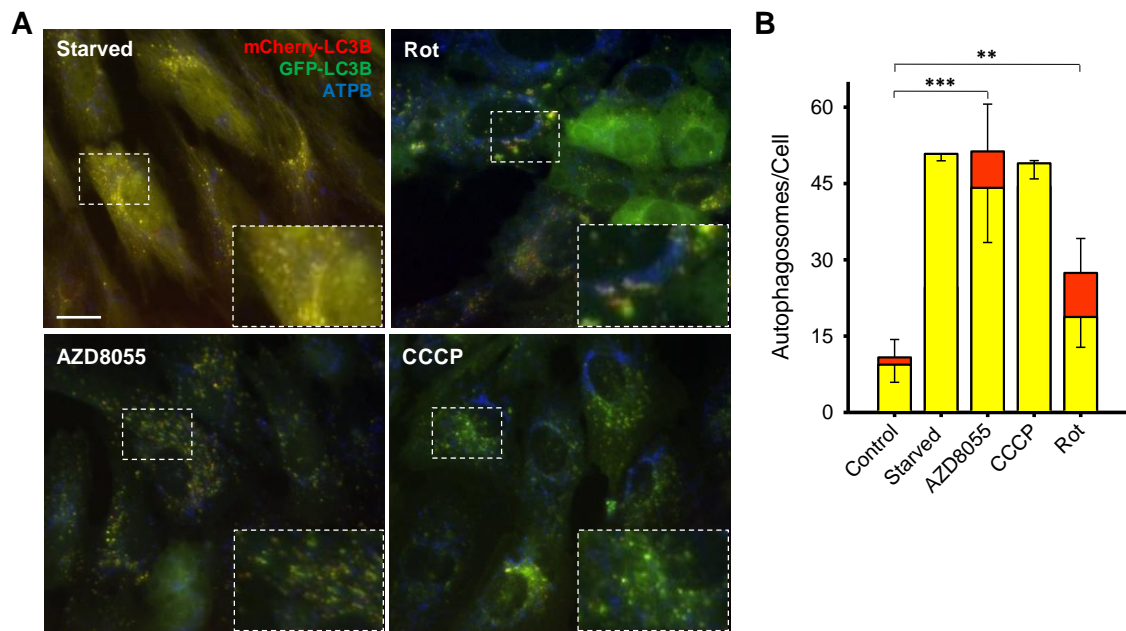


Figure 4.4. Rotenone induces less, larger puncta but more LC3B mitochondrial localization in human RPE1 cells. (A) Representative images of mCherry-GFP-LC3B colocalization analysis with ATPB-stained mitochondria and (B) tandem LC3B puncta quantitation are shown in response to AZD8055 (1 nM; 2 h), rotenone (5 μ M; 4 h) or CCCP (20 μ M; 4 h) treatments, or to nutrient deprivation conditions in RPE1 cells. Yellow bars represent red/green autophagosomes; red bars represent red-only autolysosomes (control = vehicle; upward error bars are SD of the red-only puncta and downward error bars are SD for green/red puncta; **P = 0.001 to 0.01, ***P < 0.001). Quantitation of >400 cells/condition across n = 3 experiments. Bar: 20 μ m.

Although the above data were useful in determining the mCherry-GFP-LC3B response to both non-selective and selective autophagy inductions in RPE1 cells, a comparison with another LC3/GABARAP family member was needed to see whether this response was specific for LC3B in this human cell line. mCherry-GFP-GABARAPL1 expressing RPE1 cells were therefore treated with rotenone and CCCP as mitophagy inducers and AZD8055 was also included as a bulk nonselective autophagy inducer. In control non-treated cells, most of the mCherry-GFP-GABARAPL1 fluorescent signal was cytoplasmic with a few small puncta (**Figure 4.5**). The strongest responses in terms of number of autophagosomes were observed for AZD8055 and CCCP treatments (**Figure 4.5B**). Importantly, GABARAPL1 also localized to the outer mitochondrial membrane in response to rotenone administration in human RPE1 cells. This differs from the previous results with the other GABARAP subfamily proteins, GABARAP and GABARAPL2, which were not recruited to mitochondria after rotenone

treatment in U87MG glioma cells (**Figure 4.2**). Thus, human Atg8 orthologs may play different roles during damage-induced mitophagy in different cell types.

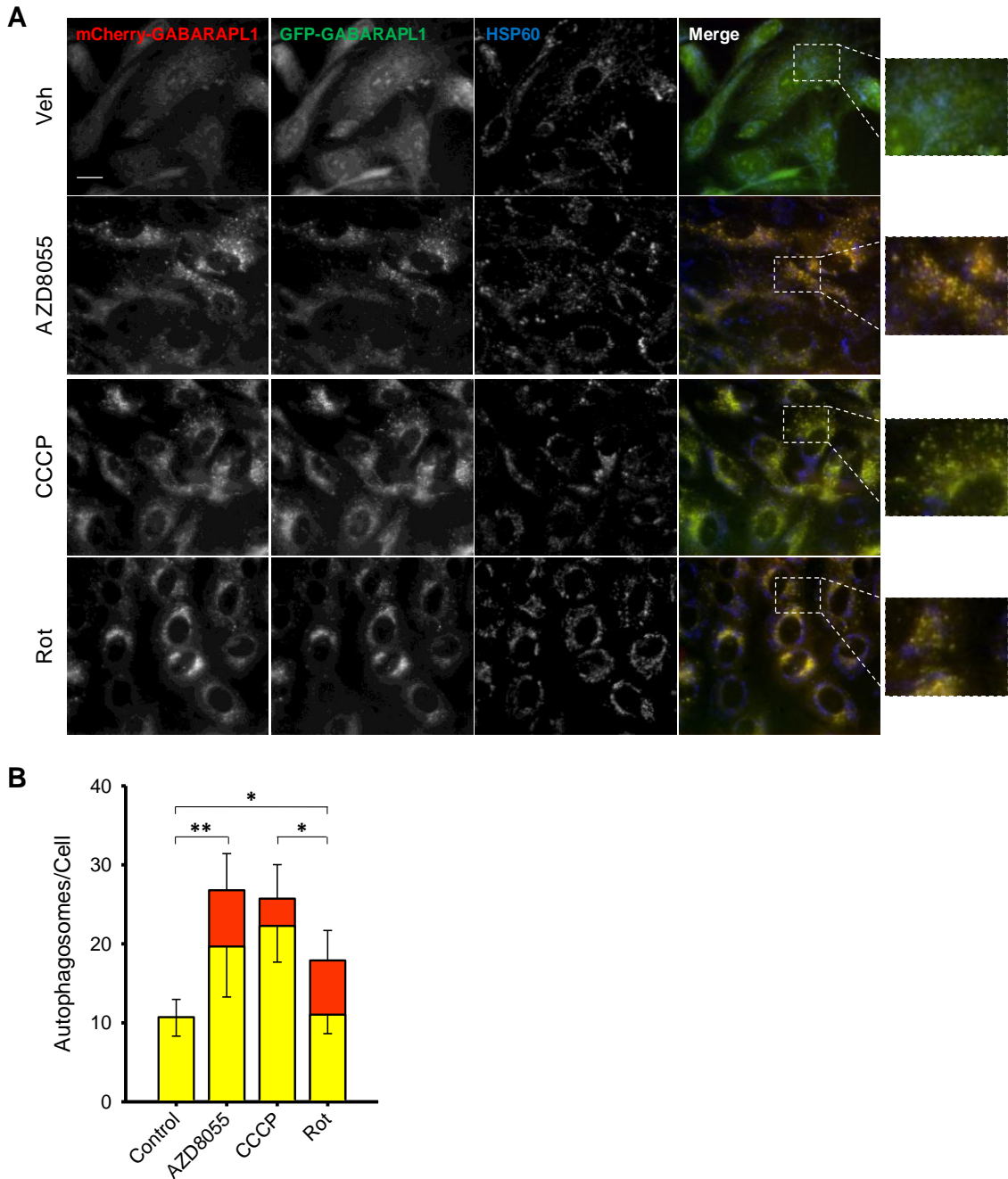


Figure 4.5. Rotenone-induced tandem GABARAPL1 mitochondrial recruitment in RPE1 cells. (A) Colocalization analysis of mCherry-GFP-GABARAPL1 with HSP60-stained mitochondria in response to AZD8055 (1 nM; 2 h), CCCP (20 μ M; 4 h) or rotenone (5 μ M; 4 h) treatments in RPE1 cells. (B) mCherry-GFP-GABARAPL1 puncta quantitation of >400 cells/condition across $n = 3$ experiments are shown. Yellow bars represent red/green autophagosomes; red bars represent red-only autolysosomes (control = vehicle); upward error bars are SD of the red-only puncta and downward error bars are SD for green/red puncta; * $P = 0.01$ to 0.05 , ** $P = 0.001$ to 0.01). Bar: 20 μ m.

Next a RPE1 cell-line stably expressing GFP-ATG5 was used. ATG5 is a key component of an E3-like ATG12-ATG5-ATG16 protein complex that catalyzes conjugation of LC3 to lipids (Matsushita *et al.*, 2007). ATG5 plays a crucial role in the early stages of autophagosome formation controlling autophagic vesicle formation and expansion. ATG5 localizes to the PAS or “isolation membrane”, together with ATG12 and ATG16L1 (Mizushima *et al.*, 2003; Mizushima *et al.*, 2001; Suzuki *et al.*, 2001), making this target a useful potential marker for the isolation membrane or assembly site. When the isolation membrane elongates and fuses to form the autophagosome, ATG5 detaches from the membrane. Therefore, GFP-ATG5 can be detected only on nascent autophagosomes, but not on completely formed ones (Mizushima *et al.*, 2001). In the context of this study, the rationale for the assessment of ATG5 intracellular localization was to see whether translocation of CL to the OMM would cause the recruitment of assembly sites, as detected by the mitochondrial localization of GFP-ATG5-positive structures during rotenone-induced mitophagy. Moreover, both early and late autophagosomal structures were labeled using immunostaining for LC3B in RPE1 cells exposed to AZD8055, CCCP or rotenone (**Figure 4.6**). Labeling of endogenous LC3B with antibodies is often used to identify autophagosomes and to quantify autophagy (number of puncta by fluorescence imaging or LC3B cytoplasmic/membrane-bound ratio by immunoblotting). Both methods can be used to measure autophagy at steady state or to monitor autophagy flux (Klionsky *et al.*, 2008). As shown in **Figure 4.6B**, CCCP and the mTOR catalytic inhibitor AZD8055 caused a significant increase in GFP-ATG5 puncta numbers (4.4 and 6.8-fold increase, respectively) with respect to the non-treated control cells. In contrast, under the same conditions 5 μ M rotenone administration caused a lower increase in GFP-ATG5 puncta numbers. LC3B puncta signals were also increased and dispersed by AZD8055 treatment in GFP-ATG5 expressing cells (**Figure 4.6**), as in mCherry-GFP-LC3B expressing RPE1 cells (**Figure 4.4**). Furthermore, after rotenone-induced mitophagy, LC3B also localized to mitochondria in this cell line. Thus as shown above in **Figure 4.4** for mCherry-GFP-LC3B, the data in GFP-ATG5 expressing RPE1 cells indicate that rotenone is a weaker autophagy inducer than AZD8055 or CCCP. However, GFP-ATG5 colocalization with mitochondria was difficult to determine due to GFP-ATG5 puncta being far less frequent (<5 per cell) than LC3B ones.

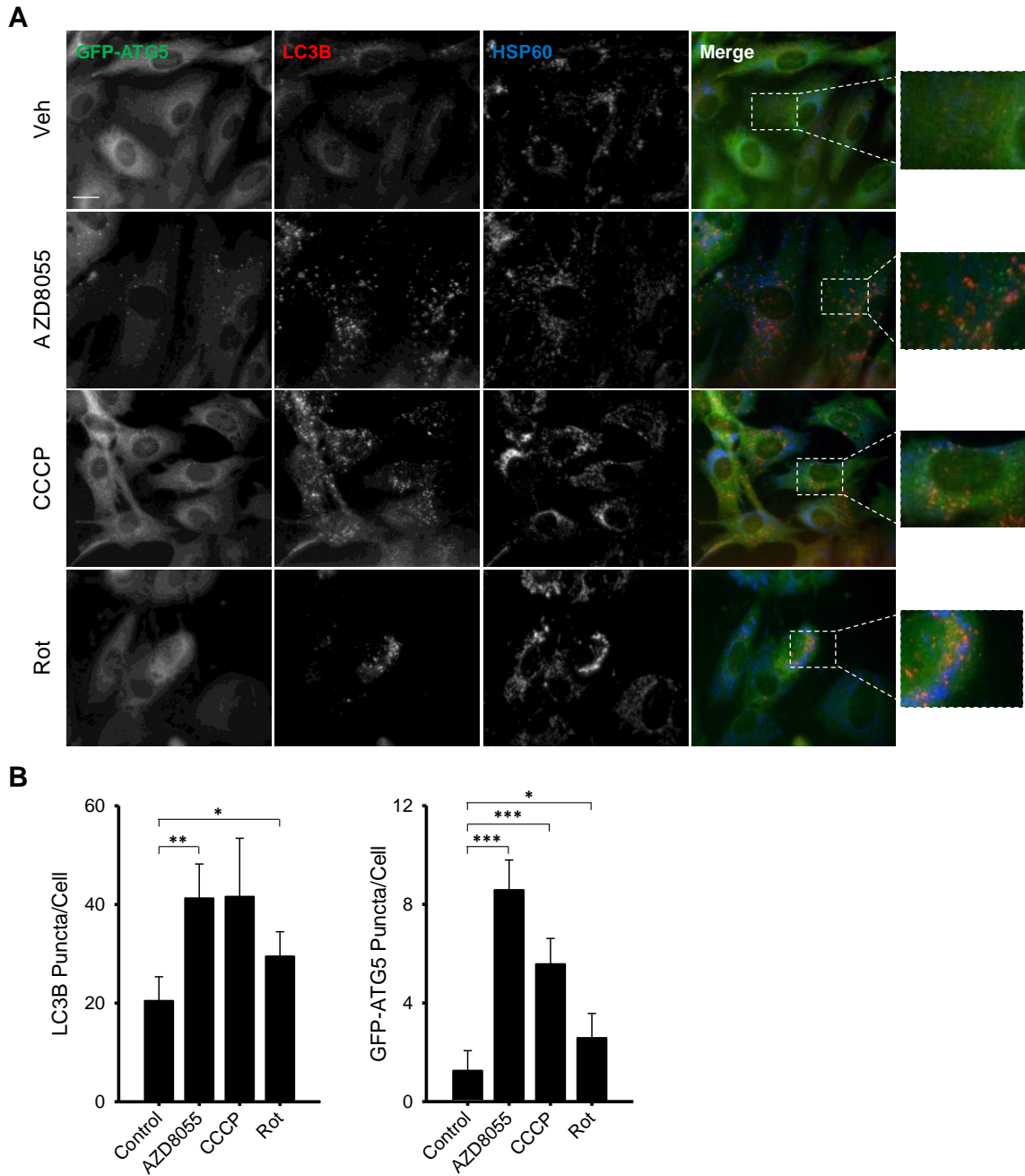


Figure 4.6. GFP-ATG5 as an early marker for preautophagosome localization after rotenone-induced mitophagy in RPE1 cells. (A) Colocalization analysis of GFP-ATG5 and LC3B with HSP60-stained mitochondria in response to AZD8055 (1 nM; 2 h), CCCP (20 μ M; 4 h) or rotenone (5 μ M; 4 h) treatments in RPE1 cells. (B) Measurements of LC3B and GFP-ATG5 puncta of >400 cells/condition across $n = 3$ experiments are shown (control = vehicle; error bars are SD; * $P = 0.01$ to 0.05 , ** $P = 0.001$ to 0.01 , *** $P < 0.001$). Bar: 20 μ m.

Taken together, the data obtained using three different fixed RPE1 cell-lines showed that mCherry-GFP-LC3B, mCherry-GFP-GABARAPL1, and GFP-ATG5 puncta numbers increased after AZD8055 and CCCP treatments indicating an autophagy cell response (Figures 4.4B, 4.5B and 4.6B). Rotenone treatment also significantly increased LC3B, GABARAPL1 and ATG5 puncta numbers, albeit less dramatically. In addition, induction of selective removal of mitochondria by CCCP or rotenone caused

LC3B and GABARAPL1 mitochondrial recruitment in fixed RPE1 cells (**Figures 4.4A, 4.5A and 4.6A**). In contrast, the autophagic structures labeled for the early autophagy marker ATG5 were not clearly localized to mitochondria after mitophagy induction (**Figure 4.6A**). Remarkably, LC3B- and GABARAPL1-labeled autophagosomes were far more perinuclear and clustered after 4 h rotenone treatment compared to other treatments in human RPE1 cells. The significance of altered autophagosome localization with regard to mitophagy efficiency is unclear, although admittedly the clustering of puncta may have caused an underestimation of automated autophagosome counts in rotenone-treated cells. Autophagosomes are normally delivered to the perinuclear region for fusion with the lysosomes thus there might be greater autophagosomal degradation in cells with perinuclear clustered autophagosomes (Korolchuk & Rubinsztein, 2011).

4.3.2.2 Dynamics of LC3B- or ATG5-labeled Autophagic Structures in Rotenone-treated RPE1 Cells

As mentioned above, the cellular response to rotenone treatment depends on the dose and exposure time, cell type and prior cell conditions (Zhu *et al.*, 2004; Meurers *et al.*, 2009; Maurya *et al.*, 2016). Indeed high concentrations of rotenone or prolonged low rotenone doses are found to induce cell death in some cell types (Shaikh & Nicholson, 2009; Höglinger *et al.*, 2003; Lapointe *et al.*, 2004). To find out at which point LC3B-mediated mitophagy is susceptible to 5 μ M rotenone treatment, live-cell imaging of mCherry-GFP-LC3B or GFP-ATG5 stably expressing RPE1 cells was conducted (**Figures 4.7 to 4.9**). Time-lapse imaging of LC3B-labeled autophagosomes by wide-field or confocal imaging can be used to observe autophagosome or mitophagosome dynamics in living cells (Karanasios *et al.*, 2013; Kimura *et al.*, 2008; Maday *et al.*, 2012; Wang *et al.*, 2013; Yang & Yang, 2013). Here this technique was used to follow autophagosomes and capture uptake events of labeled mitochondria by autophagosomes during rotenone-induced mitophagy, analyzing variations at the ultrastructural level using correlative light and electron microscopy (see Section 4.3.2.3). In the following time-lapse experiments, mCherry-GFP-LC3B stably expressing RPE1 cells were transiently transfected with MTS-CFP for mitochondrial staining (**Figures 4.7 and 4.8**).

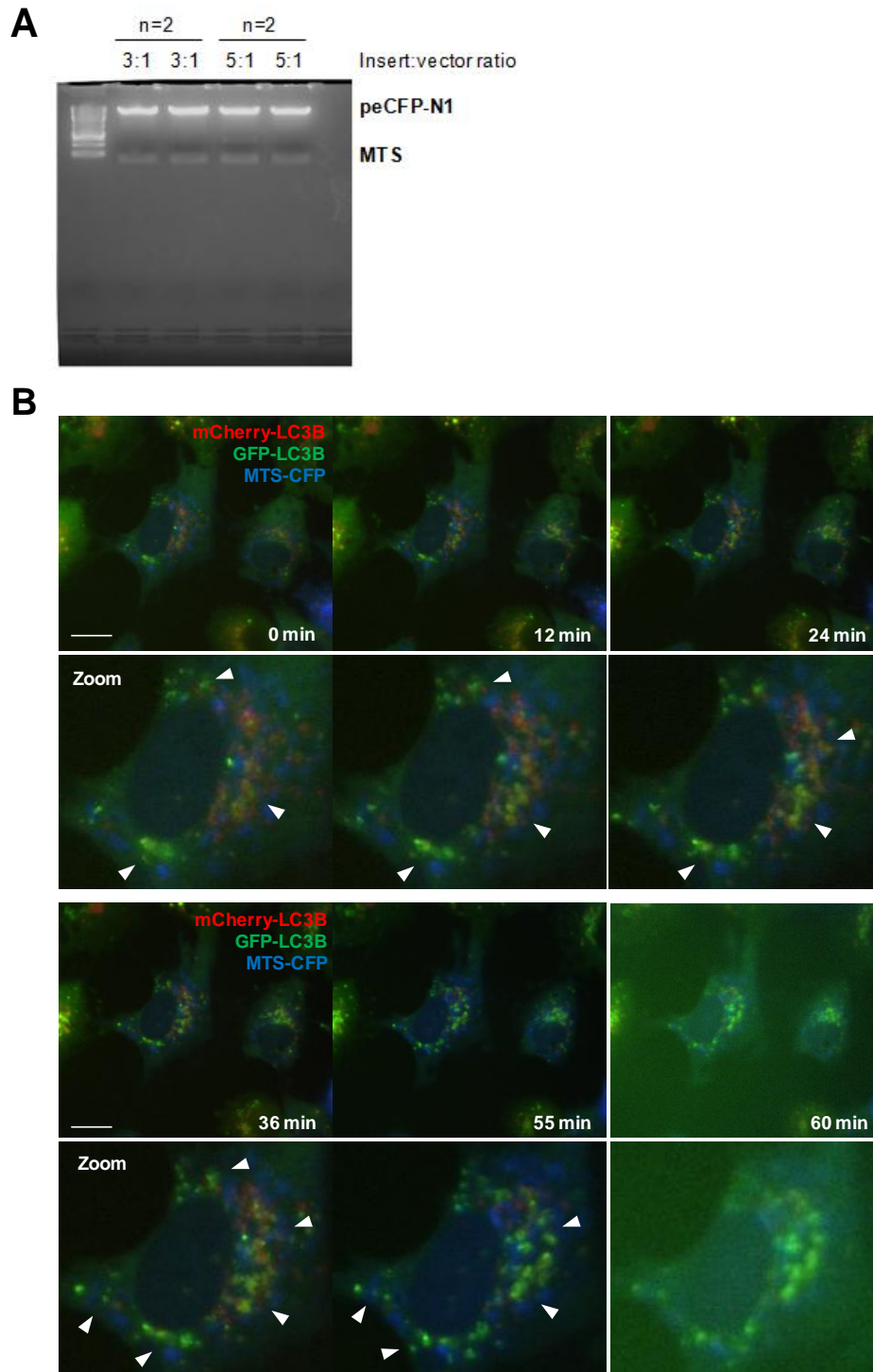


Figure 4.7. Tandem LC3B recruitment to damaged (rotenone-treated) mitochondria in RPE1 cells transfected with MTS-CFP. (A) The construct containing the MTS, MTS-peCFP-N1, was sub-cloned in this work from the construct MTS-peGFP. Confirmation of the presence of the MTS in the peCFP-N1 vector by agarose gel is shown. Various insert:vector ratios were used in the ligation reaction, and 3:1 and 5:1 ligation products containing bacteria colonies were selected. (B) mCherry-GFP-LC3B stably expressing RPE1 cells were transiently transfected using DNA samples with a 3:1 insert:vector ratio and treated with rotenone. Representative images from cell time-lapse experiments are shown (see Supplemental Movies 4.7 and 4.11). Arrow heads indicate changes observed for LC3B punctae surrounding mitochondria. The sequence starts (0 min) 1 h after rotenone addition to cells. 60 min: cells were fixed with 2% glutaraldehyde for CLEM (see Section 4.3.2.3). Bars = 20 μ m.

This sequence may suggest (i) the targeting and engulfment of mitochondria by autophagosomes, (ii) fusion events between autophagosomes and (iii) a slow turnover of autophagosomes in human RPE1 cells (note that the imaging periods were about 60 min) (**Figure 4.7B**). Moreover, as shown above for rotenone-fixed cells (see previous Section 4.3.2.1), a perinuclear localization of LC3B puncta-decorated mitochondria was observed after rotenone treatment in living RPE1 cells (see arrows in **Figure 4.7B**).

To further confirm that mCherry-GFP-LC3B RPE1 cells remove their damaged mitochondria via mitophagy and for a better ultrastructural analysis of mitophagosomes using CLEM (see next section), a complete inhibition of lysosomal proteases and therefore of autophagosome degradation was induced using the acidic protease inhibitor leupeptin (**Figure 4.8**). The lysosome is the ultimate degradative autophagic compartment in the cell and leupeptin is a naturally occurring protease inhibitor that inhibits cysteine, serine and threonine peptidases (Yang *et al.*, 2013). It blocks autophagy at the step of degradation of the lysosome-enclosed cytoplasm and causes the accumulation of autolysosomes (see **Figure 4.8**) and/or many cytoplasmic inclusions in the central vacuoles (Kominami *et al.*, 1983; Moriyasu & Inoue, 2008). The V-ATPase inhibitor BafA1 is another autophagy inhibitor that is also used to study mitophagy flux. BafA1 acts as a pharmacological inhibitor of V-ATPase, and appears to prevent autophagosome maturation by blocking lysosome acidification and hindering the fusion of autophagosomes with lysosomes (Klionsky *et al.*, 2008). In the presence of BafA1 cells are unable to deliver mitochondria to lysosomes for degradation. However, as BafA1-induced lysosomal inhibition blocks lysosome acidification, it also affects pH thus the green-red fluorescence shift would not be observed in mCherry-GFP-LC3B expressing cells.

After treatment with both rotenone and leupeptin, mitochondrial colocalization with LC3B-labeled autophagosomes was also detected and perinuclear accumulation of autolysosomes was observed (**Figure 4.8**). Thus in the presence of the lysosomal inhibitor leupeptin, mitochondrial mass reduction is blocked at the basal state and after mitophagy induction.

In summary live-cell imaging of mCherry-GFP-LC3B expressing cells revealed that 2 h rotenone treatment was sufficient to trigger LC3B translocation to the mitochondria in RPE1 cells, confirming that 5 μ M rotenone induces LC3B mitochondrial recruitment in this cell type. Interestingly, most autophagosomes were grouped in a mitochondria-rich area and persisted throughout the imaging period, about 60 min – a comparatively long time when the half-life of an autophagosome is thought to be on

average 6-9 min in several rat or mice tissues (Kovács *et al.*, 1987; Pfeifer, 1978) (Figures 4.7 and 4.8).

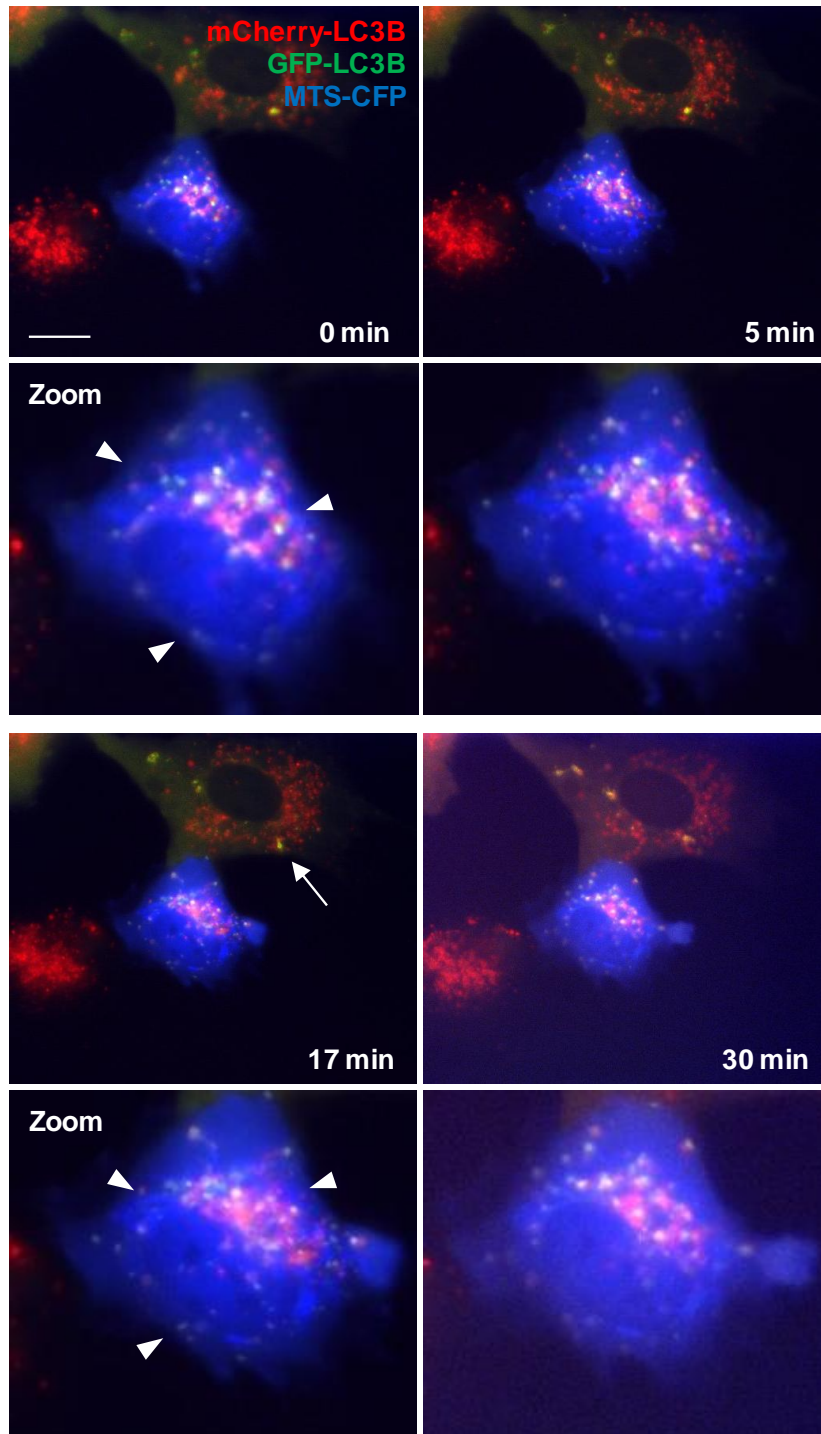


Figure 4.8. Inhibition of autophagic lysosomal degradation during rotenone-induced mitophagy in RPE1 cells stably expressing tandem LC3B. Live-cell imaging of mCherry-GFP-LC3B recruitment to mitochondria in RPE1 cells transfected with MTS-CFP and treated with 5 μ M rotenone and 20 μ M leupeptin (see Supplemental Movie 4.8). Arrow heads indicate localization of LC3B puncta-decorated mitochondria and lysosome inhibition-caused perinuclear accumulation of autolysosomes (see also arrow in the adjacent non-transfected cell). The sequence starts (0 min) 2 h after rotenone and leupeptin addition to cells. 30 min: cells were fixed with 2% glutaraldehyde for CLEM (see Section 4.3.2.3). Bar = 20 μ m.

The next step was to study early stages of autophagosomal formation in a PARK2-independent rotenone-induced mitophagy background through analysis of GFP-ATG5 dynamics at the assembly site. To identify the autophagosome assembly site, or “isolation membrane” and its possible mitochondrial location, human RPE1 cells stably expressing GFP-ATG5 and cotransfected with Mito-DsRed were used (**Figure 4.9**).

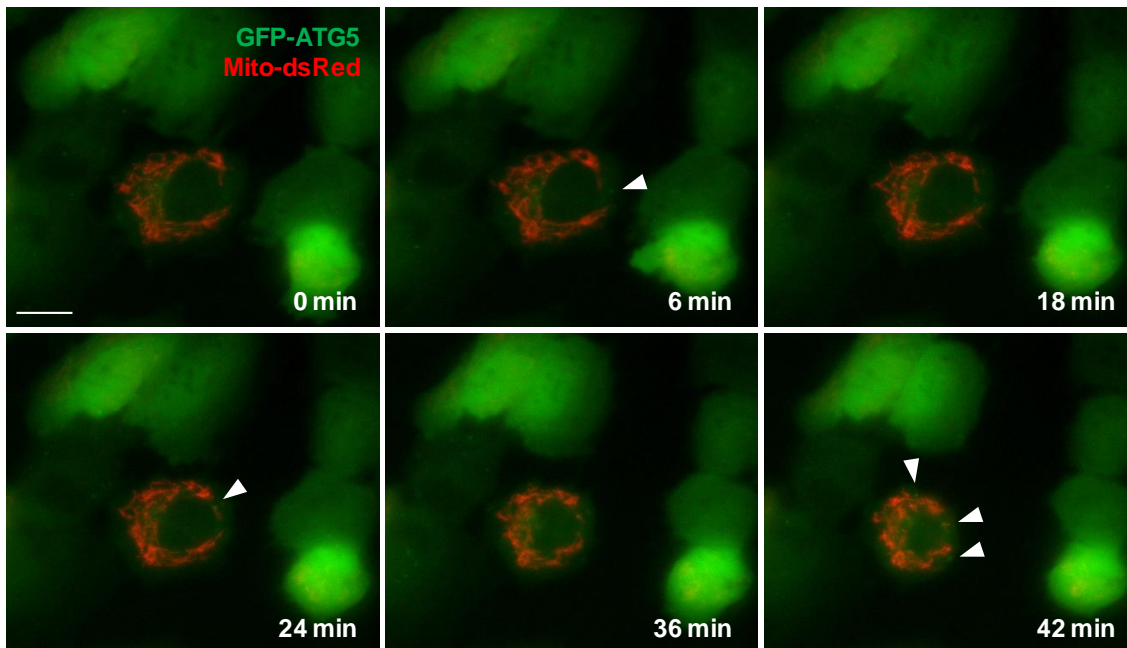


Figure 4.9. Recruitment of ATG5-positive isolation membranes to rotenone-treated mitochondria in RPE1 cells coexpressing Mito-DsRed. Arrow heads indicate cell zones where transient GFP-ATG5 punctae were observed (see Supplemental Movie 4.9). The sequence starts (0 min) 1 h 30 min after rotenone addition to cells. Bar = 20 μ m.

Unfortunately, clear evidence of GFP-ATG5 recruitment to mitochondria was not detected in rotenone-treated RPE1 cells (**Figure 4.9**), despite the previously observed mitophagy response of this cell type to rotenone administration using the same dose and exposure time (**Figure 4.7**). These results suggest that preautophagosome localization to mitochondria might not be required for LC3B attachment to the outer mitochondrial membrane via LC3B-CL interaction. Note however that the lifetime of ATG5-positive structures was shorter than LC3B punctae and that GFP-ATG5 signal was primarily cytosolic, making ATG5 recruitment to mitochondria difficult to be determined in this early, mitophagy-inducing treatment conditions involving low doses of rotenone.

4.3.2.3 CLEM for Ultrastructural Characterization of LC3B Recruitment to Mitochondria in Rotenone-treated Cells

One of the aims of this project was to examine the ultrastructure of mitochondrial or/and autophagosomal membrane remodeling after LC3 recruitment to mitochondria and to examine possible changes in mitochondrial membrane morphology or structure after LC3B binding. For that purpose, EM was used following time-lapse imaging of rotenone-treated human RPE1 cells expressing mCherry-GFP-LC3B.

Autophagy was discovered and described as a lysosomal degradation pathway using EM. The discovery of autophagy factors involved at different stages of autophagosome formation and maturation from the 1990s allowed fluorescent labeling of these compartments and opened new possibilities for study, especially concerning autophagosome dynamics. CLEM links live-cell imaging with EM and provides crucial information to understand complex cellular events in all their dimensions including temporal information (van Rijnsoever *et al.*, 2008; Spiegelhalter *et al.*, 2014; Karreman *et al.*, 2016) – e.g. ultrastructural connections between phagophores and endoplasmic reticulum (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Ylä-Anttila *et al.*, 2009) or the colocalization of several proteins on the same autophagosomal structure (Ravikumar *et al.*, 2010). In this technique living cells are fixed at a selected time point of the time-lapse experiment following examination of the cellular architecture using EM imaging. In the context of this project, CLEM was used to study rotenone-induced mitophagy as it allowed identification and characterization of the mitochondria engulfed by autophagosomes and sent for degradation through this pathway. It was also a good alternative to EM to explore the stepwise events during rotenone-mediated cardiolipin exposure and mitophagy. Moreover, the prior immunofluorescence inspection of the sample – in this case using the autophagosome-specific antigen, mCherry-GFP-LC3B – allowed us to identify early and late autophagosomes involved in this pathway. Finally, CLEM provided us information about the ultrastructure of whole rotenone-treated RPE1 cells (e.g. distorted ER, pinocytic events, mitochondria and ER contact sites, transitional ER, lysosomal degradative compartments, or multivesicular bodies).

As mentioned above samples are fixed at a selected time point for CLEM. Aldehyde fixation is a widely used EM fixation technique adapted to most tissues and used in many autophagy studies (Eskelinen *et al.*, 2011). However, it is known to be associated with several artefacts including the possible collapse of membrane structures during dehydration (van Weering *et al.*, 2010). For this project glutaraldehyde fixation was used (see **Figure 4.10** for representative images of the cell of interest and

organelles fixed with glutaraldehyde). This fixation permitted a good identification of organelles and also had the strong advantage of generating highly contrasted ultrastructure (**Figures 4.10 to 4.12**). Despite the shape of cells and their organelles being sometimes irregular upon glutaraldehyde fixation, CLEM allowed the observation and identification of many autophagosomal structures (**Figures 4.10B, 4.11B and 4.12C**) – indicating an autophagic cell response induced by rotenone administration – and also other cellular organelles presented along this section. This included Golgi apparatus, intracellular trafficking vesicles, ER or mitochondria (**Figures 4.10A and 4.12A,B**).

Identification of the membrane-bound mitochondrion involved in the mitophagic pathway was essential for the purpose of this study. Labeling for LC3B, as a specific marker of autophagic vesicles, and the use of defined ultrastructural characteristics of their content based on criteria established by previous EM studies (Eskelinen *et al.*, 2011; Kent *et al.*, 1966), allowed us to classify early and late autophagosomes and mitophagosomes. Cells treated with the electron transport chain complex I inhibitor rotenone (**Figures 4.10 to 4.13**) or rotenone and leupeptin (**Figures 4.14 and 4.15**) were analyzed to confirm the extent and kinetics of mitophagy induction and mitochondrial removal in mCherry-GFP-LC3B expressing cells.

Green and red LC3B punctae observed with FM were clearly correlated with early and late autophagosome structures by EM imaging of the same cell regions (**Figure 4.11**). The autophagosome assembly site, or “isolation membrane” is a transient structure, with a characteristic ultrastructural morphology in aldehyde-fixed samples in Epon resin, consisting of closely opposed double membranes that appear electron dense (**Figure 4.11B**). This should ideally be the main criterion for recognizing these organelles; however, as EM studies have repeatedly mentioned (Eskelinen *et al.*, 2011), the two lipid bilayers delimitating autophagosomes cannot always be distinguished. Therefore, autophagosomes were also routinely identified by their cytoplasmic and/or organelle content and by FM observation of LC3B puncta. A large proportion of autophagosomes contained undefined cytoplasmic content, but larger organelles such as mitochondria could also be seen within (or close to) these compartments (**Figures 4.10B and 4.11B**). Supporting the observation made from light microscopy images that rotenone induced larger LC3B puncta compared to other autophagy inducers (**Figures 4.4A and 4.7B**), a portion of the autophagosomes observed by EM were larger and/or with varied content, possibly generated by homotypic fusion of several autophagosomes, or heterotypic fusion of autophagic and endocytic compartments, and could therefore be considered as amphisomes (**Figure**

4.11). Finally, together with the labeling for LC3B red puncta, the late autophagic compartments or autolysosomes, which represent the latest stage of this pathway, were identified by their fully degraded content (less electron dense and without the double membrane) (Figures 4.10B, 4.11B and 4.12C).

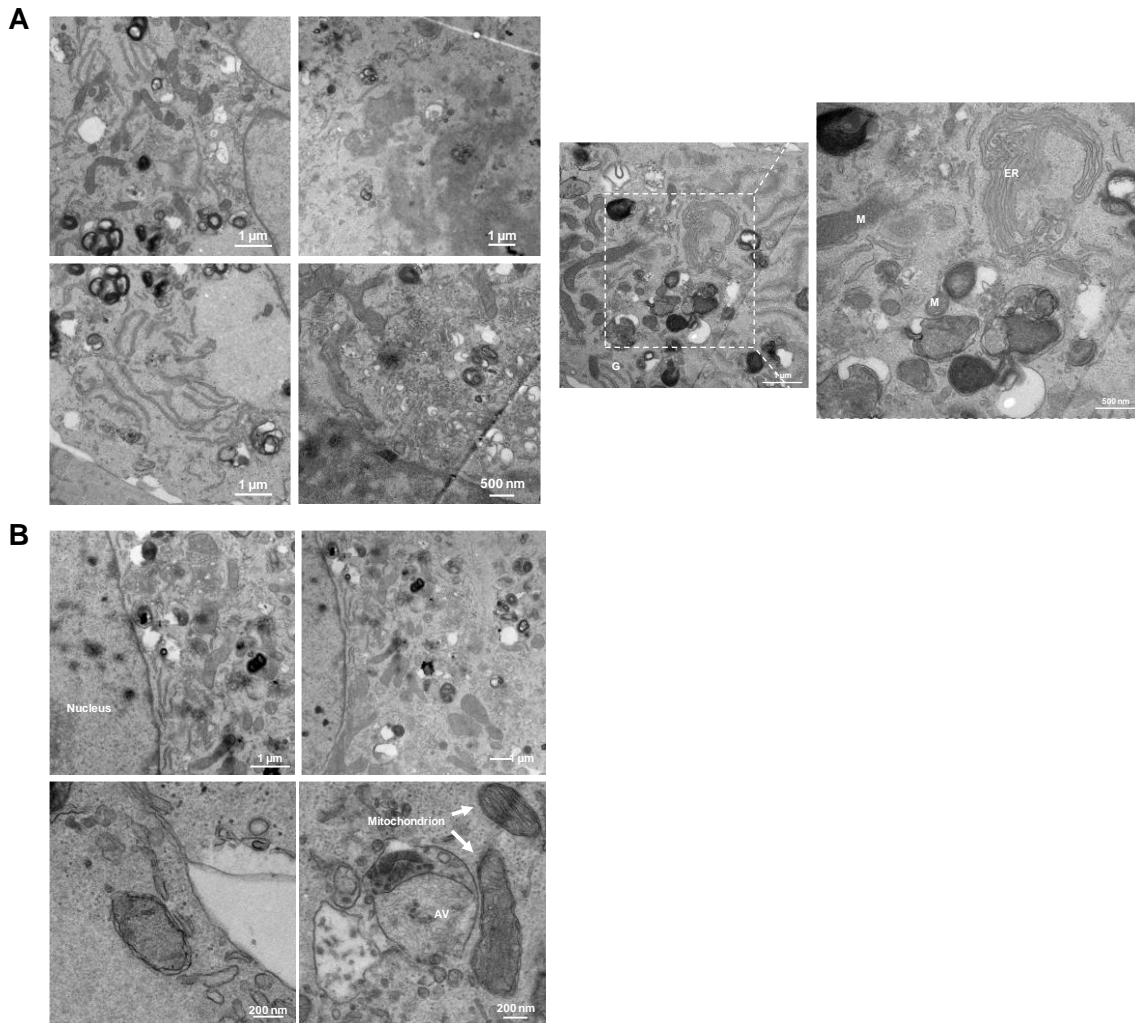


Figure 4.10. Ultrastructure of rotenone-treated human RPE1 cells expressing mCherry-GFP-LC3B fixed with glutaraldehyde. (A) Representative images of rotenone-treated mCherry-GFP-LC3B expressing cells following fixation with 2% glutaraldehyde. Glutaraldehyde-fixed cellular components were highly contrasted but often distorted. A large number of organelles could be identified by their ultrastructure on EM images as shown by examples of a RPE1 cell mitochondrion (M), ER, Golgi apparatus (G) or nucleus. (B) Representative images of organelles involved in the autophagy pathway in rotenone-treated and glutaraldehyde-fixed RPE1 cells. Early and late autophagosomes were observed, in correlation with green and red LC3B punctae previously observed by FM, and some of them were close to mitochondria.

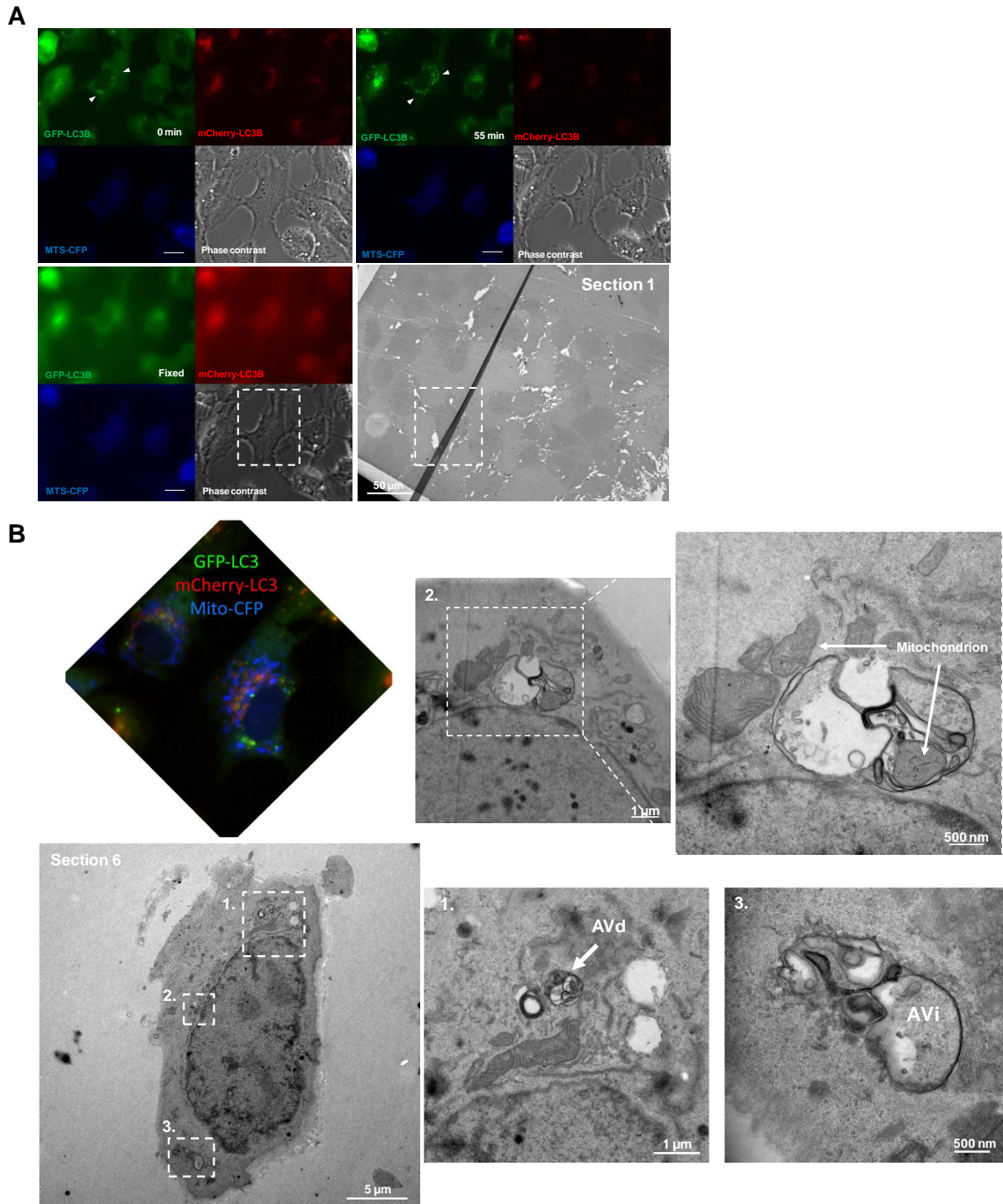


Figure 4.11. CLEM of rotenone-treated RPE1 cells stably expressing mCherry-GFP-LC3B transfected with Mito-CFP (Mito-CFP). (A) Cells were treated with rotenone for 2-3 h and, after live-cell imaging (see Supplemental Movies 4.7 and 4.11), were fixed and processed as described in Section 4.2.7. A representative image of the first section with the location of the cell of interest is shown (note white zones in the EM image due to the first sample section being thinner than later sections). Bars = 20 μm. (B) CLEM of rotenone-treated tandem LC3B expressing cell. The cell of interest is shown in the same orientation in both FM and EM images. Several cell regions were selected (1-3) for EM imaging considering live-cell imaging results for LC3B-positive structures and mitochondria.

Distorted ER structures were also observed by EM imaging suggesting ER stress induced by rotenone treatment (**Figure 4.12A**). Excessive ROS production is the major mediator of ER-stress among numerous pathological conditions (Hotamisligil, 2010). ER-stress response is afflicted with an adaptive signaling cascade known as the UPR (Hotamisligil, 2010). Excess ROS generation mediates unfolded or misfolded protein aggregation, which stimulates the prolonged UPR (Hotamisligil, 2010). ER-stress mediated prolonged UPR leads to general translational attenuation and hindrance in ER-associated degradation of unfolded or misfolded proteins (Hotamisligil, 2010; Xu *et al.*, 2005). This phenomenon promotes accumulation of high molecular weight ubiquitinated proteins and impaired UPS (Xu *et al.*, 2005). PDI is a chaperone protein that remains in the ER-lumen and assists in the maturation and transport of unfolded protein via thiol disulfide exchange. Previous work has shown that rotenone can lead to S-nitrosylation of PDI (catalytically inactive form), initiating misfolded protein aggregation (Uehara *et al.*, 2006). Moreover, Pal and coworkers have also shown that rotenone induces calcium-dependent oxidative stress in the ER in SHSY-5Y cells (Pal *et al.*, 2014), which correlates with observations in rotenone-treated human RPE1 cells (**Figure 4.12A**).

In the particular cell shown in **Figure 4.13**, a large number of autophagic processes were observed. However, the mitochondrion appeared relatively undamaged suggesting that damaged mitochondria had been already removed. Indeed, after mitophagy initiation mitochondria are rapidly degraded by autolysosomes. Therefore to be sure of analyzing cells in the mitophagy stage previous to mitochondrial degradation by autolysosomes, mitophagy and mitochondria removal were examined using CLEM in leupeptin-treated cells (**Figures 4.14 and 4.15**). Multivesicular bodies were observed that could be fused autophagosomes (autophagic structure) or endosomes. In addition, an autophagosome with mitochondria inside was also observed which could be a late mitophagosome (fused autophagosomes, internal vesicles) or an early mitophagosome (content similar to cytoplasm) (**Figure 4.13**). Finally, vacuoles and late autophagosomes located in the vicinity of the nucleus, and lysosomal degradative compartments (black circular dots) were identified.

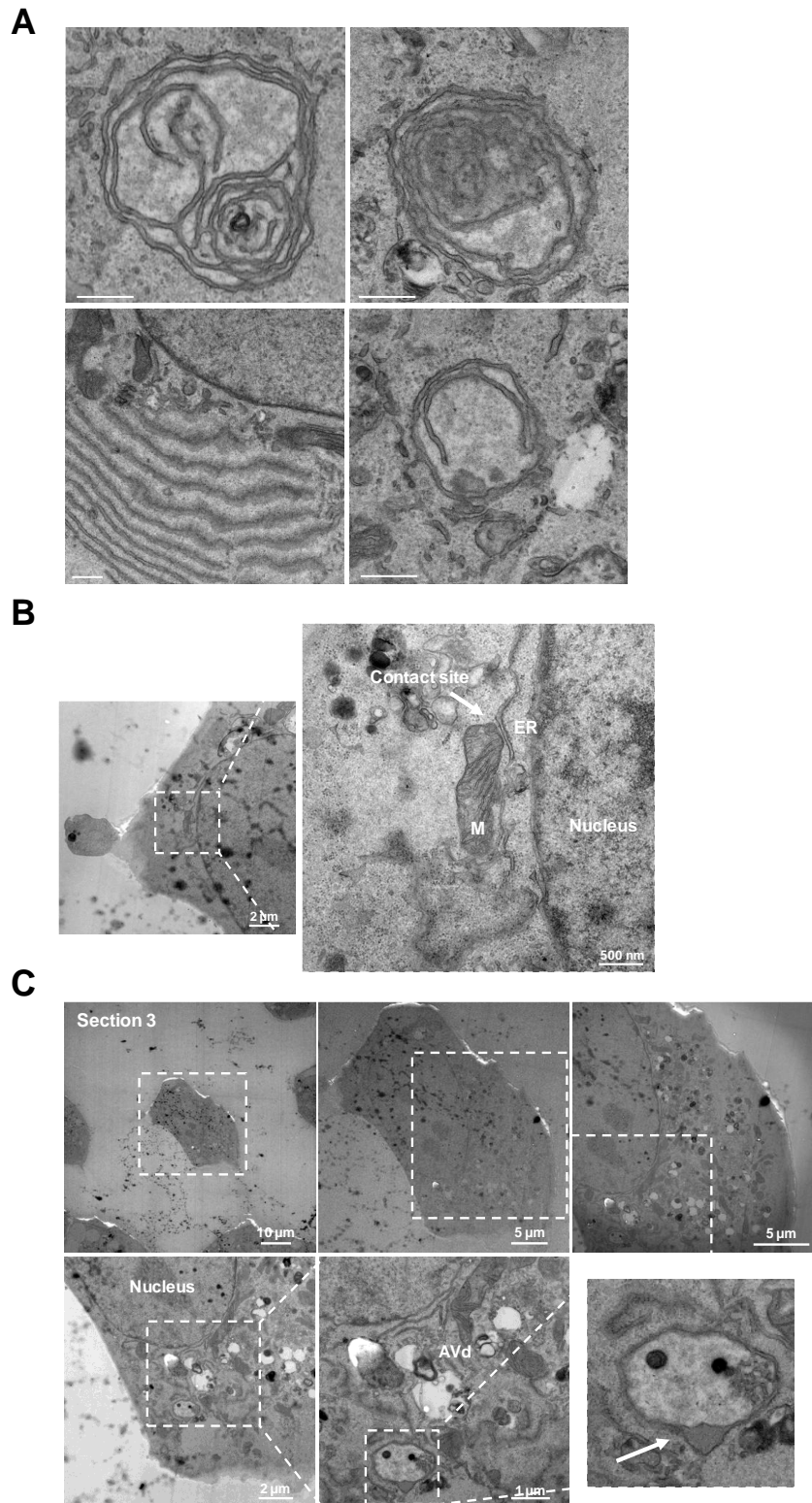


Figure 4.12. Representative images of different structures observed in rotenone-treated RPE1 cells. (A) Rotenone-mediated ER stress in human RPE1 cells. Representative images of distorted ER organization seen in rotenone-treated RPE1 cells are shown. Bars = 500 nm. (B) Representative image of a mitochondria (M)-ER contact site. (C) Representative images of the third section. Black deposits were observed formed during sample processing for EM. An autophagosome-like double membrane structure is also shown that eventually was not classified as an autophagosome due to the zone indicated with an arrow.

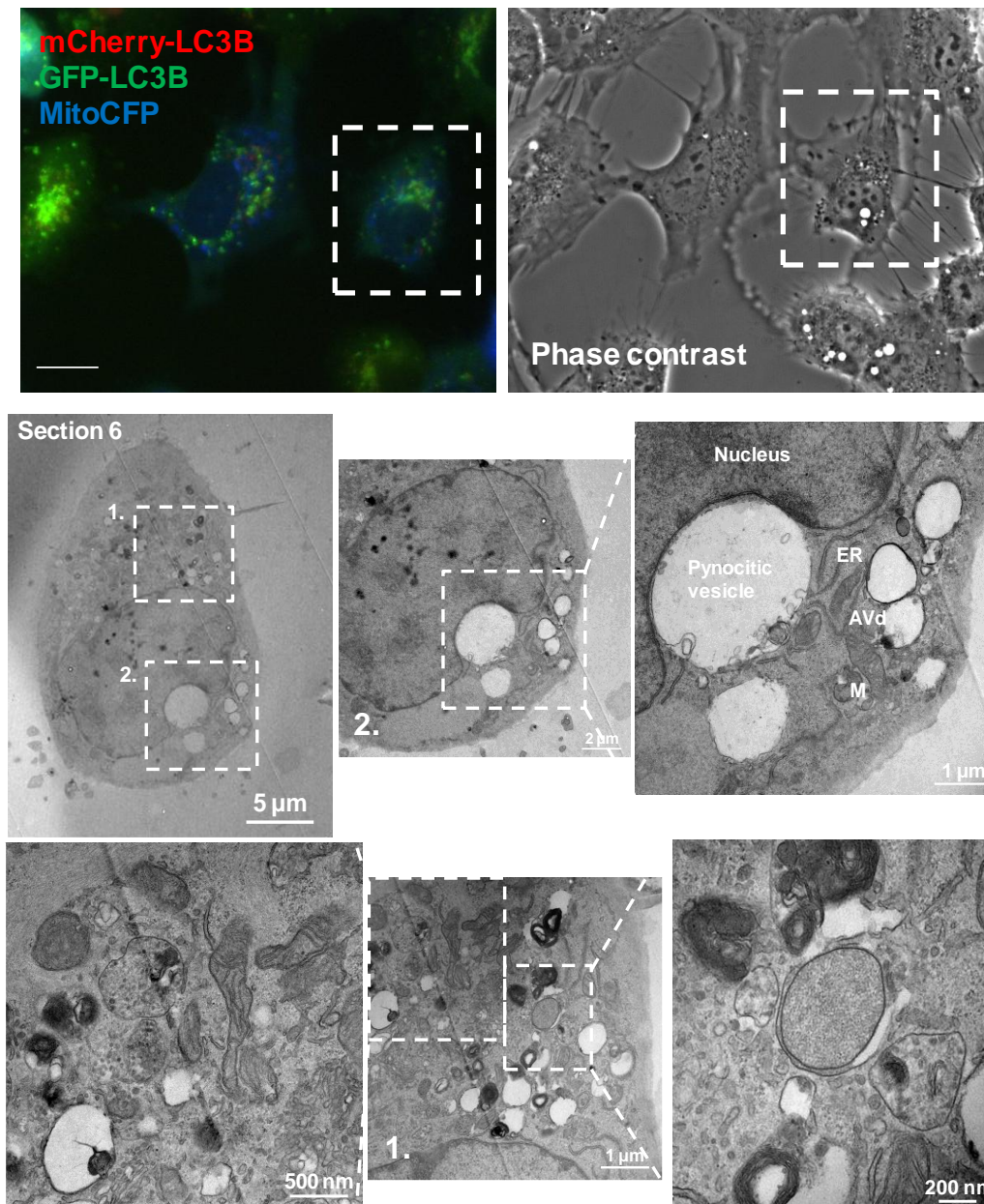


Figure 4.13. CLEM of rotenone-treated tandem LC3B expressing human RPE1 cells transfected with MTS-CFP. Cells were treated with rotenone for 2-3 h and after live-cell imaging were fixed and processed as described in Section 4.2.7. Bar = 20 μm . Representative image of the sixth section of the cell of interest is shown. The cell of interest is shown in the same orientation in both FM and EM images. (1) Multivesicular bodies, Golgi apparatus, AV with mitochondria inside and transitional ER and (2) pinocytic vesicles, mitochondrion (M), ER, early autophagosomes, AVd and lysosomal degradative compartments were observed.

As expected, many lysosomal structures were observed in rotenone- and leupeptin-treated cells, reflecting LC3B-labeled autolysosomes (red LC3B puncta) observed previously by light microscopy (**Figure 4.14**) – leupeptin inhibits lysosomal degradation but does not affect lysosome formation. The use of ethanol in sample processing for CLEM causes cell dehydration and in some cases lysosomes are less electron dense (white vesicles). HPF is a very useful alternative fixation technique to

avoid distortions in ultrastructure of specimens and introduces minimal artefacts (Studer *et al.*, 2008).

Double membrane vesicles were also observed in some cases with another membrane inside these structures, which could be mitochondria, or cytoplasmic material (**Figure 4.14**). In some sections parallel lines were observed probably caused by an occasional defect in the diamond knife used to cut the block face for sections (see arrow heads in **Figures 4.14 and 4.15**).

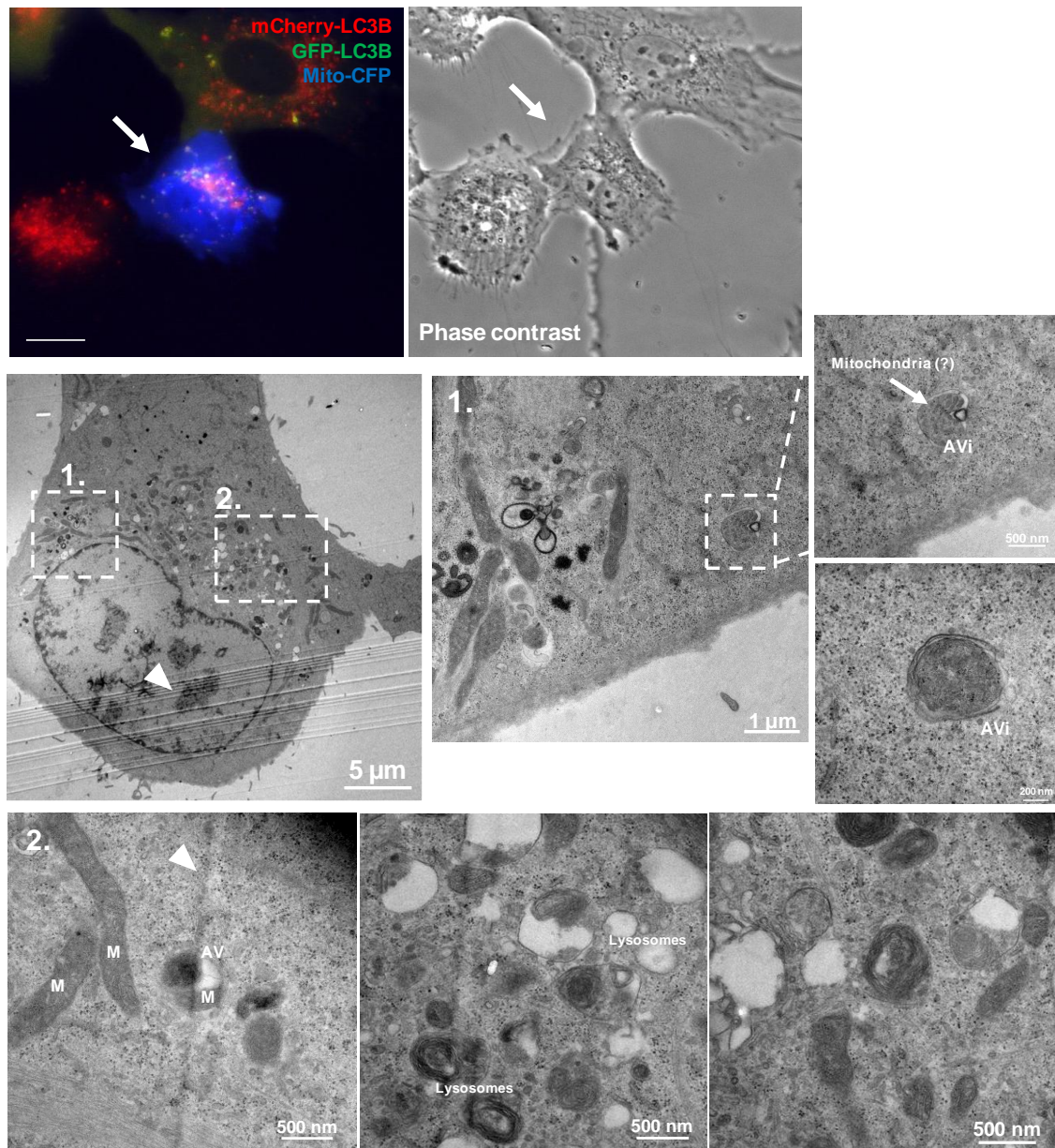


Figure 4.14. CLEM of rotenone- and leupeptin-treated RPE1 cells stably expressing mCherry-GFP-LC3B and transfected with MTS-CFP. Cells were treated with rotenone for 2-3 h and processed as described in Section 4.2.7. Arrows indicate which cell was examined by EM imaging. Bar = 20 μ m. Representative images of (1) early autophagosomes (with double membrane), (2) an autophagosome engulfing a mitochondrion (M), and lysosomes are shown.

In the CLEM experiment shown in **Figure 4.14**, EM analysis was limited by the fact that it was not a flat cell and therefore some information was lost through serial sectioning. To circumvent this problem the adjacent flatter cell was next examined by EM and many double-membrane autophagic structures and autolysosomes were observed containing different cytoplasmic materials (**Figure 4.15**). In some cases it was hard to identify the intravesicular content in the autophagic compartments, as shown e.g. by an early autophagosome that was close to rough ER decorated with ribosomes (**Figure 4.15B**). Since ribosomes (black dots) were not observed within this compartment it could not be determined whether it was an ER- or mitochondria-containing autophagosome. In addition, a possible mitochondrial fission event was observed (**Figure 4.15B**). It is generally accepted that mitochondrial fission is a prerequisite for mitophagy in many mammalian cell types (MacVicar & Lane, 2014; Ni *et al.*, 2015; Hsu & Shi, 2016; Twig *et al.*, 2008; Frank *et al.*, 2012). Indeed, it would be expected for an elongated mitochondrion of over 5 μm in length to be chopped up prior to engulfment by 0.5 μm diameter autophagosomes (Gomes & Scorrano, 2011). But it could also be the result of different heights along the section upon sample processing.

Together, CLEM results confirmed that rotenone notably affects the behavior of cellular membranes. Autophagosome assembly sites in the vicinity of mitochondria observed by EM (**Figures 4.10, 4.11, 4.14 and 4.15**) clearly correlated with mitochondria-rich regions where numerous GFP-LC3B puncta were observed by light microscopy (**Figure 4.7B**). Moreover, large compartments (autophagosomes with large organelles and amphisomes) possibly formed by fusion of several autophagic compartments observed by EM imaging (**Figure 4.11B**) closely correlated with large LC3B green/red punctae observed by fluorescence microscopy (**Figure 4.7**). Finally, mitochondrial degradation was blocked by co-treatment with the acidic protease inhibitor leupeptin and rotenone, and consequently, a greater number of mitophagosomes were observed in cells co-treated with the two reagents (**Figures 4.14 and 4.15**) as compared to rotenone-only treated cells (**Figures 4.11 to 4.13**).

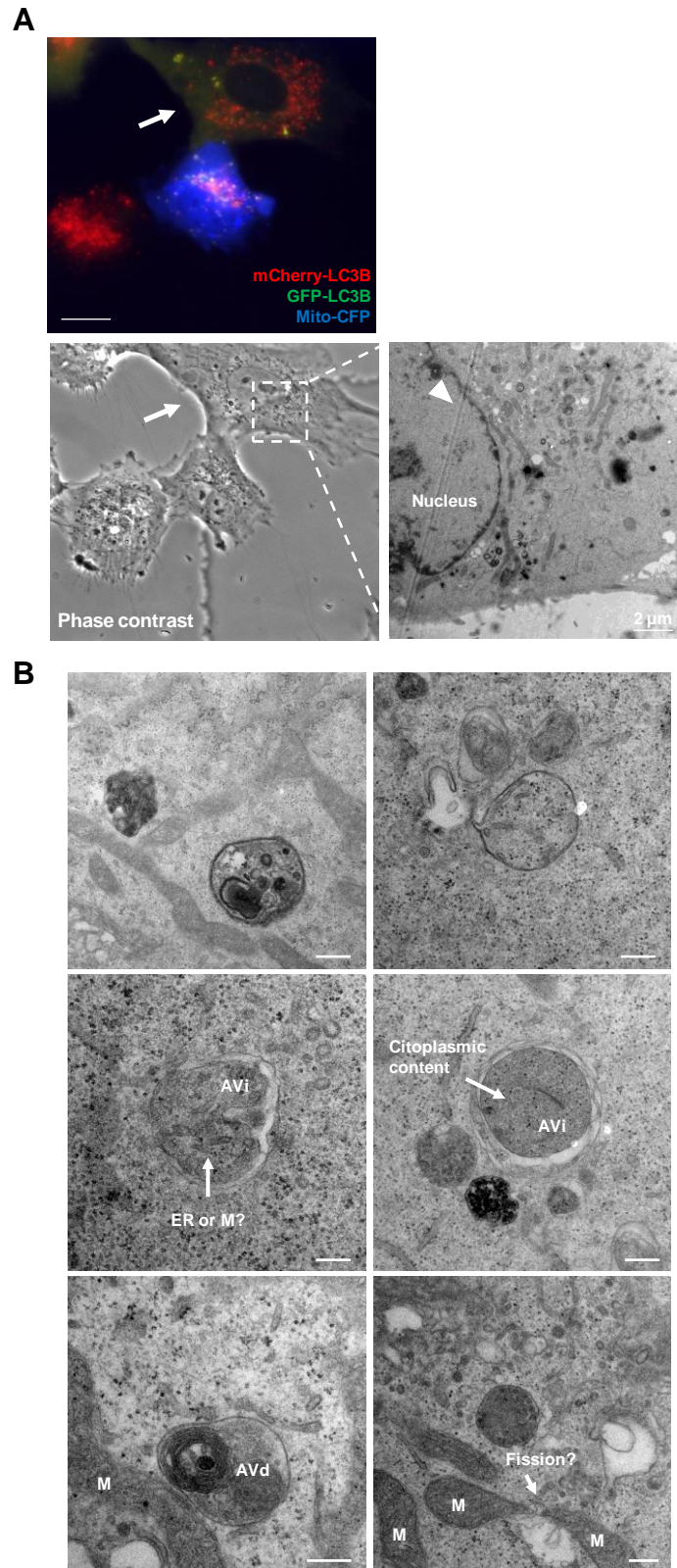


Figure 4.15. CLEM of rotenone and leupeptin-treated RPE1 cells stably expressing mCherry-GFP-LC3B and transfected with MTS-CFP. (A) Cells were treated with rotenone for 2-3 h and processed as described in Section 4.2.7. Arrows indicate the cell that was examined by EM imaging. Bar = 20 μ m. **(B)** Examples of isolation membranes (AVi) and late autophagosomes (AVd) adjacent to mitochondria (M), and a possible mitochondrial fission event are shown. Note that the isolation membrane is very electron dense. Bars = 200 nm.

4.3.3 LC3B and GABARAPL1 Intracellular Location during Mitophagy in iPSC-derived Cortical Neurons

Mitochondria play a very important role in neurons supplying energy to sustain neuronal activity and are highly exposed to stress conditions such as high Ca^{2+} influx or oxidative stress over time (Grenier *et al.*, 2013). The majority of mitochondria are located at distal axons, whereas lysosomes occur in the soma. Synapses represent regions of high energy demand and protein turnover; they contain abundant mitochondria and polyribosomes which make them more susceptible to the consequences of dysfunctional autophagy (Lee *et al.*, 2013; Nixon, 2013; Aufschnaiter *et al.*, 2016; Vidal *et al.*, 2014). In addition, the fact that neurons are postmitotic and do not replicate in general might predispose them to the accumulation of toxic proteins and damaged organelles that would become diluted through cell division in replicating cells.

Damaged mitochondria accumulate with age and neuronal autophagy is essential for the turnover of dysfunctional mitochondria in the pathogenesis of Parkinson disease, in which the failure to induce mitophagy may underlie the selective dopaminergic neuronal loss. Thus, stimulating the proper induction of mitophagy in neurons may serve as a therapeutic target to slow disease progression in PD (Gautier *et al.*, 2008; Greene *et al.*, 2003; Son *et al.*, 2012). Previously, recessive mutations in the ubiquitin E3 ligase PARK2 had been identified as a cause of early onset, familial PD. PARK2 is shown to be selectively recruited to damaged mitochondria, that have low membrane potential in mammalian cells (Narendra *et al.*, 2010a; Geisler *et al.*, 2010). However, the precise role of PARK2 in mitochondria is unknown. Moreover, so far, whether damaged mitochondria have to be translocated to the soma to be degraded remains unclear. Ashrafi and coworkers have proposed that they might be locally sequestered in autophagosomes before their retrograde transport to the soma or may even be degraded outside the soma (Ashrafi & Schwarz 2013; Ashrafi *et al.*, 2014).

Exposure to rotenone, a widely used pesticide, increases the risk of developing neurodegenerative disorders (Pal *et al.*, 2011; Uehara *et al.*, 2006). Rotenone is known to inhibit complex 1 of the mitochondrial respiratory chain, and to regulate Nox activity, increasing oxidative stress in neuronal cells and leading to neurodegeneration (Uehara *et al.*, 2006; Xia *et al.*, 2007; Zhou *et al.*, 2012). As already mentioned, rotenone has also been implicated as a potential inducer of CL externalization to the OMM in primary rat cortical neurons and human neuroblastoma cells (SH-SY5Y) (Chu *et al.*, 2013). The OMM-localized CL acts as a mitochondrial receptor for LC3 triggering mitophagy *via* a

pathway that may be different from the PINK1/PARK2 pathway upstream of the damage mechanisms (Chu *et al.*, 2013; Chu *et al.*, 2014). Indeed, the LC3-CL pathway shows less than 15% depolarization in mitochondria, and in PARK2-dependent mitophagy PARK2 is found to be recruited, in a PINK1-dependent fashion, to depolarized mitochondria (Narendra *et al.*, 2008; Narendra *et al.*, 2010a; Rüb *et al.*, 2016).

As most information about selective autophagy pathways has been established in non-neural cell lines, mitophagy triggered by LC3/GABARAP family proteins in neurons needs to be further examined. This project aimed at studying LC3B and GABARAPL1 recruitment to mitochondria during rotenone-induced mitophagy in cortical neurons to gain further insight into the ability of different human Atg8 orthologs to mediate selective mitochondrial targeting in neural cells. The extreme sensitivity to *ex vivo* manipulation and very limited availability of mature human neurons, as well as the limited differentiation potential of neural progenitors, are significant constraining factors in the study of neuronal processes such as aging or degeneration. Therefore, the *ex vivo* use of mature neurons generated from induced-pluripotent stem cells became a good alternative approach. Adult cells converted “back to pluripotency” also termed hiPS, are pluripotent cells derived from adult donors which allow both *ex vivo*, highly reproducible studies of specific diseases, as well as transplantation back into the patient, free of host-versus-graft reaction. Nistor and colleagues have adapted the current protocols for differentiating human pluripotent cells towards neuronal lineages (Chambers *et al.*, 2009, 2012; Shi *et al.*, 2012), generating an improved protocol for neuronal differentiation from hiPSs (Nistor *et al.*, 2015). In this study, iPSC-derived cortical neurons were then transduced with a lentiviral system carrying GFP-LC3B or GFP-GABARAPL1 in a p_{xl}g3 vector to follow GFP-tagged autophagosomes upon rotenone exposure. Expression of LC3 tagged at its N terminus with a fluorescent protein (e.g. GFP), is frequently used to monitor autophagosome numbers and dynamics using direct fluorescence imaging, especially in living cells (Klionsky *et al.*, 2008). In this case, GFP is observed on forming and maturing autophagosomes but not autolysosomes, as LC3 is thought to be removed from the outer membrane of autophagosomes during maturation (Tanida *et al.*, 2004a), and, as mentioned above, the GFP fluorescence bound to the inner membrane of autophagosomes gets quenched by acidification of these compartments (autolysosomes: ~pH 4.7, whereas GFP has a pKa of 6.0) (Shaner *et al.*, 2004).

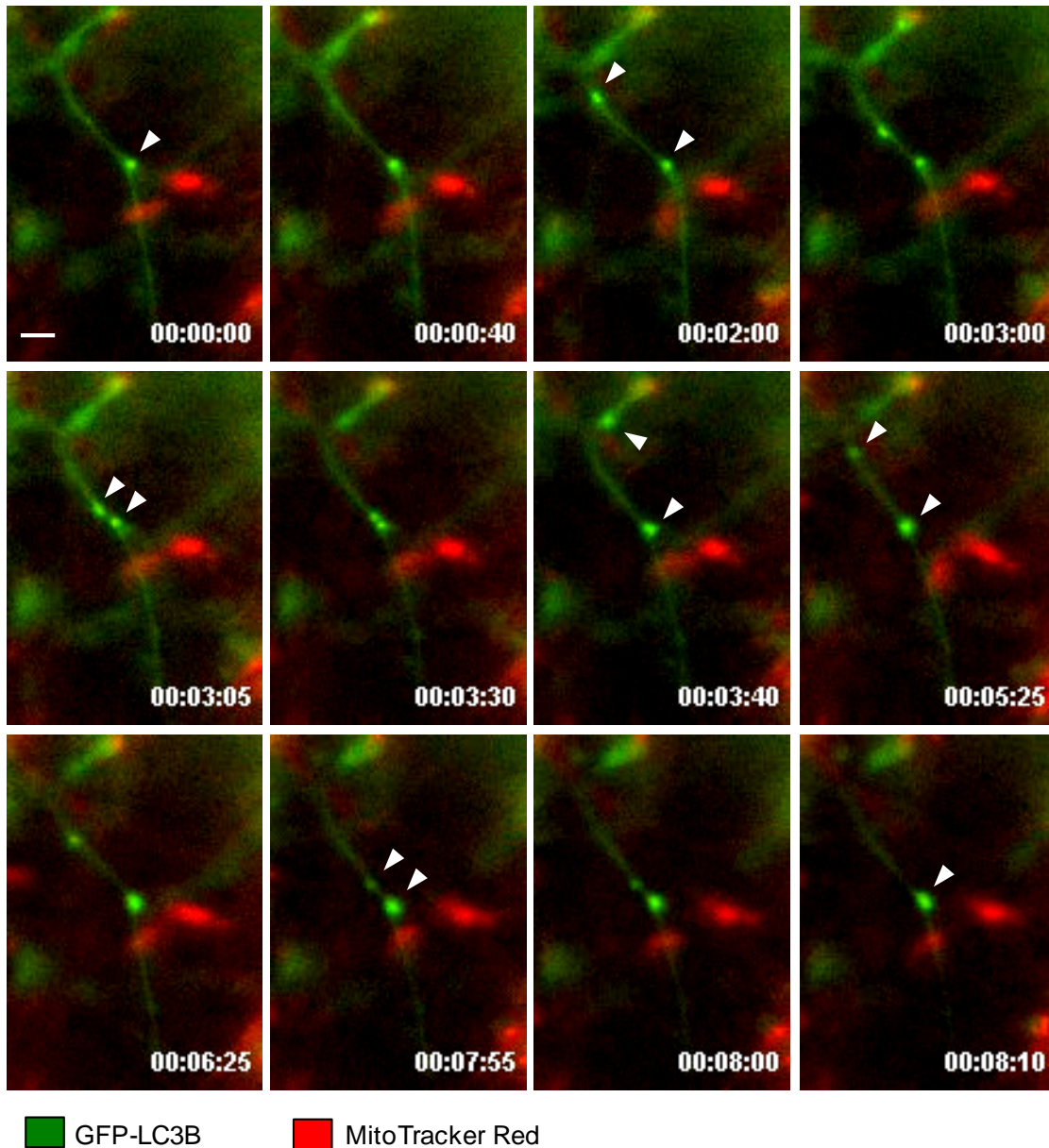


Figure 4.16. Autophagosome fusion events in iPSC-derived human cortical neurons expressing GFP-LC3B after 5 μ M rotenone treatment. Cortical neurons were transduced with the autophagosomal marker GFP-LC3B using lentiviruses. Representative images are shown of the distribution of GFP-LC3B (cytoplasmic and bound to autophagosomal membranes/puncta – arrow heads) in a neuronal axon at the indicated filming time points (see Supplemental Movie 4.16). Bar = 2 μ m.

A preliminary study using iPSC-derived human cortical neurons stably expressing GFP-LC3B or GFP-GABARAPL1 was performed in the presence of a mitochondrial marker (MitoTracker[®] Red) with the aim of precisely determining LC3B- or GABARAPL1-labeled autophagosome localization and possible recruitment to damaged mitochondria at various stages of the rotenone-induced mitophagy process. Time-lapse imaging of the described iPSC-derived cortical neurons allowed visualization of fusion processes of LC3B-positive structures upon rotenone treatment,

leading to the autophagosome growth as indicated by larger GFP-LC3B puncta (**Figure 4.16**). Unfortunately, in this particular experiment the mitochondrial marker (MitoTracker[®] Red) was unspecific and had a high background fluorescence (**Figure 4.16**). After rotenone administration to neurons expressing GFP-LC3B, most of the GFP-LC3B fluorescent signal was cytoplasmic with a few small puncta (**Figures 4.16 and 4.17**). Nonetheless, consistent with the previous results in U87MG glioblastoma cells and fixed and living RPE1 cells indicating that LC3B-positive autophagosomes are recruited to mitochondria after rotenone treatment, autophagosomes (GFP-puncta) were observed in the vicinity of mitochondria and seemed to follow mitochondria along the neuronal axon (**Figure 4.17**).

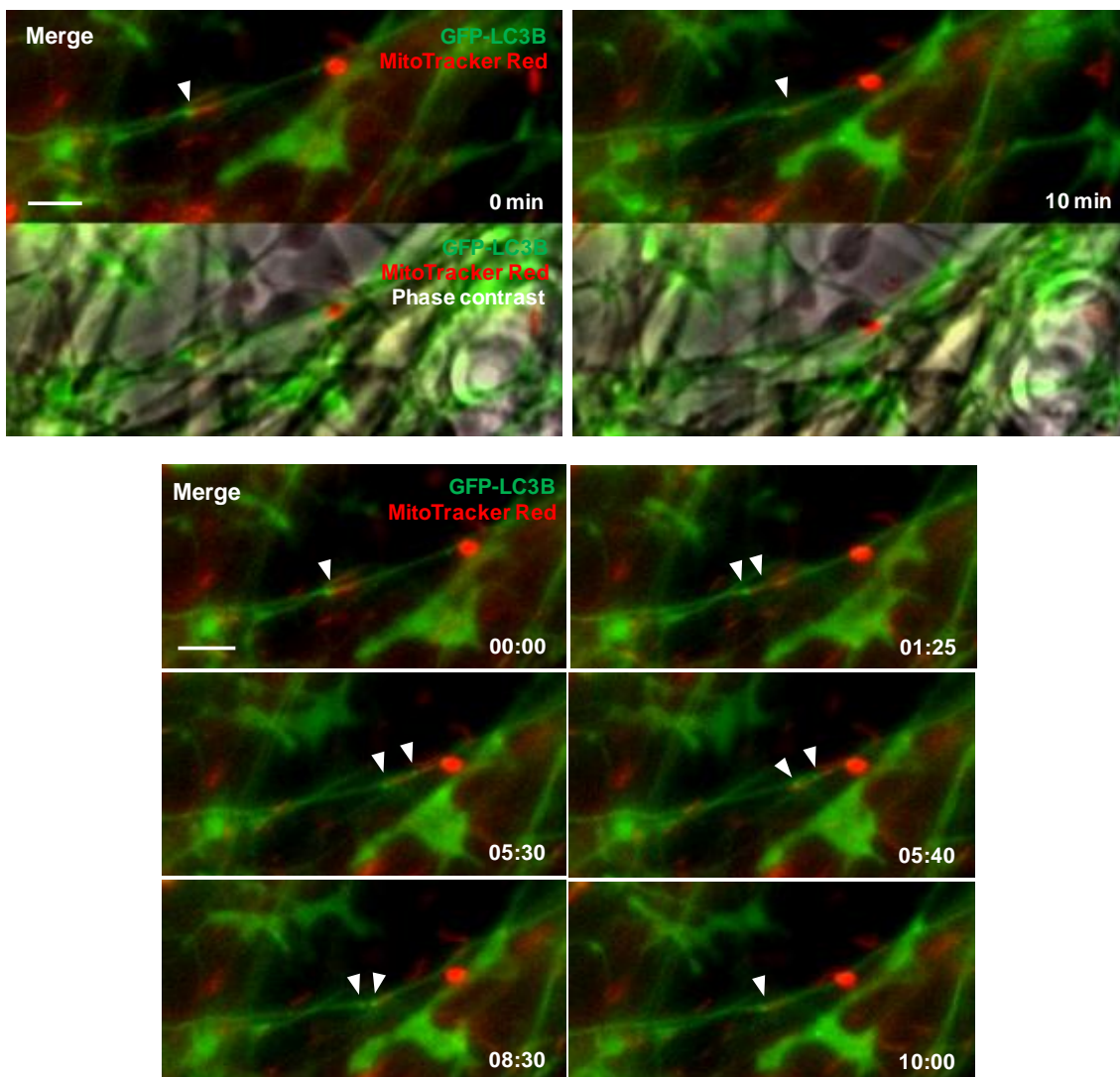


Figure 4.17. Autophagosome dynamics in GFP-LC3B expressing iPSC-derived human cortical neurons treated with 5 μ M rotenone. Cortical neurons were virally transduced with GFP-LC3B and filmed 3 days later by time-lapse imaging. Mitochondria were stained with MitoTracker[®] Red CMXRos. Pictures follow several autophagosomes (arrow heads) possibly engulfing mitochondria in one neuronal axon at the indicated filming time points during a period of 10 min (see Supplemental Movie 4.17). Bars = 4 μ m.

Live-cell imaging experiments with GFP-LC3B may suggest the targeting and engulfment of mitochondria by autophagosomes (**Figure 4.17**), fusion events between autophagosomes (**Figure 4.16**), and a rapid turnover of autophagosomes in human cortical neurons (note that the imaging periods were of about 10 min). Conversely, under these conditions, 5 μ M rotenone treatment of cortical neurons virally transduced with GFP-GABARAPL1 had no effect on the number of GABARAPL1 puncta or mitochondrial recruitment (data not shown). This differs from previous results in human RPE1 cells that GABARAPL1 localized to outer mitochondrial membrane in response to rotenone administration (**Figure 4.5**).

This study using iPSC-derived cortical neurons was started late in this project (so that there was limited time to pursue it), but it could provide an interesting final read-out for future experiments following modulation of mitophagy in human cortical neurons. Overall, some obstacles need to be overcome, e.g. decrease the LC3B background fluorescence optimizing the protocol for GFP-LC3B lentiviral transduction, using formaldehyde fixation instead of methanol fixation, or using a photoactivatable GFP, to confirm and quantify the results obtained in cortical neurons and compare them with other LC3/GABARAP family proteins.

4.4 Discussion

4.4.1 LC3B, but not GABARAP or GABARAPL2, Translocation to Mitochondria in U87MG Glioblastoma Cells

Data presented in the previous chapter described novel molecular mechanisms underlying the association of the autophagy protein LC3 with CL-containing membranes (Antón *et al.*, 2016). Using several compositionally defined *in vitro* reconstituted systems LC3B has been shown to avidly interact with CL in CL-enriched model membranes (**Figures 3.5 to 3.9**). Moreover, site-specific NBD labeling coupled to NBD fluorescence spectroscopic analysis supported the model proposed by Chu and colleagues in which the N- and C-terminal regions would bind mitochondria and autophagosomal membranes respectively (Chu *et al.*, 2013; Antón *et al.*, 2016). Consistent with this, results presented in this work show that LC3B translocates to mitochondria upon mild rotenone treatment in U87MG glioblastoma cells, resulting in targeted removal of damaged mitochondria through the action of lysosomes (**Figure 4.1**). Nevertheless, nutrient deprivation did not elicit translocation of LC3B to mitochondria or targeting of mitochondria to lysosomes. These results are consistent

with reports indicating that in higher eukaryotes mitochondria are in fact spared from autophagy during starvation. In yeast however mitochondrial degradation occurs upon nutrient depletion and/or mitochondrial damage and there is no experimental evidence that mitophagy selects damaged, but not intact, mitochondria for degradation (Kiššova *et al.*, 2007). Moreover, yeast proteins mediating this process have no mammalian orthologs. Therefore the molecular mechanisms involved in selective mitophagy appear to be fairly different in yeast and mammals.

Furthermore, unlike LC3B, GABARAP and GABARAPL2 do not translocate to mitochondria upon mitophagy induction in U87MG cells (**Figure 4.2**), suggesting different regulatory mechanisms between orthologs. Even though GABARAP is able to interact with CL *in vitro*, though with less affinity than LC3B, it does not show mitochondrial localization after rotenone treatment (Antón *et al.*, 2016). Thus the possibility remains that its capacity to interact with CL simply relies on its structural homology with LC3B, in such a way that translocation of GABARAP to mitochondria would occur via a different mechanism.

4.4.2 Dynamics of LC3B-, GABARAPL1- and ATG5-positive Autophagic Structures during Mitophagy in RPE1 Cells

The aim of this study was not only to observe, but also to extensively quantify and structurally analyze mitochondrial targeting and degradation processes in several human cell lines. The average outcome of rotenone administration in RPE1 cells expressing mCherry-GFP-LC3B or -GABARAPL1 was a significant increase in puncta numbers compared to nontreated control cells, and a mitochondrial recruitment of both human Atg8 orthologs. These data suggest that rotenone-induced CL externalization triggers mitophagy via the recruitment of both proteins leading to the removal of damaged mitochondria (**Figures 4.3 to 4.7**). In addition, experiments with fixed human RPE1 cells indicate that the mTOR catalytic inhibitor AZD8055 exposure has the strongest influence on the autophagic cell response, which is similar to that of CCCP on PARK2-dependent mitophagy. LC3B and GABARAPL1 puncta numbers are also significantly increased after rotenone treatment but this causes a weaker cell response and a lower increase in puncta numbers as compared to other inducers (**Figures 4.4 to 4.6**). Furthermore rotenone treatment leads to larger LC3B punctae while in nutrient deprivation conditions LC3B-labeled structures are smaller and dispersed throughout the cytoplasm of RPE1 cells (**Figures 4.3 and 4.4**). This observation could indicate (i) that LC3B-positive autophagosomes formed after rotenone-treatment contain mitochondria (a large organelle inside autophagosomes, larger puncta), or (ii) that

ATG4-mediated LC3B delipidation is dysfunctional under conditions of rotenone administration, therefore LC3B would not only be inside the autophagic vesicle (small and bright puncta) but it would also localize outside the autophagosome (larger puncta). It should also be considered that when using mCherry-GFP tagged LC3B or labeling of endogenous LC3B in immunofluorescence microscopy, differences in puncta size might also be due to the space occupied by the antibodies (**Figures 4.4 and 4.6**). The latter could also be the reason for differences observed in LC3B puncta quantitation between tandem tagged LC3B expressing cells and endogenous LC3B labeled by antibodies. However, in both cases similar cell response patterns were observed with the different autophagy and mitophagy inducers (**Figures 4.4B and 4.6B**).

Identification and positional analysis of the autophagosome assembly site, or “isolation membrane” using GFP-ATG5 expressing human RPE1 cells suggest that rotenone causes a low but significant increase in ATG5 puncta numbers (**Figure 4.6B**), however a significant mitochondrial recruitment was not observed for ATG5-positive preautophagosomes (**Figures 4.6A and 4.9**). These data suggest that the PAS recruitment to mitochondria may not be required for the selective mitochondrial targeting mediated by LC3B and/or GABARAPL1 during mitophagy and therefore the molecular machinery for autophagosome assembly is not likely to be localized in the mitochondria, at least at this stage of the process. Hence, experiments with soluble LC3B and CL-enriched vesicles described in the previous chapter (Antón *et al.*, 2016), together with that observed for GFP-ATG5 expressing cells, suggest rather that both soluble and autophagosome-bound LC3 forms would be recruited to mitochondria after CL translocation for targeting of damaged mitochondria in RPE1 cells. However these observations have to be considered carefully due to the low number of ATG5-positive structures present and measured (less than 5 puncta per cell for cells treated with rotenone) (**Figure 4.6B**). The ATG5-positive autophagosome assembly site being a transient structure (ATG5 punctae were shorter-lived than LC3B punctae), and the early, mitophagy-inducing treatment conditions involving low doses of rotenone used would make GFP-ATG5 positive with isolation membrane puncta hardly detectable in rotenone-treated RPE1 living cells (**Figures 4.6 and 4.9**).

CLEM was another method of choice to study how the recruitment process takes place during mitophagy, with the purpose of first studying the process dynamics by time-lapse imaging and subsequently ultrastructurally indentifying interesting events after adapting cell samples to this method (**Figures 4.10 to 4.15**). Since the study of early autophagosome, late autophagosome or mitophagosome morphology could not be pursued by fluorescent-labeling of resident LC3B, CLEM approach was expected to

be more informative. This study confirmed that green and red LC3B puncta observed by light microscopy can be closely correlated with early and late autophagosome structures by EM imaging in the same cell regions (**Figure 4.11**). Rotenone-induced larger LC3B puncta formation observed by immunofluorescence (**Figures 4.4, 4.7 and 4.8**) was supported by the observation in EM images that a fraction of the autophagosomes were larger and/or exhibited heterogeneous content (**Figures 4.10, 4.11 and 4.15**). In addition, in rotenone- and leupeptin-treated cells, a large number of lysosomal structures were also observed reflecting red LC3B punctae previously observed by fluorescence microscopy (**Figures 4.8, 4.14 and 4.15**). Finally, GFP-LC3B puncta located to mitochondria-rich regions (**Figure 4.7B**) appear to correlate with early autophagosomal vesicles observed in the vicinity of mitochondria by EM (**Figures 4.10, 4.11, 4.14 and 4.15**). In order to preserve the ultrastructure and size of cells and their organelles, the HPF technique could be used under conditions thought to introduce minimal artefacts as compared to direct chemical fixation used in this work.

4.4.3 LC3B-mediated Mitochondrial Targeting in Rotenone-treated iPSC-derived Cortical Neurons

Defective autophagy in the brain causes accumulation of damaged mitochondria and protein aggregates, leading to neurodegeneration (Lee *et al.*, 2013; Vidal *et al.*, 2014; Aufschnaiter *et al.*, 2016; Nixon, 2013). In contrast to the protonophores FCCP or CCCP (Kondapalli *et al.*, 2012), that cause a severe loss of mitochondrial membrane potential in neuronal cells, sublethal doses of rotenone do not distinctly affect the mitochondrial membrane potential and induce LC3, but not PINK1/PARK2 translocation to mitochondria in rat cortical neurons and human neuroblastoma cells (Chu *et al.*, 2013). However, as previously mentioned, rotenone-induced cell response can vary depending on treatment conditions and cell type.

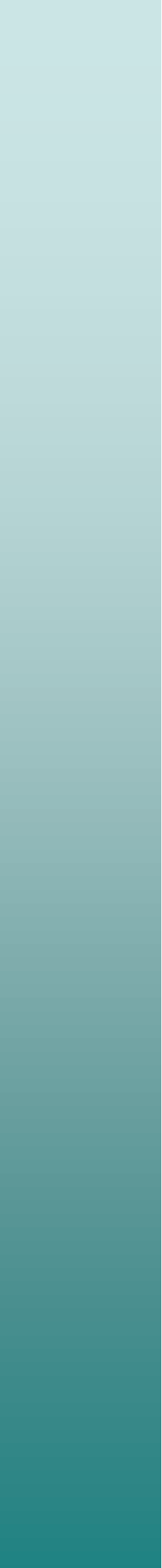
This preliminary study suggests that rotenone induces autophagy and GFP-LC3B translocation to mitochondria in iPSC-derived human cortical neurons (**Figures 4.16 and 4.17**). In addition, LC3B puncta formation observed upon rotenone treatment could not be repeated in GFP-GABARAPL1 expressing cortical neurons (data not shown). However, analysis of much larger data sets would be required to confirm the statistical significance of these results. Moreover, to further determine the involvement of the PINK1/PARK2 pathway mentioned above in this rotenone-induced mitophagy response, PARK2-deficient cells could be used. Several obstacles will need to be overcome to pursue this study of autophagosome dynamics and mitochondrial targeting by time-lapse microscopy in human cortical neurons. First, GFP-LC3B background

fluorescence in live-cell imaging of neuronal samples is higher than in RPE1 cells (**Figures 4.16 and 4.17**). This high background fluorescence makes it harder to trace the formation and location of GFP-LC3B puncta. Furthermore, to continue the study of LC3B mitochondrial recruitment and to obtain quantitative data of the process, preliminary experiments were performed with fixed neurons using GFP-LC3B lentiviral transduction or LC3B immunostaining. However, as a result of the LC3B background fluorescence, few GFP-LC3B punctae were detected after rotenone or CCCP treatment in fixed neural cells (data not shown). Second, *ex vivo* cultured cortical neurons proved to be very sensitive to e.g. light exposure, addition of toxins or immunolabeling, influencing either their distinctive morphological features of cortical neurons or their health and viability, a strong limitation for live-cell imaging techniques. Moreover, neuronal cell cultures are built up by neurons as a 3D network, and conventional microscopy collects data from individual planes and cannot therefore simultaneously image neurons across different focal planes and fields of view. Finally, mitochondrial labeling rapidly appeared diffuse and in some cases was non-specific (**Figures 4.16 and 4.17** – in red). A more reliable method of mitochondrial labeling would be necessary – e.g. co-transduction of fluorescently-labeled mitochondrial markers such as MTS-CFP, previously used in this project for LC3B colocalization analysis with mitochondria in RPE1 cells (**Figure 4.7**).

In conclusion this study has confirmed that rotenone-induced CL externalization promotes LC3B translocation to mitochondria and the subsequent induction of mitophagy in U87MG glioblastoma cells and human RPE1 cells, while other proautophagy stimuli lead to nonselective autophagy. However the specific factors, distinct from bulk autophagy, required for individual mitochondria to be distinguished within the mitochondrial network are still to be determined. Moreover data described in this chapter indicate that specific LC3/GABARAP protein family members are recruited to mitochondria in a cell-type dependent manner that could also be stress-specific (Antón *et al.*, 2016). There is still an underlying layer of complexity when it comes to LC3/GABARAP family protein recruitment, which is found to be executed by multiple protein and lipid receptors, and remains a matter of investigation.

Chapter 5

Novel mechanisms for ceramide-mediated cancer cell death: Lethal autophagy versus lethal mitophagy



CHAPTER 5:

Novel mechanisms for ceramide-mediated cancer cell death: Lethal autophagy versus lethal mitophagy

5.1 Introduction

There are several types of autophagy, among which are microautophagy, chaperone-mediated autophagy and macroautophagy. Macroautophagy, hereafter named autophagy, was first described in mammals, where it was observed that complete organelles could be sequestered into double-membrane vesicles called autophagosomes. The autophagosome fuses with lysosomes, after which the sequestered content is degraded rapidly (Boya *et al.*, 2013; Klionsky *et al.*, 2012; Rubinsztein *et al.*, 2007). The membrane source from which autophagosomes are derived is still debated, it has been proposed that it could be derived either from *de novo* synthesized lipids or generated by vesicle budding from the ER, Golgi apparatus or endosomes (He & Klionsky, 2009; Juhasz & Neufeld, 2006), or the plasma membrane (Ravikumar *et al.*, 2010). In particular, an ER-derived structure termed the omegasome has been proposed as an origin of the phagophore membrane (Juhasz & Neufeld, 2006; Reggiori & Klionsky, 2005). Autophagy is the major source of nutrients and biosynthetic precursors when the supply of nutrients to cells is insufficient (Boya *et al.*, 2005; Green & Levine, 2014; Liu & Levine, 2015; Marino *et al.*, 2014). However, stimulation of autophagy can also have a cytotoxic effect. For example, several anticancer agents activate autophagy-associated cell death (Green & Levine, 2014; Liu & Levine, 2015; Marino *et al.*, 2014; Galluzzi *et al.*, 2015). However, the molecular

mechanisms that determine the outcome of autophagy activation for the survival or death of cancer cells remain to be clarified (reviewed in Galluzzi *et al.*, 2015).

Furthermore, specific autophagy can take place in the presence of nutrients, to remove damaged organelles or to assist in cell differentiation (i.e. in the erythrocyte maturation process), or simply to direct organelle replacement. For instance, in the selective process called mitophagy, cells turn over mitochondria through autophagy for quality control and to adapt to cellular needs (Kiššova *et al.*, 2004; Tal *et al.*, 2007; Youle & Narendra, 2011). Defects in mitophagy (mitochondria-specific autophagy) have been linked to multiple human pathological states including neurodegeneration, e.g. Parkinson disease (Valente *et al.*, 2004), myopathies, and cancer (Aufschnaiter *et al.*, 2016; Kubli & Gustafsson, 2012; Lu *et al.*, 2013; Nixon, 2013).

An unresolved question is how the autophagosome membrane forms and elongates. The most widely accepted hypothesis proposes *de novo* formation from a PAS, from which the adhesion and fusion of new membranes would be produced. Autophagosome elongation would be catalyzed by specific proteins and would involve their interaction with membrane lipids. Approximately 35 Atg proteins have been identified in mammals, which are essential in the initial stages of the PAS formation and autophagosome elongation (Boya *et al.*, 2013; Rubinsztein *et al.*, 2007; Klionsky *et al.*, 2012). Enlargement of this compartment to form the autophagosome requires the participation of 2 ubiquitin-like conjugation systems, one involving the conjugation of ATG12 to ATG5, and the other requiring conjugation of phosphatidylethanolamine to LC3 (Rubinsztein *et al.*, 2007). The first discovered member of the Ubl family was Atg8 from *S. cerevisiae*. Several Atg8 orthologs are known in mammals, divided into LC3 and GABARAP subfamilies. These proteins are proteolyzed by ATG4B, causing the exposure of a C-terminal Gly. After proteolysis, LC3, GABARAP, and GABARAPL2 are activated by ATG7 (ubiquitin E1-like) and transferred to ATG3 (ubiquitin E2-like), after which they are able to conjugate with PS or PE. This protein-lipid covalent binding is essential for autophagosome elongation, but its mechanism remains unknown (Mizushima *et al.*, 2011; Landajuola *et al.*, 2016). The final outcome of autophagy activation is highly dependent on the cellular context and on the strength and duration of the stress-inducing signals.

THC, the main active component of *Cannabis sativa* (Pertwee *et al.*, 2010), exerts a wide variety of biological effects by mimicking endogenous substances — the endocannabinoids anandamide (Devane *et al.*, 1992) and 2-AG (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) that engage specific cell-surface G protein-coupled

cannabinoid receptors (Pertwee *et al.*, 2010). So far, 2 major cannabinoid-specific receptors, CNR1/CB1 (brain) and CNR2/CB2 (macrophage), have been cloned and characterized from mammalian tissues (Matsuda *et al.*, 1990; Munro *et al.*, 1993). Previous findings from the group of Velasco have unraveled that cannabinoid administration induces autophagy in different types of tumor cells, including glioma/astrocytoma and pancreatic cancer cells, whereas they do not activate this cellular process in non-transformed cells of interest (Salazar *et al.*, 2009). Pharmacological or genetic inhibition of autophagy prevents cannabinoid-induced cell death as well as apoptosis, whereas abrogation of apoptosis prevents cell death but not autophagy as induced by these agents (Salazar *et al.*, 2009). These observations allowed them to conclude that induction of autophagy is part of the mechanism by which cannabinoids promote the apoptotic death of cancer cells (Velasco *et al.*, 2012). They have investigated the molecular mechanisms responsible for the activation of autophagy upon THC administration and have found that THC binds cannabinoid receptors, leading to the stimulation of *de novo* sphingolipid synthesis and the subsequent activation of an ER stress-related signaling route that involves the up-regulation of the transcriptional co-activator NUPR1/p8 (transcriptional regulator) and its effector TRIB3 (Armstrong *et al.*, 2015; Carracedo *et al.*, 2006a; Carracedo *et al.*, 2006b; Velasco *et al.*, 2012). The stimulation of this pathway promotes in turn autophagy via TRIB3-mediated inhibition of the AKT-MTORC1 axis, which is essential for the proapoptotic and antitumoral action of cannabinoids (Salazar *et al.*, 2009, 2013).

A lipidomic study of human glioma cells after THC administration supports that the effect is related to differences in sphingolipid composition of autophagosomes, particularly an increased content of 14:0 and 16:0 dihydroceramides (Hernández-Tiedra *et al.*, 2016). Moreover, treatment with THC also promotes the release of cathepsins by autolysosomes. These effects may be due to the increased levels of those sphingolipids, which would alter the structure of autolysosomal or autophagosomal membranes, making them more unstable or permeable and allowing the release of cathepsins. On the basis of these observations, the study in this work has attempted to gain further insight into the molecular mechanism underlying the activation of autophagy-mediated cancer cell death by modeling the behavior of membranes with different ratios of dihydroceramide to test the above hypothesis. With this purpose, we have performed experiments in which ceramides and dihydroceramides were added to model liposomal membranes of controlled compositions, and their ability to induce membrane destabilization was assayed. We have also explored the implication of ceramide and dihydroceramide domain formation by generating them enzymatically

after *B. cereus* nSMase action (Hernández-Tiedra *et al.*, 2016). Using this experimental approach, we found that (i) dihydroceramide forms primarily dihydroceramide-rich rigid regions that melt at temperatures well above those generated by ceramide; (ii) dihydroceramides form rigid domains in GUV which are morphologically similar to those formed by ceramides; and (iii) dihydroceramide causes release of contents in liposomes based on PC and Chol. Release of vesicular aqueous contents induced by dihydroceramide appears to be larger and faster than that induced by ceramide. Thus, the alteration of the balance between different molecular species of ceramides and dihydroceramides in the microsomal fraction of cancer cells induced by THC administration can promote the permeabilization of autophagosomal and autolysosomal membranes leading to the release of cathepsins to the cytoplasm and the subsequent activation of apoptotic cell death.

Moreover, as mentioned in the Introduction, recent studies have shown that ceramide in the mitochondrial membrane acts as a receptor for LC3B (Sentelle *et al.*, 2012; Dany & Ogretmen, 2015). Mutagenesis and docking analyses indicated that ceramide has a higher affinity to the PE-conjugated LC3 than to soluble LC3 (Sentelle *et al.*, 2012). In the present work we examined this interaction, using the vesicle flotation approach, to test whether some of the features observed in LC3B interaction with cardiolipin (see Chapter 3) also play a role in its interaction with ceramide. Our results with the soluble form of the protein strongly support the idea that previous lipidation of the autophagic protein is essential for LC3-Cer interaction, modulating the binding capacity of the protein.

5.2 Materials and Methods

5.2.1 Materials

PC and PE from egg yolk were purchased from Lipid Products. Bovine heart CL (90% TLCL, 5% TOCL, 5% unknown), DOPE, Chol/Ch, Cer16/C16Cer, C16dhCer, Cer18, Cer24:1 and egg SM were from Avanti Polar Lipids. dhSM (86% C16dhSM) was synthesized by Gemma Fabrias (Department of Biomedical Chemistry, iQAC, Barcelona) from egg SM (Avanti Polar Lipids). SMase (EC 3.1.4.12) from *Bacillus cereus* and o-phenanthroline were supplied by Sigma-Aldrich. ANTS and DPX were supplied by Molecular Probes. Anti-LC3 monoclonal antibody was from MBL International (Medical & Biological laboratories Co. Ltd.).

5.2.2 Recombinant Protein Expression and Purification

A plasmid for expression of the human Atg8 ortholog LC3B was kindly provided by Dr. I. Tanida. Note that this Atg8 ortholog is a truncated form ending in the reactive C-terminal Gly such that no ATG4-mediated preprocessing is necessary. LC3B was purified from soluble fractions of bacterial extracts obtained in the absence of detergents, and was >90% pure as evaluated by Coomassie Blue-stained SDS-PAGE. *E.coli* BL21(DE3) cells were transformed with the LC3B plasmid and cells were grown to OD₆₀₀ 0.8 and induced with 0.5 mM IPTG (Sigma-Aldrich) for 3 h at 37°C. Following centrifugation at 4,500 x g for 15 min, the pellet was resuspended and sonicated in breaking buffer [10 mM PBS, 20 mM Tris-HCl, pH 7, 150 mM NaCl, supplemented with freshly prepared 1 mg/ml lysozyme (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), bacterial protease inhibitors (Roche) and 2.5 µg/ml DNase (Sigma-Aldrich)]. After removal of cellular debris by centrifugation at 30,000 x g for 30 min at 4°C, the sample supernatant fraction was incubated with 1 ml glutathione Sepharose 4B (GE Healthcare) for 3 h at 4°C to bind GST-tagged proteins. Then PreScission Protease (GE Healthcare) was added at 100 units/ml in a 2-bed volume of PreScission Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) freshly prepared with 1 mM DTT and cleavage was performed for 4 h at 4°C. Cleaved protein was eluted and concentrated. Recombinant LC3B was stored in 20% glycerol at -80°C.

5.2.3 Liposome Preparation

The appropriate lipids were mixed in organic solution [chloroform:methanol (2:1 v/v)] and the solvent was evaporated to dryness under a N₂ stream. Then the sample was kept under vacuum for 2 h to remove solvent traces and the lipids were swollen in

a buffer solution for MLV preparation. In the present study, when preparing “complex” MLVs, i.e. those presenting several lipid components or those having lipids with high main phase transition temperatures, hydration was performed in a slightly different way. Briefly, instead of adding the aqueous solution at once, hydration was performed by adding increasing amounts of the aqueous solution while maintaining the temperature above that of the lipid with the highest main phase transition temperature, and helping dispersion by stirring with a glass rod. Finally, the generated vesicles were homogenized by passing the sample 50-100 times between two syringes through a narrow tubing (0.5 mm internal diameter, 10 cm long) at the same temperature of the hydration process.

LUVs of diameters 100–150 nm were prepared from swollen lipids, subjected to 10 freeze/thaw cycles, and then extruded using a LIPEX Liposome Extrusion System (Transferra Nanosciences) equipped with 0.1-mm pore diameter Nuclepore filters (Whatman) at 65°C (as described in Mayer *et al.*, 1986). By using a circulating water bath, the extruder was maintained at the desired temperature. Vesicle size was checked by quasi-elastic light scattering using a Malvern Zeta-Sizer 4 spectrometer (Malvern Instruments). Phospholipid concentration was measured in terms of lipid phosphorus.

5.2.4 Differential Scanning Calorimetry (DSC)

Previously degassed buffer solutions (20mM PIPES, 150mM NaCl, 1mM EDTA, pH 7.4) were introduced into both reference and sample cells of a VP-DSC high-sensitivity scanning microcalorimeter (MicroCal) to obtain the buffer reference thermogram. Then, in order to determine the specific thermodynamic parameters, at least three heating scans were performed with the lipid sample in the form of MLV. Lipid concentration of the sample was determined by phosphate analysis. In all cases, background thermograms from samples lacking lipid were subtracted from the thermograms obtained with lipid containing samples. Sample concentration was normalized and the specific parameters determined. Data treatment was performed using the ORIGIN 7.0 (MicroCal).

5.2.5 Release of Vesicle Contents

Leakage of LUV contents was assayed with the ANTS/DPX fluorescence system (Ellens *et al.*, 1985; Nieva *et al.*, 1989; Goñi *et al.*, 2003). LUVs were prepared as previously described in the following buffer: 20 mM ANTS, 70 mM DPX, 40 mM NaCl, 10 mM HEPES, 10mM CaCl₂ and 2mM MgCl₂, pH 7. A high DPX/ANTS ratio was

used to ensure complete quenching inside vesicles. The vesicle suspension was passed through a Sephadex G-25 column to remove non-entrapped ANTS and DPX molecules. A previously adjusted isosmotic buffer solution consisting of 150 mM NaCl, 10 mM HEPES, 10mM CaCl₂ and 2mM MgCl₂, pH 7 was used for this process.

Ceramide or SMase was added to vesicles and leakage was followed in terms of ANTS fluorescence in a QuantaMaster spectrofluorometer (Photon Technology International) at 37°C and 520 nm. The excitation wavelength was set at 355 nm and the band-pass was 2 nm. A 470 nm cut-off filter was placed between the sample and the emission monochromator to avoid scattering interference. Since commercial sphingomyelinase preparations may contain phospholipase C impurities, 2 mM o-phenanthroline (Sigma-Aldrich) was routinely added in all our enzyme assays. Lipid concentration was 0.3 mM and sphingomyelinase was used at 0.15 units/ml. Lipid concentration was determined by phosphate analysis. To establish the 100% leakage signal, Triton X-100 was added to a 0.1% (v/v) final concentration.

5.2.6 Sucrose Gradient Centrifugation of Liposomes

Recombinant LC3B (10 µM) was incubated with 3 mM liposomes (containing 0.05 mol percent Rho-PE for detection) for 1 h at 37°C in 200 µl PreScission buffer. 125 µl protein/lipid mix was diluted to 300 µl in PreScission buffer containing 2.4 M sucrose (Sigma-Aldrich). Then the reaction mix was transferred to a centrifuge tube. The 1.4 M sucrose layer was overlaid with 400 µl PreScission buffer containing 0.8 M sucrose and 300 µl PreScission buffer containing 0.5 M sucrose. Sucrose step gradients were centrifuged in a TLA-120.2 rotor (Beckman Coulter) at 356,160 x g for 3 h at 4°C. Four 250-µl fractions were pipetted, starting from the bottom. The T fraction, containing liposomes as indicated by the rhodamine fluorescence, and the B fraction containing the unbound protein were analyzed by SDS-PAGE and western blotting.

5.2.7 GUV Preparation

GUVs were prepared using the electroformation method described previously (Montes *et al.*, 2007), using a homemade chamber (Industrias Tecnicas ITC) that allows direct visualization under the microscope. Stock solutions of lipids (0.2 mM total lipid containing 0.3 mol% Rho-PE) were prepared in chloroform:methanol (2:1, v/v) and 5 µl of the lipid stocks were added onto the surface of platinum electrodes. Then solvent traces were removed by evacuating the chamber under high vacuum for at least 2 h. The platinum electrodes were covered with 400 µl of 25 mM HEPES and 150 mM NaCl, pH 7.5, buffer previously equilibrated at 65°C, and connected to an electric wave

generator (TG330 function generator, Thurlby Thandar Instruments) under AC field conditions (500 Hz, 0.08V for 6 min; 500 Hz, 1.0 V for 20 min, and 500 Hz, 3.0 V for 1 h 30 min) at 65°C. Vesicles remain attached to the platinum wire and are left to equilibrate for 1 h at room temperature after switching off the generator and water bath.

5.2.8 Fluorescence Microscopy

In the present work, laser scanning confocal microscopy has been applied to the characterization of lipid domains in GUVs under thermodynamic equilibrium conditions. After GUV electroformation, the chamber was placed on an inverted confocal fluorescence microscope (Nikon D-ECLIPSE C1, Nikon). The excitation wavelength was 561 nm for Rho-PE. The images were collected using band-pass filter of 593 ± 20 nm. Image treatment was performed using the EZ-C1 3.20 software (Nikon).

5.2.9 Statistical Analyses

Statistical analyses were performed by ANOVA with a *post hoc* analysis by the Student-Neuman-Keuls test.

5.3 Results

Previous reports by Velasco and coworkers show that THC triggers an early accumulation of *de novo*-synthesized sphingolipids (an event that takes place in the ER) and inhibits the transport of sphingolipids from the ER to the Golgi, which leads in turn to ER stress response and is involved in the induction of autophagy-mediated cancer cell death (Carracedo *et al.*, 2006a; Salazar *et al.*, 2009; Velasco *et al.*, 2012). To investigate the potential relevance of changes observed in the sphingolipid composition of autophagosomes and autolysosomes, and specifically of the increased dihydroceramide levels in THC-treated cells, we undertook a series of experiments to analyze the role of these lipids in model vesicles. The main objective was the verification that dihydroceramide exhibits a transition temperature similar to that of ceramide, forms rigid domains and causes release of contents in the same way as ceramides. Mass spectrometry studies of the subcellular fraction (microsomes) of human glioma cells had shown that the main change induced by THC treatment was a decrease in C16Cer and a concomitant increase in C16dhCer (Hernández-Tiedra *et al.*, 2016). Therefore, experiments were conducted using 16:0 sphingolipids. Moreover, in microsomes it had been observed that PC species predominated, together with some Chol, so PC:Chol (95:5, mol ratio) was used as the basic lipid composition.

Differential scanning calorimetry, fluorescence confocal microscopy and fluorescence spectroscopy were applied to the characterization of membranes based on egg PC with different ratios of dihydroceramide in MLVs, GUVs and LUVs at 37°C.

5.3.1 Ceramide-Domain Formation Experiments

As a first approach to investigate the molecular mechanisms responsible for the activation of autophagy-mediated cancer cell death we analyzed the formation of ceramide domains which has been previously described in our group by DSC (as different domains have different transition temperatures) and by IR spectroscopy, using both natural ceramides (brain, egg) and synthetic phospholipids. Measurements in monolayers have shown that the potential dipole may play an important role in the lateral phase separation (Goñi & Alonso, 2009). Thus, the ceramide-PC dipolar pairing would favor strong interactions while unfavorable pairings would explain phase segregation in ceramide-rich domains. Moreover, epifluorescence studies of sphingomyelin monolayers and the quantification of the area occupied by the different domains after SMase addition revealed that domain formation is related to the activity of the enzyme. It seems therefore that decoupling of the chains is an important factor

that regulates phase separation. However, the causes of domain formation can be very diverse, although the most important is probably the ability of ceramides (and sphingolipids in general) to form hydrogen bonds. While glycerophospholipids can only act as hydrogen acceptors, sphingolipids (such as sphingomyelin and ceramides) may act as acceptors and also as donors through their hydroxyl and amide groups (see **Figure 5.10**) (Goñi & Alonso, 2009).

In the present study, experiments focused on the formation of dihydroceramide domains included DSC assays that determined the thermodynamic parameters for the lipid phase transition induced by temperature changes. For that purpose, 2 mM MLV of the following mixtures were analyzed: pure ePC, ePC:C16Cer (80:20, mol ratio), and ePC:C16dhCer (80:20, mol ratio) (**Figure 5.1**).

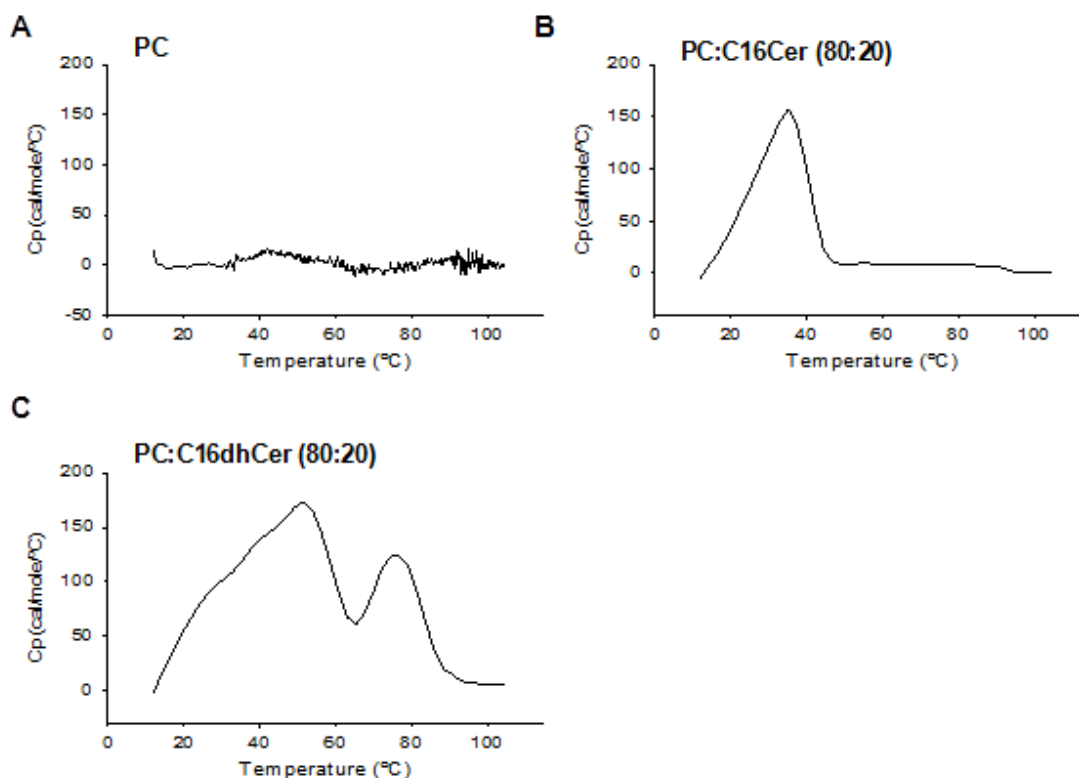


Figure 5.1. Effect of C16 ceramides and C16 dihydroceramides on the gel-to-fluid phase transition of lipid membranes. DSC thermograms of (A) pure PC, (B) PC:C16Cer (80:20, mol ratio) and (C) PC:C16dhCer (80:20, mol ratio). Representative thermograms of three independent experiments are shown.

In the control sample thermogram (PC) no peak appeared as its phase transition occurs at about -3°C , i.e. below our experimentally accessible temperature range (**Figure 5.1A**). In the PC:C16dhCer thermogram two zones can be observed: we suggest that the first is associated with dihydroceramide mixed with PC resulting in dihydroceramide-rich rigid regions that melt at $\sim 52^{\circ}\text{C}$; the same phenomenon can be observed in PC:C16Cer samples at $\sim 40^{\circ}\text{C}$ (**Figure 5.1B**). The second peak would correspond to regions primarily formed of dihydroceramide ($\sim 77^{\circ}\text{C}$) that contain small amounts of PC (**Figure 5.1C**).

This calorimetric phase transition experiments indicate that C16dhCer, prepared in a mixture with egg PC, exhibited a more complex transition (extending over higher temperatures) than C16Cer, i.e. the membrane rigidifying effect of C16dhCer is higher than that of C16Cer. These observations suggest that an enhanced proportion of dihydroceramide facilitates the formation of more rigid domains than ceramide in biological membranes, so there should be larger fluidity differences between ePC-rich areas and dihydroceramide domains than in the case of ceramide domains. This in turn would lead to the greater release ratios observed with dihydroceramide in the leakage experiments described below (see **Figures 5.5 and 5.6** and **Tables 5.1 and 5.2**).

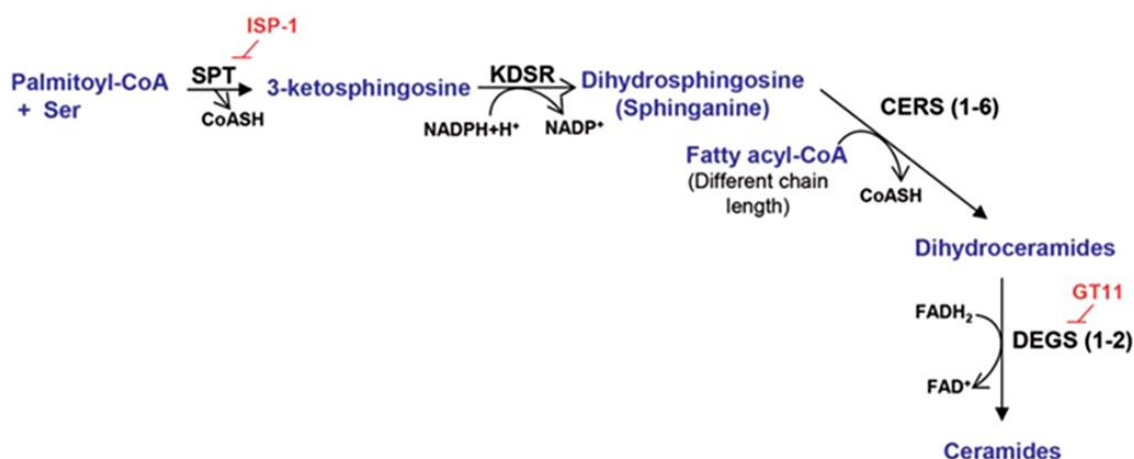


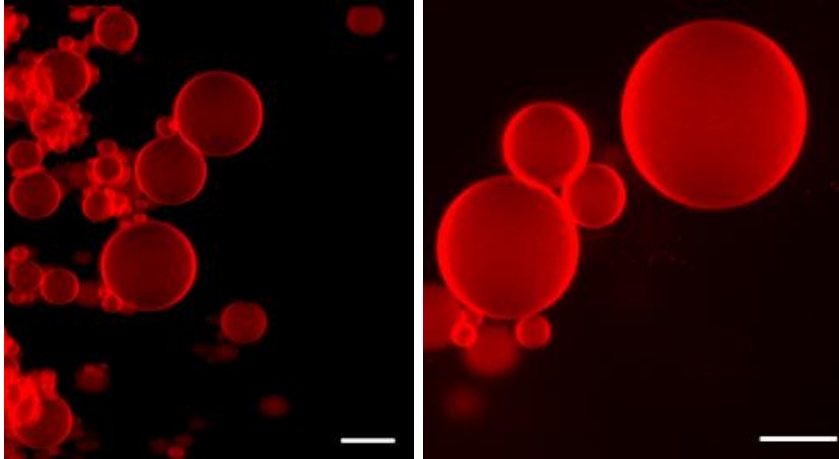
Figure 5.2. The pathway of *de novo* sphingolipid synthesis. SPT catalyzes the condensation of serine and palmitoyl-CoA to produce 3-ketosphinganine. KDSR catalyzes the reduction of 3-ketosphinganine to dihydrosphingosine (sphinganine). The next reaction is catalyzed by CERS1 to CERS6 (each isoform of this enzyme has selectivity for fatty acyl-CoAs with different chain length). CERSs convert dihydrosphingosine into the different molecular species of dihydroceramides, which are subsequently transformed into ceramides by the insertion of a 4, 5-*trans* double bond catalyzed by the enzymes DEGS1 and DEGS2. ISP-1 and GT11 are pharmacological inhibitors of SPT and DEGS, respectively.

As mentioned above, analysis of the sphingolipid composition of autophagosome-enriched fractions derived from human glioma cells have shown that PC species predominate together with some Chol and that THC treatment produces an increase in the levels of different species of dihydroceramides (Hernández-Tiedra *et al.*, 2016; see **Figure 5.2**). It should be noted that ceramide levels were 6- to 10-fold higher than those of dihydroceramides in vehicle-treated cells. Thus, the observed THC-triggered increase in dihydroceramides led to a striking modification of the ratio of both sphingolipid types in the microsomal fraction, specifically the C16Cer:C16dhCer ratio varied from 6:1 to 2:1 (Hernández-Tiedra *et al.*, 2016).

Thus, after studying by DSC phase transitions in samples with both ceramide and dihydroceramide, the formation and morphology of domains were monitored by direct confocal microscopy of individual vesicles. For this purpose, 0.2 mM GUV were electroformed attached to a platinum wire composed of PC:Chol (95:5, mol ratio) to which either 30 mol% of C16Cer:C16dhCer (6:1, mol ratio) or 30 mol% of C16Cer:C16dhCer (2:1, mol ratio) were added (73:4:23, final mol ratio) (**Figures 5.3 and 5.4**). A buffer was used (25 mM HEPES, 150 mM NaCl, pH 7.5) with which large, non-overlapping vesicles were obtained.

Morphological examination of GUVs indicated that the control GUVs (PC:Chol) failed to show lateral domain formation (**Figure 5.3**). In contrast domains can be observed in ceramide- and dihydroceramide-containing vesicles, in accordance with the results obtained by DSC (**Figures 5.3 and 5.4**). We noticed that some of the ceramide-containing vesicles lacked Cer/dhCer-rich domains, which suggests sample heterogeneity.

PC:Chol (95:5)



PC:Chol (95:5) + 30 additional mol% Cer:dhCer (2:1)

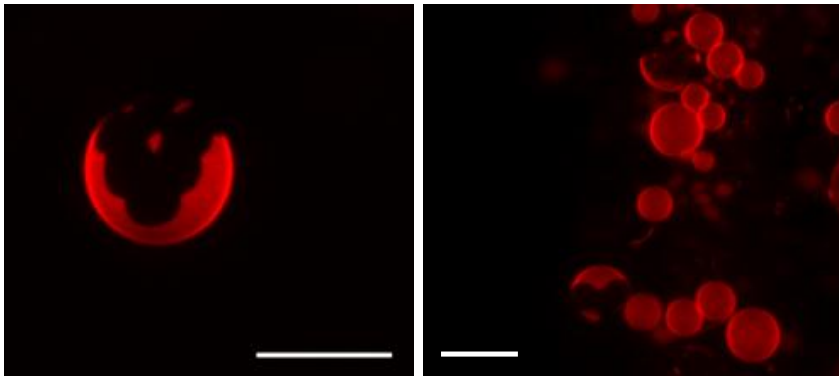


Figure 5.3. Generation of ceramide rigid domains in C16 ceramide:dihydroceramide-containing GUVs. Upper panel: a control experiment with GUVs composed of PC:Chol (95:5, mol ratio) that does not give rise to domains under these conditions. Lower panel: Ceramide rigid domains in bilayers containing PC (a fluid phospholipid), Chol, C16 ceramide, and C16 dihydroceramide (73:4:15:8, final mol ratio). Bars: 10 μm .

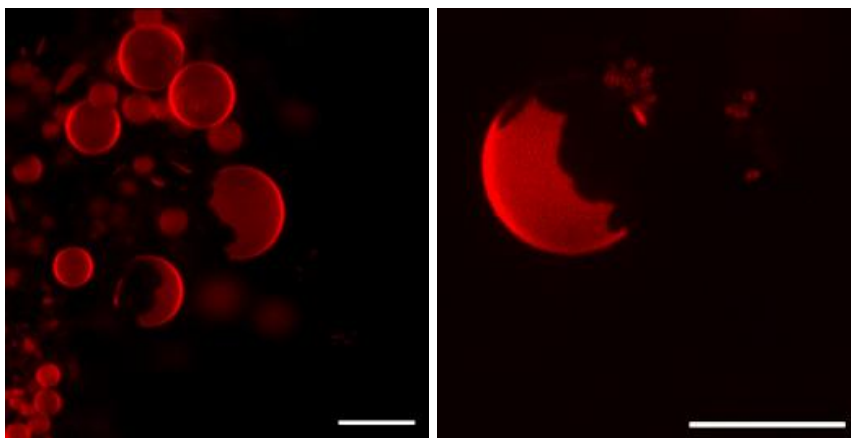
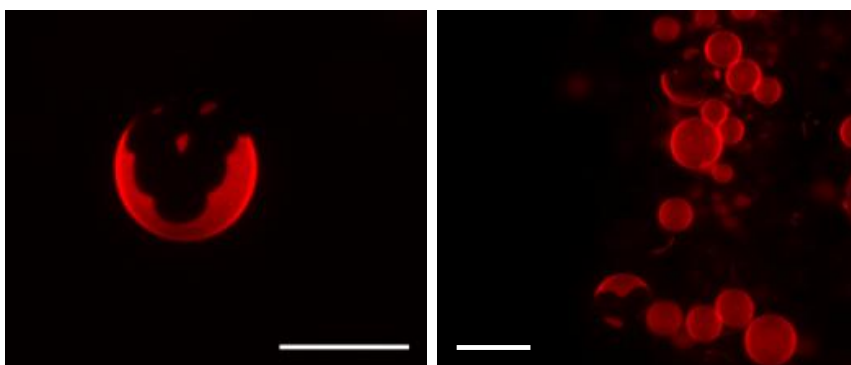
PC:Chol (95:5) + 30 additional mol% Cer:dhCer (6:1)**PC:Chol (95:5) + 30 additional mol% Cer:dhCer (2:1)**

Figure 5.4. Effect of the C16Cer:C16dhCer ratio modification in the formation and morphology of ceramide domains. Upper panel: Ceramide rigid domains in bilayers containing PC (a fluid phospholipid), Chol, C16 ceramide, and C16 dihydroceramide (73:4:20:3, final mol ratio). Lower panel: Ceramide rigid domains in bilayers containing PC (a fluid phospholipid), Chol, C16 ceramide, and C16 dihydroceramide (73:4:15:8, final mol ratio). Bars: 10 μ m.

5.3.2 Vesicle Contents Leakage Assays

Next we analyzed whether the domains observed in ceramide-enriched vesicles could contribute to membrane destabilization. Changes in membrane stability of vesicles can be determined by measuring the release of the vesicles aqueous contents. This technique measures the ability of individual molecules to permeabilize the vesicle lipid bilayer. In the present study, the permeabilising effects of ceramide and dihydroceramide were tested in LUVs by following the externalization of the well-known low molecular weight and water-soluble fluorescent dye ANTS and its quencher DPX.

As mentioned in the Introduction, ceramide can be synthesized in at least two different ways: (i) *de novo* by condensation of serine and palmitoyl-CoA forming 3-oxosphinganine, dihydroceramide in the final step being oxidized by dihydroceramide reductase to form ceramide by the introduction of a *trans* double bond in position 4-5; and (ii) by the action of enzymes involved in the hydrolysis of SM, called sphingomyelinases (Goñi *et al.*, 2012).

Six types of sphingomyelinases are considered, namely acidic, secretory, Mg²⁺-dependent neutral (integral membrane proteins in mammals and soluble proteins in bacteria), Mg²⁺-independent neutral (cytosolic), alkaline, and bacterial enzymes with both phospholipase C and sphingomyelinase activity. nSMase is involved in several cellular processes including differentiation, cell cycle arrest and programmed cell death (apoptosis) through the generation of ceramide. It has been proposed that mammalian nSMase1 is localized predominantly in microsomal fractions (ER and Golgi) (Tomiuk *et al.*, 2000). Moreover, it has been shown that when bacterial SMase is directed to mitochondria the cell undergoes apoptosis (Birbes *et al.*, 2001). These enzymes have been found in *Bacillus cereus*, *Staphylococcus aureus* (β -toxin), *Listeria ivanovii*, *Leptospira interrogans*, *Cromobacterium violaceum*, *Helicobacter pylori* and *Pseudomonas TK4*. The best known of these enzymes is the nSMase from *B. cereus*. It was the first one cloned in *E. coli* and it is a secreted enzyme of 37 kDa (306 amino acids), which depends on Mg²⁺ and is activated by TNF, ligand-bound Fas, CD-95, oxidized LDL and various chemotherapeutic agents (for a recent review see Goñi *et al.*, 2012).

In this work, ceramides and dihydroceramides were generated enzymatically by the action of *Bacillus cereus* nSMase. Alternatively ceramides and dihydroceramides were added in organic solvents to the preformed liposome suspensions (see **Figure 5.10**).

As a first approach to analyze the effect of acute increases in ceramide and/or dihydroceramide on membrane stability, ceramide and/or dihydroceramide were formed *in situ* in the membrane by addition of bacterial sphingomyelinase at 37°C (**Figures 5.5 and 5.6** and **Tables 5.1 and 5.2**). Tests were performed with 0.3 mM LUV containing different proportions of C16 sphingomyelin and C16 dihydrosphingomyelin to which 0.15 U/mL SMase was added. In all enzymatic assays 2 mM o-phenanthroline, a phospholipase C inhibitor, was added as sphingomyelinase commercial preparations may contain phospholipase C impurities. Phospholipase C induces aggregation and fusion of vesicles composed of PC:PE:Chol (2:1:1, mol ratio) (Goñi & Alonso, 2006).

Fusion induced by SMase is also possible, although the mechanism is probably different from the fusion elicited by phospholipase C.

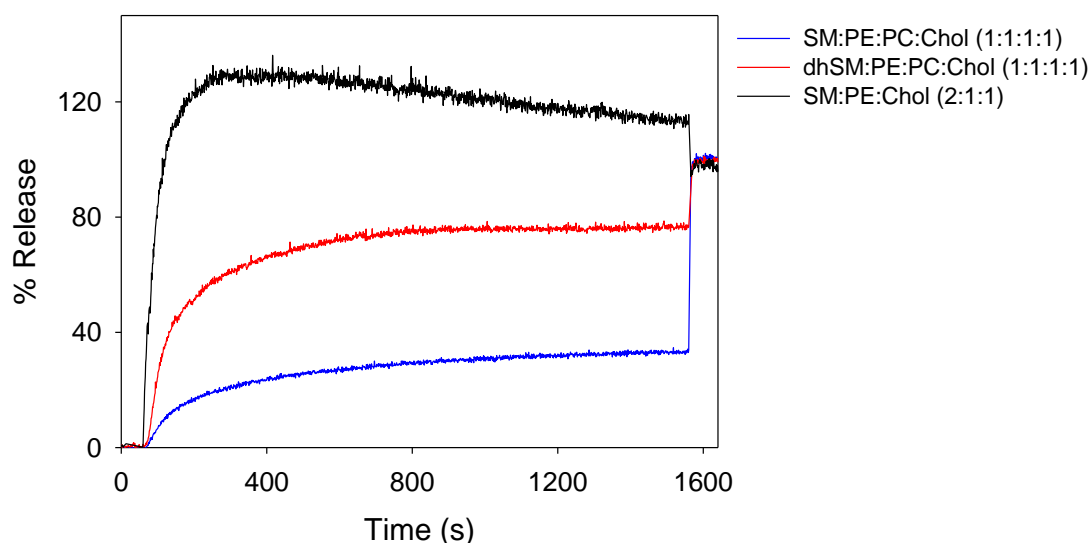


Figure 5.5. Release of vesicular aqueous contents induced by C16 ceramide and C16 dihydroceramide. Ceramides were generated by the action of sphingomyelinase on LUVs composed of SM:PE:Chol (2:1:1, mol ratio), SM:PE:PC:Chol (1:1:1:1, mol ratio); and dhSM:PE:PC:Chol (1:1:1:1, mol ratio). Representative time courses of three independent experiments are shown.

	SM:PE:Chol (2:1:1)	SM:PE:PC:Chol (1:1:1:1)	dhSM:PE:PC:Chol (1:1:1:1)
Initial slope 1	1.90	0.25	0.45
Initial slope 2	1.90	0.20	0.49
Initial slope 3	0.74	0.30	0.59
Mean	1.50	0.25	0.51
SD	0.65	0.052	0.072
Size (n)	3	3	3

Table 5.1. Individual and average values obtained from time courses of *in situ*-generated Cer- and/or dhCer-induced leakage of vesicle contents as shown in Fig. 5.5.

There are few studies regarding the effect of lipids on sphingomyelinase activity, and most of them have used model membranes as substrates. It has been shown that human recombinant SMase activity is enhanced by lysosomal lipids and that sphingomyelinase binding to lipid bilayers is enhanced by certain types of lipids

(anionic) (Goñi *et al.*, 2012). It has been observed that SMase from *B. cereus* is little active on SM bilayers in the gel state. Including egg PC or PE in the composition of the bilayer at 37°C, sphingomyelinase activity (practically zero in pure sphingolipid bilayers) increases, together with a decrease in the transition temperature of sphingomyelin (Goñi *et al.*, 2012). Thus, as glycerophospholipids are added to the membrane composition, the amount of sphingomyelin in the fluid state at 37°C also increases, making easier the enzyme hydrolysis. Therefore, sphingomyelinase requires the substrate to be in bilayer form and in the fluid state for optimum activity. Lateral organization of lipids also influences SMase activity (Fanani *et al.*, 2002) since the enzyme induces phase separation, which changes the surface topography. These topographical changes in turn act as feedback regulators in the kinetics of the enzyme.

After treatment of large unilamellar vesicles composed of SM:PE:Chol (2:1:1, mol ratio), SM:PE:PC:Chol (1:1:1:1, mol ratio) and dhSM:PE:PC:Chol (1:1:1:1, mol ratio) with sphingomyelinase, ceramides were generated in the lipid bilayers resulting from sphingomyelin hydrolysis. Vesicles were packed with soluble fluorescent probes under isotonic conditions, so that probe release could be observed. Probe release occurs simultaneously with the production of ceramides. As shown in **Figure 5.5**, increasing SM, thus ceramide ratio in the bilayer [SM:PE:Chol (2:1:1, mol ratio)], the maximum release increased. This result demonstrates that release is very sensitive to ceramide concentration in the bilayer. Moreover the release of vesicle aqueous contents induced by C16dhCer generated by SMase [dhSM:PE:PC:Chol (1:1:1:1, mol ratio)] was larger and faster than that induced by C16Cer [SM:PE:PC:Chol (1:1:1:1, mol ratio)]. In terms of initial slopes (see **Table 5.1**), SM:PE:PC:Chol mixture exhibited an initial slope of 0.25 ± 0.030 , lower than that of dhSM:PE:PC:Chol 0.51 ± 0.041 ($n = 3$; $*P = 0.019$).

Next, we prepared vesicles with lipid compositions that mimicked the microsomal and autophagosome-enriched fraction of cells treated with THC or EBSS (containing sphingomyelin and dihydro sphingomyelin in the same proportion as those of ceramides and dihydroceramides after treatment with THC or EBSS) (Hernández-Tiedra *et al.*, 2016). Addition of sphingomyelinase to these membranes showed that a higher proportion of dihydroceramides resulted in a more rapid and extensive release of aqueous contents from these vesicles (see **Figure 5.6 and Table 5.2**).

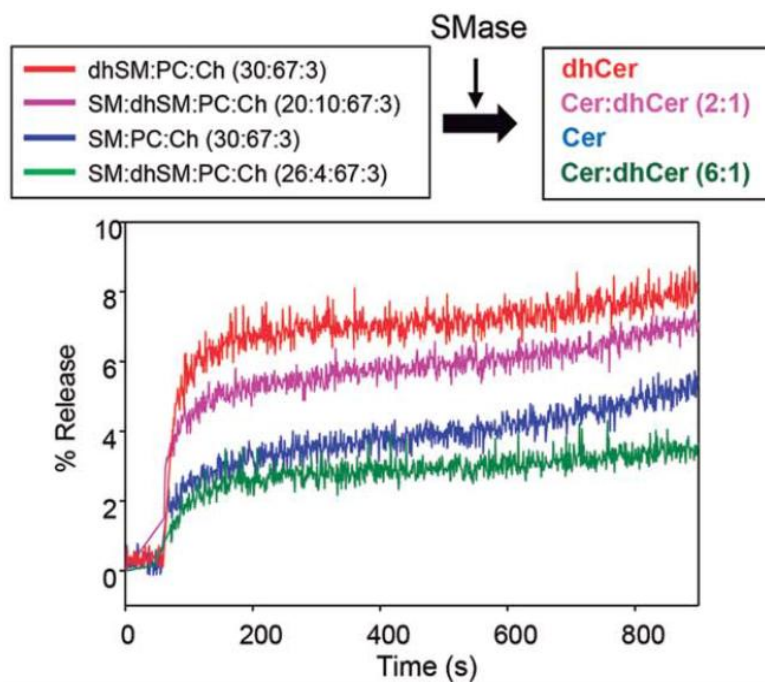


Figure 5.6. Release of vesicular aqueous contents induced by ceramides. Effect of the different proportions of C16 ceramide:C16 dihydroceramide generated by the action of sphingomyelinase on LUVs composed of dhSM:PC:Ch (30:67:3; red); SM:dhSM:PC:Ch (20:10:67:3; magenta); SM:PC:Ch (30:67:3; blue); and SM:dhSM:PC:Ch (26:4:67:3; green). A representative example of 3 closely similar experiments is shown.

	SM:PC:Chol (30:67:3)	dhSM:PC:Chol (30:67:3)	SM:dhSM:PC:Chol (26:4:67:3)
Initial slope (Mean)	0.044	0.25	0.030
SD	0.048	0.021	0.010
Size (n)	3	3	3

Table 5.2. Individual and average values obtained from time courses of *in situ*-generated Cer- and/or dhCer-induced leakage of vesicle contents as shown in Fig. 5.6.

In order to mimic these conditions, we also performed leakage assays by externally adding different amounts of ceramide and dihydroceramide at 37°C (**Figures 5.7 and 5.8**). To 0.3 mM PC:Chol (95:5, mol ratio) LUV 15 mol% and 30 mol% of C16Cer:C16dhCer (83:4:13, mol ratio and 73:4:23, mol ratio) in 6:1 and 2:1 proportions were added dissolved in DMSO or dodecane/ethanol (2:98 v/v). Results of release induced by externally-added ceramides are summarized in **Tables 5.3 to 5.6**. In all cases control experiments (0% ceramide in **Tables 5.5 and 5.6**) were performed in which only the organic solvent was added.

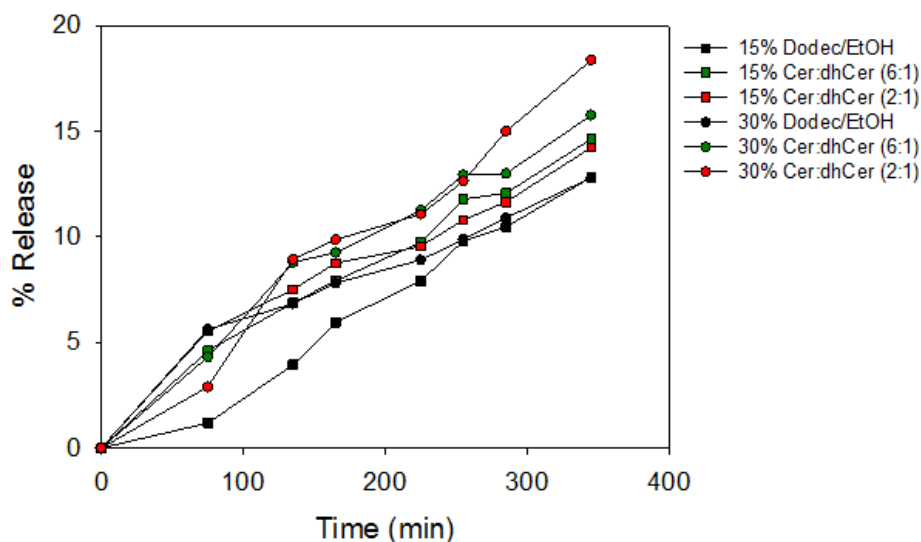


Figure 5.7. Time-course of ANTS/DPX release. Long-chain ceramides were added dissolved in dodecane/ethanol (2:98 v/v) to the preformed PC:Chol (95:5, mol ratio) vesicles.

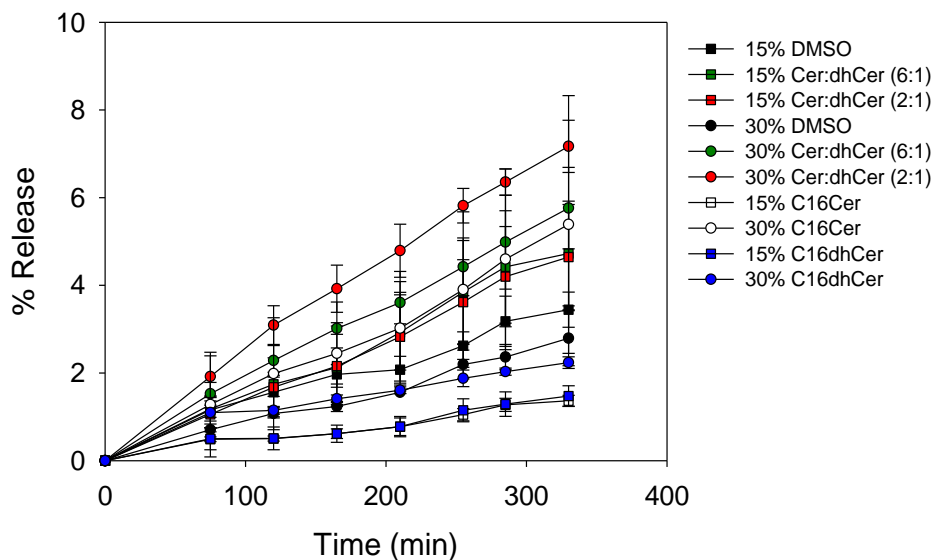


Figure 5.8. Time-course of ANTS/DPX release. Long-chain ceramides were added dissolved in DMSO to the preformed PC:Chol (95:5, mol ratio) vesicles.

When ceramides were added to preformed liposomes, the solvent was an important issue. We observed that dodecane/ethanol caused a high release of contents by itself (**Figure 5.7**). For this reason, DMSO was chosen as a more convenient solvent than dodecane/ethanol to perform this kind of experiments (**Figure 5.8** and **Tables 5.5** and **5.6**).

As shown in **Figure 5.8**, release of ANTS was observed after adding ceramides to bilayers even though at lower rates and extents than in the case of enzymatically-generated ceramides.

Regarding the release caused by externally-added ceramides dissolved in DMSO, it was found that Cer-induced ANTS/DPX release was dose-dependent (**Figure 5.8**). Furthermore mixed ceramides (C16Cer:C16dhCer), both in 6:1 and in 2:1 proportion, appeared to cause higher release than when added in the pure form.

The highest release was found for the samples containing a higher proportion of C16dhCer, i.e. 30 mol% of the Cer:dhCer 2:1 mixture (**Figure 5.8, Tables 5.3 and 5.4**). This confirms the higher permeabilizing ability of dhCer as compared to Cer, in agreement with the results obtained with *in situ* Cer generation (**Figures 5.5 and 5.6**).

Time (min)	15% DMSO	15% Cer:dhCer (6:1)	15% Cer:dhCer (2:1)	15% Cer	15% dhCer
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	1.158 ± 0.322	1.182 ± 0.277	1.056 ± 0.308	0.489 ± 0.055	0.501 ± 0.251
120	1.562 ± 0.345	1.742 ± 0.274	1.675 ± 0.671	0.505 ± 0.058	0.510 ± 0.260
165	1.969 ± 0.222	2.125 ± 0.449	2.157 ± 0.916	0.617 ± 0.109	0.616 ± 0.196
210	2.075 ± 0.306	2.926 ± 0.858	2.827 ± 1.012	0.776 ± 0.200	0.778 ± 0.232
255	2.624 ± 0.315	3.844 ± 1.177	3.618 ± 0.966	1.050 ± 0.140	1.150 ± 0.263
285	3.179 ± 0.573	4.420 ± 1.283	4.198 ± 1.142	1.274 ± 0.151	1.291 ± 0.280
330	3.445 ± 0.401	4.727 ± 1.194	4.643 ± 1.202	1.367 ± 0.125	1.474 ± 0.236

Table 5.3. Vesicle contents leakage induced by externally added 15% Cer or dhCer. Average values ± S.D. (n = 3).

Time (min)	30% DMSO	30% Cer:dhCer (6:1)	30% Cer:dhCer (2:1)	30% Cer	30% dhCer
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
75	0.706 ± 0.048	1.524 ± 0.260	1.917 ± 0.476	1.280 ± 1.195	1.097 ± 0.107
120	1.077 ± 0.102	2.286 ± 0.340	3.093 ± 0.442	1.985 ± 1.278	1.147 ± 0.090
165	1.238 ± 0.115	3.016 ± 0.134	3.923 ± 0.539	2.450 ± 1.170	1.412 ± 0.088
210	1.559 ± 0.028	3.605 ± 0.481	4.790 ± 0.606	3.022 ± 1.295	1.609 ± 0.074
255	2.194 ± 0.327	4.423 ± 0.660	5.816 ± 0.394	3.903 ± 1.773	1.878 ± 0.189
285	2.364 ± 0.288	4.985 ± 1.072	6.354 ± 0.300	4.595 ± 2.061	2.031 ± 0.082
330	2.793 ± 0.628	5.762 ± 0.929	7.170 ± 0.595	5.389 ± 2.940	2.236 ± 0.132

Table 5.4. Vesicle contents leakage induced by externally added 30% Cer or dhCer. Average values ± S.D. (n = 3).

Bilayer composition	Ceramide % in the bilayer	Initial slope (% release/min)
PC:Chol (95:5)	0%	11.5
	15% C16Cer:C16dhCer (6:1)	11.8
	15% C16Cer:C16dhCer (2:1)	10.7
PC:Chol (95:5)	0%	9.6
	30% C16Cer:C16dhCer (6:1)	12.8
	30% C16Cer:C16dhCer (2:1)	15.1

Table 5.5. Release of vesicle aqueous contents induced by ceramides dissolved in dodecane/ethanol. Vesicles contained ANTS/DPX. Total lipid concentration was 0.3 mM.

Bilayer composition	Ceramide % in the bilayer	Initial slope (% release/min)
PC:Chol (95:5)	0%	3.8
	15% C16Cer	1.4
	15% C16dhCer	1.5
	15% C16Cer:C16dhCer (6:1)	5.1
	15% C16Cer:C16dhCer (2:1)	4.5
PC:Chol (95:5)	0%	3.0
	30% C16Cer	5.6
	30% C16dhCer	1.8
	30% C16Cer:C16dhCer (6:1)	5.9
	30% C16Cer:C16dhCer (2:1)	6.5

Table 5.6. Release of vesicle aqueous contents induced by ceramides dissolved in DMSO. Vesicles contained ANTS/DPX. Total lipid concentration was 0.3 mM.

In summary data obtained from contents leakage experiments indicate that the presence of long-chain dihydroceramides induces release of contents through membranes, the release being higher and faster when dihydroceramides are enzymatically produced by SMase than when they are externally added.

Lysosomal membrane permeabilization produces cell death as a consequence of the release of lysosomal proteases to the cytoplasm (Aits & Jäättelä, 2013; Boya & Kroemer, 2008). Therefore, considering the above-described membrane permeabilizing effect of dihydroceramide, Velasco's group investigated whether the cell death promoting activity of THC relies on a sphingolipid-dependent induction of LMP. In line with this idea, treatment with THC produced an increase in cytosolic CTSB and CTSL activity and caused the appearance of CTSB in the cytosol of both U87MG cells and the melanoma cell line SK-MEL-28, these events being prevented by the pharmacological inhibition of *de novo* sphingolipid synthesis (Hernández-Tiedra *et al.*, 2016). Moreover, they found that THC-induced CTSB release was abrogated in U87MG and SK-MEL-28 cells and in oncogene-transformed MEFs in which autophagy had been genetically inhibited (Hernández-Tiedra *et al.*, 2016), indicating that autophagy stimulation is required for THC induced LMP. Collectively these observations suggest

that the increase in dihydroceramide autophagosomal content that triggers THC leads to autolysosomal membrane destabilization, LMP and cathepsin release (see **Figure 5.9**).

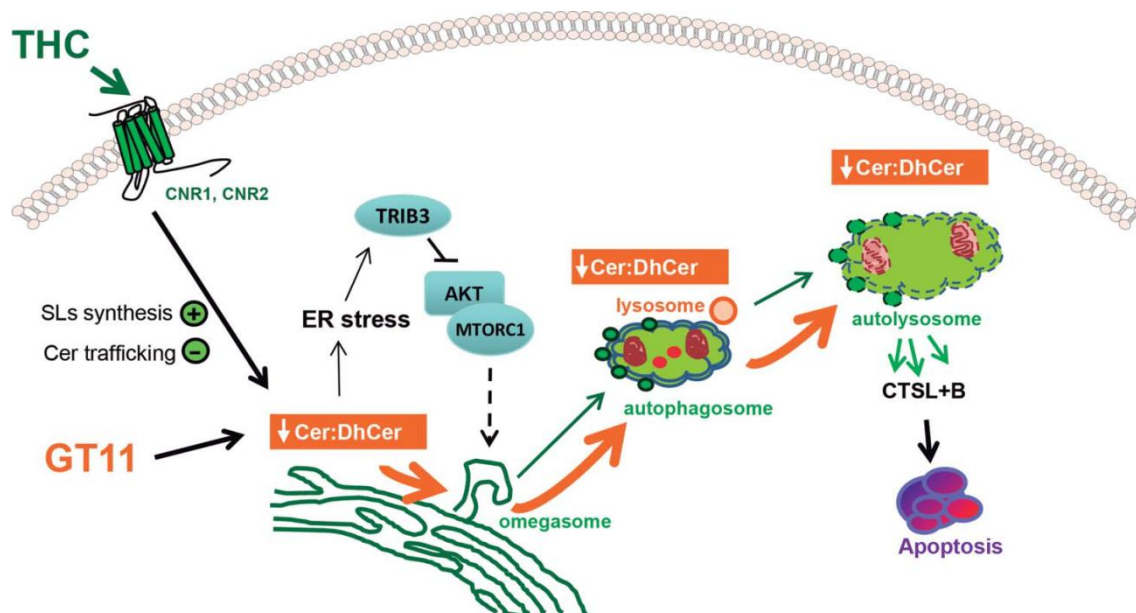


Figure 5.9. Proposed model of a mechanism by which the intracellular increase of dihydroceramide would promote glioma cell death. THC binding to CNR1 and CNR2 stimulates *de novo* synthesis of ceramide and inhibits the transport of ceramide from the ER to the Golgi inducing a modification on the ER sphingolipid composition. This event triggers: (i) the induction of an ER stress response that leads to a TRIB3-dependent inhibition of the AKT-MTORC1 axis and the subsequent induction of autophagy and (ii) a modification of the ceramide to dihydroceramide (Cer:dhCer) ratio in the ER. The DEGS1 inhibitor GT11 produces a similar decrease on the ratio Cer:dhCer. The alteration in the Cer:dhCer ratio triggered by THC or GT11 is transmitted to autophagosomes and autolysosomes, thus modifying the permeability of the membranes, facilitating LMP, cathepsin release and the subsequent activation of apoptosis and cell death (Hernández-Tiedra *et al.*, 2016).

LMP triggers the activation of the mitochondrial apoptotic pathway although it can also lead to necrotic cell death (Aits & Jäättelä, 2013; Boya *et al.*, 2003; Serrano-Puebla & Boya, 2015). In agreement with Velasco and coworkers previous findings showing that autophagy is upstream of apoptosis in the mechanism of cannabinoid induced cell death (Salazar *et al.*, 2009), they found that treatment with THC induced apoptosis and did not lead to a significant increase in necrotic cell death (Hernández-Tiedra *et al.*, 2016). Moreover, THC treatment promoted mitochondrial CYCS release (an event that is closely associated with the activation of the intrinsic apoptotic pathway), an event which was prevented by the pharmacological inhibition of sphingolipid biosynthesis and CTSB activity (Hernández-Tiedra *et al.*, 2016). Likewise, genetic inhibition of autophagy prevented THC-induced CTSB and CYCS release.

Furthermore, pharmacological inhibition of cathepsins prevented THC-induced cell death. Therefore THC-induced autophagy appears to promote LMP and the subsequent activation of the mitochondrial apoptotic pathway in a sphingolipid biosynthesis- and autophagy-dependent manner.

Taken together, the data described in this section support the notion that an increase in *de novo*-synthesized sphingolipids, specifically a decrease in the ceramide: dihydroceramide ratio (similar to that induced by THC in the microsomal and autophagosome-enriched fraction of live cells), leads to the formation of specific membrane domains and to a subsequent destabilization (increased permeability) of the membrane. In collaboration with Velasco and coworkers we found that this is an important factor in the activation of cytotoxic autophagy by THC (Hernández-Tiedra *et al.*, 2016) and therefore pharmacological manipulation of dihydroceramide levels could be used as a strategy to stimulate autophagy-mediated *in vivo* cancer cell death.

5.3.3 LC3B Interaction with Ceramide-containing Membranes

Cer18 has been recently reported as a receptor for anchoring LC3-II to mitochondrial membranes, through direct interaction of Cer18 with LC3-II, but not with LC3-I (Sentelle *et al.*, 2012; Dany & Ogretmen, 2015). Based on these data, we decided to study the molecular mechanisms of Cer-LC3B interaction which leads to LC3B-II autophagosomes targeting to mitochondrial membranes. For this purpose, the binding of the soluble form of LC3B to ceramides with different fatty acid chain lengths (see **Figure 5.10**) was examined using the flotation assay, in which lipids maintain a certain bilayer curvature and could form domains.

We observed that LC3B-I did not interact with membranes containing ceramide (**Figure 5.11**). Thus while LC3 lipidation appears to be important for Cer18 interaction, it is not required for LC3B binding to CL (see Chapter 3). This may suggest that ceramide interaction involves the central hydrophobic domain of LC3, which has similarities to the CERT domain that binds C16- and C18-ceramides, while CL would bind the N-terminal domain, as predicted by docking analysis (Sentelle *et al.*, 2012; Chu *et al.*, 2013; Antón *et al.*, 2016). Moreover, LC3B-I binding was not detectable neither with Cer18 nor with Cer16 or Cer24:1 (**Figures 5.10 and 5.11**).

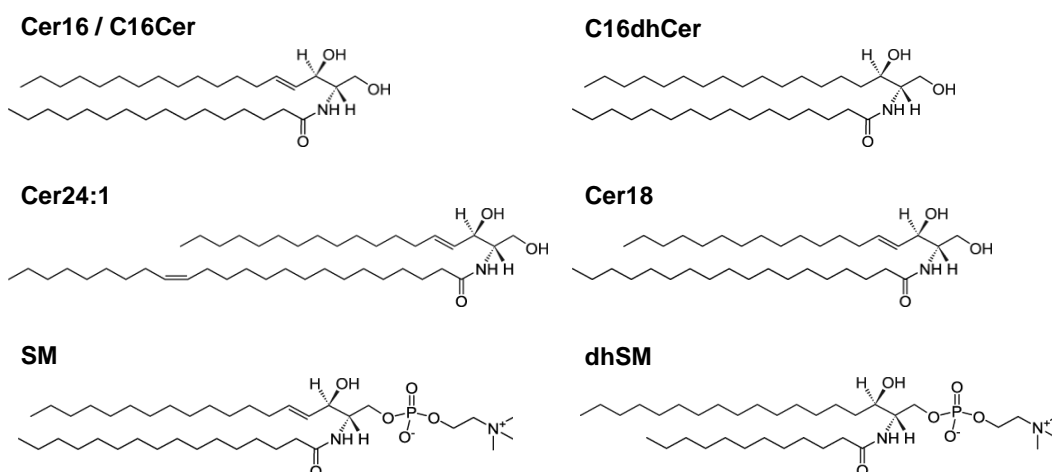


Figure 5.10. Structure of sphingolipid metabolites used in this thesis. Cer16 or C16Cer, N-palmitoyl-D-erythro-sphingosine; Cer18, N-octadecanoyl-D-erythro-sphingosine; Cer24:1, N-nervonoyl-D-erythro-sphingosine; C16dhCer, N-palmitoyl-D-erythro-sphinganine; SM, sphingomyelin from egg chicken (systematic name of predominant species, N-palmitoyl-D-sphingomyelin); dhSM, Dihydrosphingomyelin (representative structure of commercial C12dhSM, N-lauroyl-D-erythro-sphinganylphosphorylcholine). Note that dhSM used in this work was synthesized by G. Fabrias and was 86% C16dhSM. Images provided by Avanti Polar Lipids.

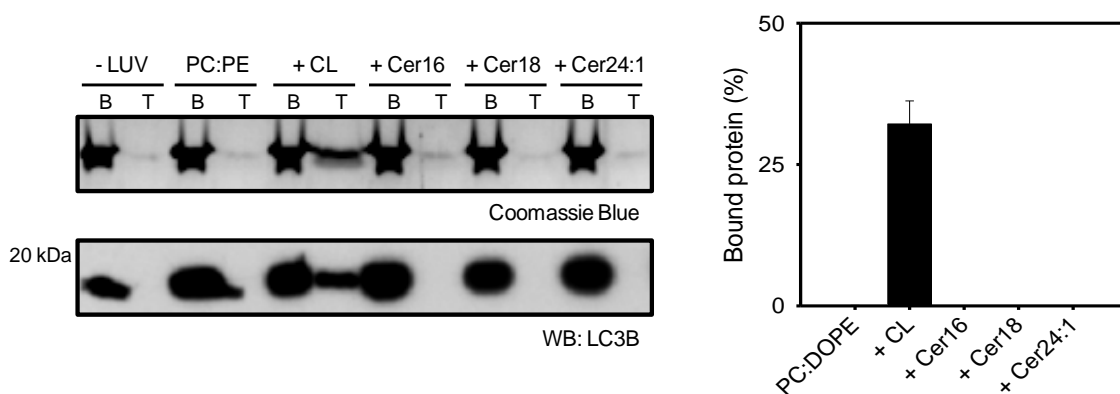


Figure 5.11. LC3B binding to ceramides of different fatty acid chain lengths. 10 μ M LC3B was incubated with 3 mM LUVs composed of PC:DOPE (80:20 mol ratio), PC:DOPE:CL, PC:DOPE: Cer16, PC:DOPE: Cer18 or PC:DOPE: Cer24:1 (50:20:30 mol ratio). The liposome-bound protein fraction was analyzed after flotation in a sucrose density gradient by SDS-PAGE/immunoblot analysis and quantified by densitometric integration of the dots. Molecular mass is shown in kDa on the left-hand side. Data shown as mean \pm SEM from at least 3 independent experiments.

5.4 Discussion

Velasco and coworkers described previously that cannabinoid action (specifically THC) induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells (Salazar *et al.*, 2009). In agreement with the notion that autophagosomal membranes are derived, at least in part and under many cellular settings, from the ER (Juhász & Neufeld, 2006; Axe *et al.*, 2008), and that the enzymes involved in the synthesis of ceramides are located in this organelle, they have also found that changes induced by THC in the ER sphingolipid composition are transmitted to the autophagosomes during the process that gives origin to the phagophore/omegasome, and, in turn, to the autolysosome (Hernández-Tiedra *et al.*, 2016).

To investigate the molecular mechanisms that determine the cytotoxic outcome of this autophagy activation, in collaboration with the above group, we constructed a model system for autophagy-mediated cancer cell death in which we compared the effect of different ratios of ceramide and dihydroceramide that are modified after THC administration. Our findings show that THC-triggered changes in the ER sphingolipid composition (especially the increase in dihydroceramide:ceramide ratio) play a crucial role in the stimulation of autophagy-mediated cancer cell death by THC. Specifically, data support the hypothesis that dihydroceramide-promoted destabilization of autolysosomes is based on their ability to (i) form dihydroceramide and dihydroceramide-rich regions that melt at temperatures well above those generated by ceramide, (ii) form rigid domains, and (iii) cause release of vesicular aqueous contents that is more extensive and faster than that induced by ceramide (Hernández-Tiedra *et al.*, 2016). However, the precise regulatory mechanisms by which binding of THC to cannabinoid receptors triggers these changes in the sphingolipid metabolism of cancer cells have not been clarified as yet.

In parallel ceramide has also been proposed to induce lethal mitophagy, anchoring LC3B-II-autophagosomes to mitochondrial membranes, leading to tumor suppression (Sentelle *et al.*, 2012; Dany & Ogretmen, 2015). Based on this observation we performed experiments in order to characterize the association of the autophagy protein LC3 with ceramide-containing membranes (Antón *et al.*, 2016).

5.4.1 Dihydroceramide Accumulation Leads to Autolysosome Destabilization during Lethal Autophagy

In this set of experiments we examined in more detail the observation that, in cannabinoid-induced autophagy and subsequent apoptosis, the proportion of dihydroceramides (lacking the 4-5 double bond of sphingosine) was considerably increased (Salazar *et al.*, 2009; Hernández-Tiedra *et al.*, 2016). Since apoptosis is accompanied by the release of proteins, specifically cathepsins, to the cytosol, it was tempting to test the capacity of dihydroceramides, as compared to that of ceramides, to increase membrane permeability. The above authors had observed a change in the ceramide/dihydroceramide ratio from 6:1 to 2:1 in the cannabinoid-induced autophagy-apoptosis. Thus a study was warranted comparing the membrane properties of ceramides and dihydroceramides, in particular their respective effects on membrane permeability (Hernández-Tiedra *et al.*, 2016).

Contents release assays described in this chapter show that, apart from ceramides, dihydroceramides also induce reorganization of the lipid bilayers, whatever the method used to insert them into the membrane (**Figures 5.5 to 5.8** and **Tables 5.1 to 5.6**). However there are significant differences in the kinetics and release rate, depending on the experimental procedures used. Therefore there are two main issues in this context: the mechanism of membrane restructuring and the concordance between different experimental methods.

When measuring release some methodological aspects must be taken into account. For example, dodecane/ethanol produces more efflux than DMSO probably for reasons related to the solubility of ceramides (**Figures 5.7 and 5.8**). But even in controls release is higher by using dodecane/ethanol as solvent. Moreover, the fact that ceramides generated by the action of sphingomyelinase results in a greater and more rapid release as compared with ceramides introduced in an organic solvent is also important (**Figures 5.5 and 5.6**). This, coupled with the large release observed at higher proportions of dihydroceramide suggests that the dihydroceramide enzymatic production is a localized process that occurs whenever a sphingomyelinase molecule binds to the membrane and goes along the lag phase (Ruiz-Argüello *et al.*, 2002). The rapid hydrolysis generates a dihydroceramide-rich microdomain and therefore an inter-domain interface through which the release occurs. In contrast addition of dihydroceramides in an organic solvent would result in a more regular distribution of the lipid in the bilayer and thus release would be a secondary process which would occur as a result of lateral segregation of dihydroceramides and domain formation.

An important mechanism that explains release is the lateral segregation of ceramide-rich domains in the membrane plane (Huang *et al.*, 1996). Ceramides have melting temperatures much higher than SM (80°C versus 40°C), so at 37°C, dihydroceramide-rich domains constitute rigid isolated islands in a fluid lipid sea (Goñi & Alonso, 2009). This fact has been corroborated in this work, both with calorimetric studies of ceramide and dihydroceramide mixtures and with GUV images in which dihydroceramide domains were observed with morphology similar to those formed by ceramide (**Figures 5.1 to 5.4**). It is known that the coexistence of gel and fluid domains is a condition that allows release to occur (Ruiz-Argüello *et al.*, 1996). Therefore, the appearance of interfaces between dihydroceramide-rich and -poor regions could be responsible for the observed release of vesicle contents. The fact that release induced by enzymatically-generated dihydroceramides is much faster supports the hypothesis that the lateral segregation of dihydroceramide-rich domains is one of the causes of release.

Local changes in the concentration of different species of sphingolipids (and specifically of ceramides) produce membrane permeabilization through the formation of rigid structures in biological membranes (Goñi *et al.*, 2014; Vieira *et al.*, 2010). Data presented in this section show now that an increase in dihydroceramide proportion strongly enhances this effect. Moreover from the above results using model vesicles, the conclusion can be reached that a local increase in the dihydroceramide:ceramide ratio (similar to that induced by THC in the microsomal and autophagosome-enriched fraction of U87MG cells) leads to the formation of specific membrane domains and to increased permeability of biological membranes (Hernández-Tiedra *et al.*, 2016). Membrane restructuring produced by dihydroceramides could be explained taking into account their tendency to induce lateral segregation. From this point of view the release of vesicular solutes would not occur through well-structured channels but preferably through transient and irregular interfaces between dihydroceramide-rich and -poor domains or through local points of destabilization. It has been recently shown that manipulation of the activity of SMPD1 (acid lysosomal; a hydrolytic enzyme located primarily in the lysosomes) leads to LMP and stimulation of cancer cell death (Aits & Jäättelä, 2013; Petersen *et al.*, 2013), suggesting that changes in the sphingolipid composition of lysosomes can affect the stability of this organelle. Results obtained in collaboration with Velasco's group now show that autophagy is required for THC-induced LMP and support the idea that the fusion of dihydroceramide-enriched autophagosomes with lysosomes leads in turn to a local increase in the proportion of dihydroceramides in specific subdomains of autolysosomes and lysosomes, thereby

leading to membrane destabilization, LMP and the subsequent release of cathepsins into the cytoplasm of cancer cells.

5.4.2 LC3B-Ceramide Interaction for Mitochondrial Targeting during Lethal Mitophagy

It has been shown that the selective targeting of mitochondria by LC3B-II-containing phagophores occurs through direct interaction between ceramide and LC3B-II (Sentelle *et al.*, 2012; Dany & Ogretmen, 2015). The globular domain of LC3B was found to be structurally similar to the ceramide-binding domain of COL4A3BP44 (that can also bind C16dhCer) (Kumagai *et al.*, 2005). It is therefore tempting to speculate that, in addition to regulating membrane stability and the activity of the above-described ER stress-related signaling pathway, local changes in the content and subcellular distribution of C16dhCer or other dihydrosphingolipids might be able to modulate autophagy via selective binding to LC3B or other autophagy regulatory proteins.

Here using several compositionally defined *in vitro* reconstituted systems we show that the soluble form of LC3B does not interact with ceramide (**Figure 5.11**). These results are consistent with the molecular docking analyses of LC3 upon binding to Cer-containing membranes implicating LC3 N-terminal, opposite to where PE is conjugated, in the interaction of the protein with Cer (Sentelle *et al.*, 2012). Indeed, docking simulations indicate that conjugation of LC3-I to PE hides a low-affinity ceramide-binding sites allowing ceramide to bind selectively to the opposite end of the protein. Thus, as observed for CL (see Chapter 3), the C terminus of LC3 would be crosslinked to the autophagosome through the conserved glycine residue after protein binding to the outer mitochondrial membrane. Furthermore, we show that association of soluble LC3B with ceramides does not change with the ceramide fatty acid chain length (**Figures 5.10 and 5.11**). This is in agreement with the proposed importance of the mitochondrial localization of endogenous ceramides, and not their fatty acid chain length *per se*, to determine their distinct biological actions during mitophagy (Sentelle *et al.*, 2012).

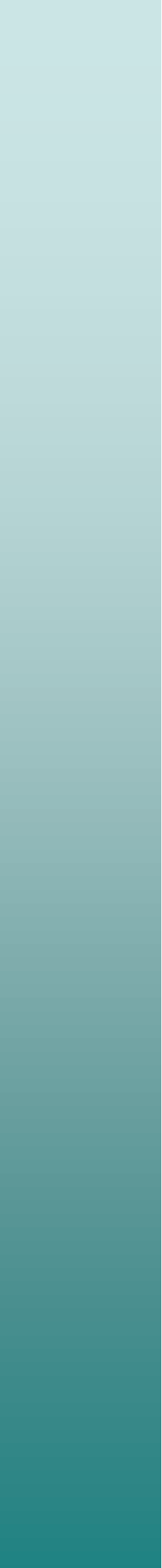
As mentioned above, THC increases sphingolipid synthesis in the ER, eventually contributing to autophagy-mediated cancer cell death (Salazar *et al.*, 2009; Hernández-Tiedra *et al.*, 2016). It is worth noting that ceramides had been previously implicated in autophagy-associated cell death via induction of lethal mitophagy (Sentelle *et al.*, 2012; Dany & Ogretmen, 2015). THC-induced autophagy-mediated

LMP also leads to cell death via stimulation of the mitochondrial apoptotic pathway rather than necrotic cell death (Hernández-Tiedra *et al.*, 2016). However, a significant increase in mitophagy upon treatment with THC was not found indicating that this mechanism is not responsible for the stimulation of autophagy-mediated cell death in response to treatment with this cannabinoid. These findings are in agreement with previous results showing that autophagy is upstream of apoptosis in the mechanism of cannabinoid induced glioma cell death (Salazar *et al.*, 2009) and with the notion that LMP can activate apoptosis (Aits & Jäättelä, 2013; Boya *et al.*, 2003). Moreover, mitochondrial localization of endogenous ceramides and previous lipidation of LC3 have been suggested to be essential for LC3-ceramide association during mitophagy (Sentelle *et al.*, 2012), the latter being in agreement with our experimental data (Antón *et al.*, 2016). In the previous chapter we have also shown that THC induces potent bulk autophagy but no mitochondria were targeted to degradation in U87MG cells (Antón *et al.*, 2016). THC-induced, autophagy-mediated cell death is mediated by ceramide accumulation which stimulates the ER stress-related signaling route (Salazar *et al.*, 2009; Hernández-Tiedra *et al.*, 2016). However, during bulk THC-promoted autophagy, direct LC3 interaction with ceramide has not yet been described.

In summary, findings described in this chapter support the concept that the stimulation of autophagy-mediated cancer cell death by THC relies on a modification of the sphingolipid composition of the endoplasmic reticulum of glioma cells that is transmitted to autophagosomes and autolysosomes thereby leading to lysosomal membrane permeabilization, cathepsin release and the subsequent activation of apoptotic cell death (Hernández-Tiedra *et al.*, 2016) (**Figure 5.9**). Moreover, we have described novel molecular mechanisms underlying human LC3B interaction with ceramide that regulate lethal mitophagy, leading to tumor suppression (Antón *et al.*, 2016). We consider that these observations further support the biological relevance of sphingolipid metabolites in the regulation of autophagy and emphasize the potential therapeutic implications of modulating the levels of dihydrosphingolipids such as dihydroceramides for the treatment of cancer. Furthermore, defining the molecular details of sphingolipid-protein binding such as the putative ceramide-LC3B-II complex, will help to understand the mechanisms by which ceramide regulates lethal versus survival autophagy.

6. Kapitulua

Laburpena eta Ondorioak



6. Kapituluua:

Laburpena eta Ondorioak

Laburpena

Ugaztun zeluletan autofagia ausazko edo aukeratutako material zitoplasmatikoen eta organuluuen degradaziorako bidezidor lisosomiko nagusia da (Mizushima eta Komatsu, 2015; Mochida *eta lank.*, 2015). Gero eta gaixotasun gehiago autofagiaren inhibizio edo faltarekin lotuta egoteak, adib. gaixotasun neurodegeneratiboak (Nixon, 2013; Aufschnaiter *eta lank.*, 2016), autofagiak prozesu fisiologiko askotan funtsezko eginkizunak dituenaren seinalea da. Eginkizun horietako bat elikagaiak eta aurrekari biosintetikoak hornitzea da (Green eta Levine, 2014; Marino *eta lank.*, 2014). Gainera, prozesu hau ez dago soilik zelulen biziraupenarekin lotuta, baita heriotza zelularrarekin ere (Galluzzi *eta lank.*, 2015), honen adibide da autofagia bidezko minbizi-zelulen heriotza (Salazar *eta lank.*, 2009; Velasco *eta lank.*, 2012).

Autofagian, isolatze-mintz edo fagoforo izenez ezagutzen den mintz bikoitzeko egitura bat eratzen da. Horren ostean, mintz egitura gehiago lotu eta fusionatuko liriteke eta proteina erregulatzailleen errekrutatze sekuentziala gertatuko litzateke, autofagosoma izeneko organulua eratzeko (Shibutani eta Yoshimori, 2014). Autofagosoma gero lisosomekin fusionatzen da, edukiaren degradazio eta birziklapenerako (Klionsky *eta lank.*, 2012; Shen eta Mizushima, 2014). Giza LC3/GABARAP familiako proteinak eraldatuak dira autofagosomaren mintzan PS edo PE lipidoekin elkartzeko (Kabeya *eta lank.*, 2000; Tanida *eta lank.*, 2002). Proteina hauek, autofagosomaren eraketarako eta degradatu beharreko materialaren aukeraketarako ezinbestekoak dira (Shpilka *eta lank.*, 2011; Ktistakis eta Tooze, 2016).

Antzekotasun estruktural handia izan arren, LC3/GABARAP familiako kideek autofagosomarekiko konjugazioan (estresak aktibatutako erregulazioa, besikularen kurbaduraren menpekoea), lotura-bikoteetan, adierazpenean (transkripzioaren erregulazio bereizgarriaren bidez) eta itzulpen-osteko erregulazioan desberdinak dira, ondorioz, bakoitzak funtzio espezifikoak betetzen ditu (Landajuola *eta lank.*, 2016; Alemu *eta lank.*, 2012; Schaaf *eta lank.*, 2016).

Mitofagia bidezidor autofagiko hautakor bat da, zeinetan zelulek mitokondrioak birziklatzen dituzte kalitate kontrolerako eta beharizan metaboliko aldakorrei moldatzeko (Hamacher-Brady *eta* Brady, 2016; Randow *eta* Youle, 2014). Mitofagia akastunak, mitokondrio kaltetuen eta proteina agregatuen pilaketa eta ROSen gehiegizko sorrera eragiten du, ondorioz, mota askotako baldintza patologikoak eraginez, horien artean daude neurodegenerazioa, adib. Parkinson-en gaixotasuna (Aufschnaiter *eta lank.*, 2016; Vidal *eta lank.*, 2014), minbizia (Lu *eta lank.*, 2013) eta miopatiak (Kubli *eta* Gustafsson, 2012). LC3/GABARAP familiako proteinek, kaltetutako mitokondrioen ezagutzean eta ezabapenean ezinbesteko funtzioa betetzen dute, proteina egokitzaille edo lipido espezifikoekiko elkarrekintzen bidez (Hsu *eta lank.*, 2015; Maguire *eta lank.*, 2016). Azkenaren adibide dira kardiopolipina eta zeramida, zeinek mitokondrioaren mintzan LC3Brentzat degradaziorako-materialen hartzaile gisa jarduten dute, azkenik mitokondrio kaltetuen ezabapena hautakorra eraginez (Chu *eta lank.*, 2014; Dany *eta* Ogretmen, 2015).

Hala ere, mitofagian eta autofagia bidezko heriotza zelularrean zeharreko LC3/GABARAP proteinen errekrutatzean kaltetutako mitokondrioetara, zenbait lipidoen inplikazio-maila eta erregulazio mekanismo espezifikoak oraindik argitu gabe daude. Hori dela eta, tesi lan honetan, mitofagiaren eta autofagia hilgarriaren bidezidorretan zehar, degradaziorako-materialaren hartzaile gisa, lipido espezifikoaren garrantzia eta oinarri molekularren, eta LC3/GABARAP familiako kide bakoitzaren mitokondriarako errekrutamendua gertatzeko mintzaren ezaugarrietan daukaten eraginaren inguruko ezagumenduan aurrerapausuak eman nahi izan dira. Helburu horretarako, teknika biofisiko kuantitatiboak, lipido-besikulak erabiliz, eta biologia zelularreko teknikak aplikatu izan dira, (i) Atg8aren giza ortologo desberdinen kardiopolipinarekiko elkarrekintza fisikoa ikertzeko, eta elkarrekintza horiek mintzaren testuinguruan daukaten ondorio estruktural eta funtzionalak zehazteko, (ii) rotetonak eragindako mitofagian zehar, LC3/GABARAP proteina bakoitzak mitokondrioetara erakarriak izateko mekanismo erregulatzaile gisa, elkarrekintza honen eginkizuna azaltzeko eta (iii) THCK aktibatutako minbizi-zeluletako heriotza autofagikoan, zeramidek izan

dezaketen jokaera deskribatzeko eta mitofagiak gauzatutako tumore supresioan zeharreko LC3B-zeramida elkarrekintza aztertzeko.

CLaren OMMrako Kanporaketaren osteko LC3/GABARAP Familiako Proteinen Mitokondriorako Errekrutazea Estresarekiko Espezifikoa eta Zelula Motaren Araberakoa da

Eukarioto garatuetan Atg8 proteina familiak kide ugari izateak, autofagia hautakor eta ez-hautakorrean zehar funtzio bereizgarriak eta degradaziorako materialaren aukeraketa ahalbidetzen ditu (Stolz *eta lank.*, 2014; Schaaf *eta lank.*, 2016). Ikerketa estrukturalak, LC3/GABARAP familiako kideetan, zenbait elkarrekintza bikote edo proteina hartzaileekin lotzeko lehentasuna zehazten duten ezaugarri espezifikoek garrantzia azpimarratu dute (Alemu *eta lank.*, 2012; Joachim *eta lank.*, 2015). Mitofagiaren bidezko mitokondrioen ezabapena hautakorrean, zenbait proteina erregulatzailerik hartzaile espezifiko gisa jarduten dute, horrela, Atg8aren ortologoak mitokondrioetara erakarriz eta mitokondrioak degradaziora eramanez. Mintz mitokondrialaren depolarizazioaren ostean, bide alternatibo bat ere aktibatua izan daiteke, zeinetan PINK1ek espezifikoki PARK2 kaltetua dagoen mitokondriora erakartzen duen (Lazarou *eta lank.*, 2015). Gainera, zenbait ikerketetan deskribatu den moduan, mitokondrioen autofagia hautakorrean zehar, zenbait lipidok, degradatu beharreko materialaren hartzaile gisa jarduten dute (Chu *eta lank.*, 2013; Sentelle *eta lank.*, 2012). Horien artean, elektroien garraio kateko 1-konplexuaren inhibitzailea den rotenonak, kardiolipinaren OMMrako kanporaketa eragiten duela erakutsi da. Kanporatutako kardiolipina honek, LC3rentzako seinale gisa jardun eta kaltetuta dauden mitokondrioen hautaketa eta ezabapena eragiten du (Chu *eta lank.*, 2013). Agente despolarizatzaile indartsuek ez bezala, adibidez CCCP (Kondapalli *eta lank.*, 2012), rotenonak ez du mitokondrioren mintz potentziala nabariki aldarazten eta PINK1/PARK2 bidezko mitofagiarekiko independentea da (Chu *eta lank.*, 2013).

Tesi lan honetan aurkeztutako LC3B-CL elkarrekintzaren inguruko emaitza kuantitatiboak erakusten dutenez, LC3Bk lipido di-anionikoen artean CLrekin elkarreragiteko lehentasuna dauka eta LC3B-CL elkarrekintza, indar elektrostatikoetan (**3.5–3.12 Irudiak, eta 3.14 Irudia**), baita CLk mintzean eragindako aldaketa espezifikoetan ere oinarritzen da. Adibidez, CL daukan asegabetasun maila altuak, mintzak elastikotasun handia edukitzea eragiten du, honek, LC3B mintzean lotzea eta txertatzea ere erraztuko luke (**3.8F Irudia**). Gainera, LC3Bren gainazaleko poltsiko

basikoetan dauden zenbait hondar, elkarrekintzarako ezinbestekoak direla aurkitu da (**3.16 Irudia**), proteinak duen batzeko gaitasuna modulatzeko dutelarik (**3.10 Irudia**). NBDren markaketa guneratuak, NBDren fluoreszentsian oinarritutako analisi espektroskopikoekin batera, LC3Bren N- eta C-muturreko eskualdeak mitokondrioaren eta autofagosomaren mintzetara, hurrenez hurren, lotuko litzatekeela deskribatzen duen eredu bat indartzen dute (**3.9 Irudia**) (Chu *eta lank.*, 2013; Antón *eta lank.*, 2016). Gainera, LC3B eta CL duten mintzen arteko elkarrekintza ingurune azido batean erraztua izango litzateke (**3.8D Irudia**), hau (i) pH zitოსolikoaren jaitsiera lokala eragingo lukeen kaltetutako mitokondrioen ATP sintasaren inhibizioaren, (ii) azido laktikoaren hartiduran zehar aktibatutako mitofagiaren, (iii) LC3ren karga positiboagoa bilakatzearen, edo (iv) CLan aberastutako mikrodomeinuen sorreraren ondorioz gertatuko litzateke (Lemmin *eta lank.*, 2013; Khalifat *eta lank.*, 2011). Gainera, LC3Bk, CL daukaten besikula txiki eta kurbatuagoetara lotzeko lehentasuna dauka (**3.8E eta 3.13B Irudiak**), hau, mitokondrioen zati txikiagoak modu eraginkorrago batean ezagutzeko mekanismo erregulatzailerik bat izango litzateke. Mekanismo honetan, autofagosomen bidezko degradazioa errazteko, mitokondrioak maneiagarriagoak diren zatietan bihurtuak izango lirateke (Twig *eta lank.*, 2008).

Aipatutako emaitzekin bat etorritik, U87MG giza glioma zeluletan, RPE1 zeluletan eta iPSC-tik deribatutako neurona kortikaletan, rotenonak eragindako CLaren OMMrako translokazioak (Chu *eta lank.*, 2013), LC3B mitokondrioetara translokatzeko eta ondoren mitokondrioak lisosometara eramanak izatea eragiten du. Hau ez da gertatzen ordea ausazko autofagia aktibatzen duten THC edo AZD8055 tratamenduen edo elikagaien gabeziaren eraginez (**4.1, 4.3, 4.4, 4.16 eta 4.17 Irudiak**). Fluoreszenteski markatutako LC3B era egonkor batean adierazten duten rotenonarekin tratatutako RPE1 zelulen analisi ultraestrukturalak CLEM teknika erabiliz, egitura autofagosomiko ugari inguratutako mitokondrioak erakutsi zituen. Honek, mitokondrioen hautaketa eta ezabapena, eta autofagosomen arteko fusio gertaerak ematen ari zirela adierazten zuen (**4.11 eta 4.13 Irudiak**). EM tomografia edota fluoreszenteski markatutako CL erabiliz, LC3 mitokondrioetara lotzearen ondorioz gertatu daitekeen mitokondrioen mintzaren birmoldaketaren ultraestruktura eta CL mikrodomeinuetan antolatua egoteko aukera ikertu litezke. RPE1 zelulak rotenona eta proteasa lisosomalen inhibitzailea den leupeptinarekin egindako kotratamenduak, LC3Bekin markatutako egitura lisosomiko ugarien eraketa eragiten du (**4.14 eta 4.15 Irudiak**). Hala ere, giza RPE1 zeluletan, ATG5ekin markatutako mihizadura guneak edo isolatze-mintzak mitofagiaren aktibazioaren ostean ez dira mitokondrioetan kokatzen (**4.6 eta 4.9 Irudiak**). Emaitza hauek, deskribatutako LC3Baren forma

zitosolikoaren eta CLan aberastuta dauden besikulen arteko elkarrekintzarekin batera (**3.5–3.12 Irudiak**), CLren OMMrako translokazioaren osteko LC3Bren errekrutamendu mitokondrialerako, aurretik LC3Bren lipidazioa gertatzea ez dela beharrezkoa iradokitzen dute. Prozesuaren urrats desberdinekiko espezifikoak diren fluoreszente-markatutako autofasosomaren beste markatzaile batzuen kokapen intrazelularrak, mitokondrioetarako faktore autofagikoen erakarpen sekuentziala eta baita LC3ren errekrutatzea autofagosomaren eraketaren aurretik edo ostean gertatzen den argituko luke. Gainera, esperimentu gehiagok ATG5 basatiarekin (ingelesez *wild type*) berreskuratutako ATG5-gabeko zeluletan, ATG12rekin lotu ezin diren eta autofagosomaren eraketan huts egiten duten ATG5 mutanteekin, LC3ren lipidazioa/delipidazioa saihesteko ATG4ren forma mutanteekin, edo ATG7 siRNArekin tratatutako zeluletan, zeina LC3 PErakin elkartzeko beharrezkoa den, hipotesi hau egiaztatzen lagunduko lukete. Autofagosomaren eraketarako ezinbestekoak diren faktore horien maila gutxituak edo forma mutanteak dauzkaten zeluletan LC3Bren kokapen zitoplasmatikoak, LC3ren lipidazioak rotenonak eragindako mitofagian funtsezko eginkizuna duela adieraziko luke.

LC3/GABARAP familiako proteinek, LIR daukaten proteina ezberdinen errekrutatze hautakorrerako espezifikotasun desberdinak izateaz gain (Weidberg *eta* *lank.*, 2010) lipidoak lotzeko afinitatean ere desberdinak dira. Izan ere, LC3B eta GABARAP proteinek ez bezala, GABARAPL2 nekez lotzen da CL daukaten mintzetara (**3.10–3.16 Irudiak**). Gainera, mitokondrioen ezabapen espezifikoa gauzatzeko, zelula mota guztiek ez dute mekanismo bera jarraitzen. Izan ere, U87MG glioma zeluletan, rotenona bidezko tratamenduaren ostean, LC3Bren kasuan ez bezala (**4.1 Irudia**), ez GABARAP ezta GABARAPL2 ere ez dira mitokondrioan kokatzen (**4.2 Irudia**). Gainera, rotenonaren eraginpean jarritako RPE1 zelulek, erantzun autofagiko ahula erakusten dute baina rotenonak bai LC3 zein GABARAPL1 mitokondrora errekrutatzea eragiten du (**4.3–4.7 Irudiak**). Hala ere, rotenonarekin tratatutako iPSCetatik eratorritako giza neurona kortikaletan, LC3B baina ez GABARAPL1 punteaketa mitokondrioan kokatzen da (**4.17 Irudia**). GABARAPL1ek mitofagian zuzenean laguntzen duen edo autofagiaren aktibitate orokorra modulatz mitofagian eragiten duen sakonago aztertua izan behar da. Bestalde, *in vitro* ikusitako elkarrekintzaren eta mitokondrionako translokazioaren arteko korrelazio biologiko bat soilik LC3B proteinak erakusten zuela kontuan hartuta, LC3 proteinaren A, B eta C isoformen CLrekiko elkarrekintzak eta ondoko mitokondrioen aukeraketa konparatzeak berebiziko informazioa emango luke.

Zeramida Autofagian eta Mitofagia Bidezko Minbizi-Zelulen Heriotzean: Esfingolipidoen Funtzio Berriak ERan eta Mitokondrioan

Esfingolipido ezberdinak, eta zeramidak, dihidrozeramidak eta esfingosina 1-fosfatoa hain zuzen ere, minbizi-zeluletan autofagia erregulatzen dutela proposatu da, batez ere, prozesu zelular hau erregulatzen duten seinalizazio bidezidorren eragile gisa (Jiang eta Ogretmen, 2013; Li *eta lank.*, 2014). Velasco eta lankideek, THC kanabinoideak giza glioma zeluletan ERaren esfingolipido konposaketan aldaketak eragiten dituela erakutsi dute. Aldaketa hauek autolisosometara transmitituak dira eta ERaren estresarekin lotutako bidezidor bat aktibatzen dute, azkenik autofagia bidezko zelulen heriotza eraginez (Salazar *eta lank.*, 2009; Salazar *eta lank.*, 2013). Tesi lan honetan, autofagia bidezko heriotza zelularren sistema-eredu bat erabilia izan da, autofagiaren bidezidor honen eragin zitotoxikoaren gertaera molekularrak ikertzeko. Kanabinoideek aktibatutako autofagia hilgarri honetan zehar dihidrozeramiden proportzioa nahiko igotzen dela, eta hau katepsinen askapenarekin batera datorrela dioten ikerketetan oinarrituz (Salazar *eta lank.*, 2009), tentagarria zen dihidrozeramidek mintzaren iragazkortasuna handitzeko daukaten ahalmena, zeramidek daukatenarekin konparatuz, aztertzea.

Edukien askapen saioek, kanpotik gaineratutako baita *de novo* sintetizatutako zeramidak eta dihidrozeramidak bigeruzak lipidikoaren berrantolaketa eragiten dutela erakutsi dute (**5.5–5.8 Irudiak** eta **5.1–5.6 Taulak**). Hala ere, *in situ* sortutako zeramidek, disolbatzaile organiko batean gaineratutako zeramidekin konparatuz, besikulen eduki urtsuaren askapen azkarragoa eta handiagoa eragiten dute. Bestalde, dihidrozeramidek eragiten duten askapena, zeramidek eragiten dutena baino handiagoa eta azkarragoa da (**5.5–5.8 Irudiak**). Emaidza hauek, esfingomielinasaren ekintzaz sortutako dihidrozeramidak dihidrozeramidan aberatsak diren mikrodomeinuak eratzen dituela iradokitzen dute, eta beraz, askapena gertatzen deneko domeinuen arteko interfaseak. Dihidrozeramidak disolbatzaile organiko batean gaineratzen direnean, ordea, bigeruzan daukaten banaketa homogeenagoa dela eta, lehenengo dihidrozeramiden albo banaketa eta domeinuen eraketa beharrezkoak dira, askapena bigarren mailako prozesu bat izanik (Goñi *eta lank.*, 2014). Bestalde, zeramida eta dihidrozeramida nahasturak dituzten besikula ereduarekin egindako kalorimetria bidezko ikasketek eta mikroskopia konfokalak erakusten dute, dihidrozeramidan aberatsak diren zonaldeen eraketa, zeintzuk zeramidarenak baino tenperatura askoz altuagoetan urtzen diren, eta dihidrozeramidak eragindako domeinu lipidiko zurrunen eraketa, zeramidak eratzen dituenen antzeko morfologia dutenak (**5.1–5.4 Irudiak**). Emaidza hauek, Velasco eta lankideen ikasketekin batera, THCK aktibatutako autofagia bidezko

minbizi-zelulen heriotzean zehar, minbizi-zelulen frakzio mikrosomalean zeramida eta dihidrozeramida espezie molekular ezberdinen arteko oreka aldatzeak, azpidomeinu espezifikoek eraketa eta mintz autolisosomalak iragazkor bihurtzea eragiten duela erakusten dute. Azken gertaera honek, ondorengo minbizi-zelulen zitoplasmako katepsinen askapenean laguntzen du eta kanabinoideek aktibatutako autofagiak zelularen heriotza eragitea zehazten du (**5.9 Irudia**; Hernández-Tiedra *eta lank.*, 2016). Hala ere, THC kanabinoideen hartzailetara lotzeak minbizi-zelulen esfingolipidoen metabolismoan aldaketa hauek eragitea erregulatzen duten ekintza mekanismo zehatzak ez dira oraindik argitu. Bestalde, ikerketa lan honetan erabilitako teknika esperimentalak, dihidrozeramidak masa molekular baxuko molekulen askapena eragiten duela erakusten du. Beraz, hasierako hipotesia egiaztatzeko – hau da, glioblastoma zelulak THCrekin tratatu ostean autolisosometan ematen den katepsinen askapena, ERaren dihidrozeramiden proportzioa handitzearen ondorioz gertatzen dela – katepsina batzuen antzeko masa molekularra daukaten fluoreszeinarekin markatutako dextranoen askapen saioak burutu ahalko litzateke.

Aurreko zenbait ikerketek erakutsi dute, zeramidek autofagiarekin lotutako heriotza zelularren bidez tumoreen supresioan parte hartzen dutela, mitokondrioan autofagosomei lotutako LC3Brentzako degradaziorako-materialaren hartzaile hautakor gisa jokatuz, eta ondorioz, mitofagia bidezko heriotza zelularra eraginez (Sentelle *eta lank.*, 2012; Dany *eta* Ogretmen, 2015). Beraz, aurretik deskribatutako ERaren estresarekin lotutako seinalizazio bidezidorean gertatzen diren eta mintzaren egonkortasuna erregulatzen duten dihidroesfingolipidoen konposaketa eta banaketa azpizelularren aldaketa lokalez gain, minbizi-zelulen erregulazio mekanismo berri bat izango litzateke zeramiden bidezko autofagiaren modulaziorako. Aurretik egindako proteina-lipido elkarrekintza honen hurbilketa molekularren azterketek, proteinaren N-muturra parte hartzen duela iradokitzen dute, autofagosomaren mintzean PE lotzen duenaren kontrako gunea izanik (Sentelle *eta lank.*, 2012). Gainera, aurretiko LC3ren lipidazioa eta zeramida endogenoen kokapen mitokondrialak, baina ez beraien gantz azido kateen luzeera *per se*, mitofagian zeharreko ekintza biologiko desberdin honetarako funtsezkoa izango litzateke (Sentelle *eta lank.*, 2012). Aipatutakoa LC3Bren forma zitostolikoak zeramidekin ez elkarrengaitzatzea, eta gantz azido kateen luzeerak proteina eta zeramida duten mintzen arteko loturan eraginik ez izatearekin bat dator (**5.10 eta 5.11 Irudiak**). Emaitza hauek, LC3B-CL elkarrekintzaren inguruan tesi lan honetan aurkeztutako emaitzekin batera, mitofagian zehar lipido-hartzaile hauen bidez proteinak mitokondrioetara erakarriak izan ostean, C-muturra autofagosomara lotua egongo litzatekeela aditzera ematen dute (Antón *eta lank.*, 2016).

Aurretik deskribatutako THCK aktibatutako autofagiaren bidezko heriotza zelularrean, glioma-zelulen heriotza ere mitokondrioaren bidezidor apoptotikoaren bidez gauzatzen da (Hernández-Tiedra *eta lank.*, 2016). Hala ere, U87MG glioma zeluletan THCK ausazko autofagia indartsua eragiten du, baina ez dira aurkitzen degradaziora eramandako mitokondriorik (**4.1 Irudia**). Beraz, mekanismo hau ez da kanabinoide honen tratamenduari erantzunez ematen den autofagia bidezko heriotza zelularren aktibazioaren arduraduna. Gainera, THCK aktibatutako ERaren estresarekin lotutako seinalizazio bidezidorrean, baliteke dihidrozeramidak mintzean bere forma librean jokatu beharrean, zenbait proteinekin konplexuak eratuz jardutea. Hala ere, ausazko autofagia prozesu honetan, oraindik ez da aurkitu proteina eta zeramiden arteko lotura zuzenik (adib. LC3 edo autofagiaren erregulatuzailea den beste proteinaren bat). Aurkikuntza hauek, autofagia bidezko biziraupena eta heriotza zelularra erregulatzeko esfingolipidoen metabolito espezifikoek eta autofagiaren proteina erregulatuzaileek erabiltzen dituzten mekanismo molekular espezifikoak eta garrantzia biologikoa ulertzen lagunduko lukete. Gainera, aurkikuntza hauek, autofagiaren aktibazioa eta tumoreen hazkuntzaren inhibizioa sustatzeko esfingolipidoen konposaketaren (eta batez ere, zeramiden eta dihidrozeramiden zenbait espezieen mailen) manipulazio farmakologikoa terapeutikoki erabilia izan beharko litzatekeela defendatzen dute. Edozein kasutan, beste estimulu batzuen aurrean organuluaren esfingolipido konposaketan emandako antzeko aldaketek autofagiaren aktibazioan lagunduko luketen argitzeko ikerketa lan gehiago beharko lirateke.

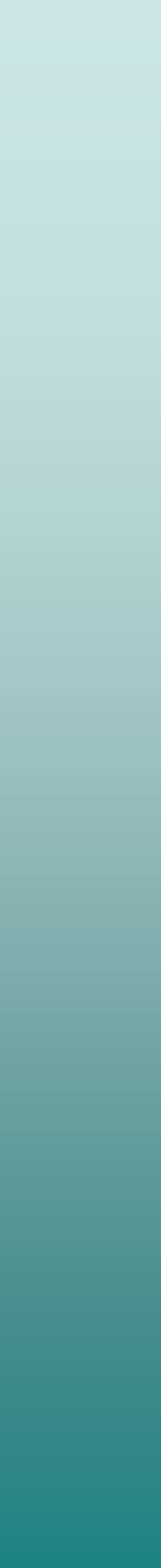
Laburbilduz, tesi lan honen aurkikuntzek, mitofagian zeharreko LC3/GABARAP familiako proteinen mitokondrioetarako errekrutatzearen azpiko mekanismo molekularren eta giza zelula lerro ezberdinetan Atg8aren giza ortologoaren funtzio espezifikoaren inguruko argibideak ematen dituzte. Kardiolipinak kaltetutako mitokondrioaren hautaketarako hartzaile gisa daukan funtzioa, indar ionikoa, bigeruzaren jariakortasuna eta mintzaren kurbadura bezalako faktoreen bidez erregulatuta dagoela argia da. Proteina-lipido elkarrekintza elektrostatikoa hau, mitofagiarekiko espezifikoa izango litzateke eta ez litzateke gertatuko autofagia babesgarri ez-hautakorrean edo THCK aktibatutako heriotza zelular autofagikoan. Emaitzek, Atg8aren giza ortologo bakoitzak mintz-ereduetan CLri era ezberdin batean erantzuten diola ere iradokitzen dute, beraien gainazaleko potentzial elektrostatikoa ikusitako ezberdintasunekin bat datorrena. Gainera, LC3/GABARAP familiako kide bakoitzak, zelula-motaren arabera den mekanismo baten bidez (estresarekiko espezifikoa ere izan litekeena), mitokondriora erakarriak dira (Antón *eta lank.*, 2016; Antón *eta lank.*, prestatzen).

Azkenik, tesi lan honetan deskribatutako aurkikuntzek, THCK gauzatutako autofagia bidezko minbizi-zelulen heriotzaren aktibazioa, ERan gertatzen den dihidrozeramiden pilaketan oinarritzen dela iradokitzen dute, zeina autolisosomen mintzak iragazkor bihurtzea, eta ondorioz, heriotza zelular apoptotikoaren aktibazioa eragiten duen (Hernández-Tiedra *eta lank.*, 2016). Bestalde, LC3Bren lipidazioak, mitokondrioaren mintzan proteinak zeramida lotzeko daukan gaitasuna modulatzeko du (Antón *eta lank.*, 2016), mitofagia bidezko heriotza zelularra eta tumoreen supresioa erregulatuz.

Ondorioak

- 1) LC3B proteinaren eta kardiolipinaren arteko elkarrekintza, kaltetutako mitokondrioen aukeraketa espezifikoa ahalbidetu ahalko luketen mintzaren ezaugarri espezifikoen bidez erregulatuta dago, hauen artean indar ionikoa, bigeruzaren jariakortasuna eta mintzaren kurbadura aurkitzen dira. LC3/GABARAP familiako proteinen arteko ezberdintasun estrukturalak CLarekiko erantzun espezifikoa ahalbidetzen dute mintz-ereduetan.
- 2) CLak degradaziorako-materialaren hartzaile gisa jotzen du mitofagiarako espezifikoa den mekanismo batean. Mekanismo hau baliteke autofagia babesgarri ez-hautakorrean edo THCK eragindako heriotza zelular autofagikoan ez gertatzea. LC3/GABARAP familiako kide desberdinak giza zelula motaren eta baldintza metabolikoen edo estres baldintzen arabera mitokondriora erakarriak dira. RPE1 zeluletan, rotenonak aktibatutako mitofagian zehar, ATG5 proteinarekin markatutako mihiztadura gunek ez dira mitokondrioetan aurkitzen.
- 3) Zeramidek autofagia bidezko minbizi-zelulen heriotzean eginkizun garrantzitsua betetzen dute: (i) mintzetan dihidrozeramiden jokaera eta ezaugarriak zeramiden ezaugarrien oso antzekoak dira, dihidro deribatuak bigeruz lipidikoak iragazkor bihurtzen eraginkorrangoak direla izan ezik. Dihidrozeramiden pilaketak autolisosomen ezegonkortzea eragiten du heriotza zelular autofagikoan zehar. (ii) Aldez aurreko LC3B proteinaren lipidazioa ezinbestekoa da proteinak mintz mitokondrialean zeramidarekin elkarreragiteko, mitofagia bidezko heriotza zelularrean zehar proteinak duen mitokondriora batzeko gaitasuna erregulatzen duelarik.

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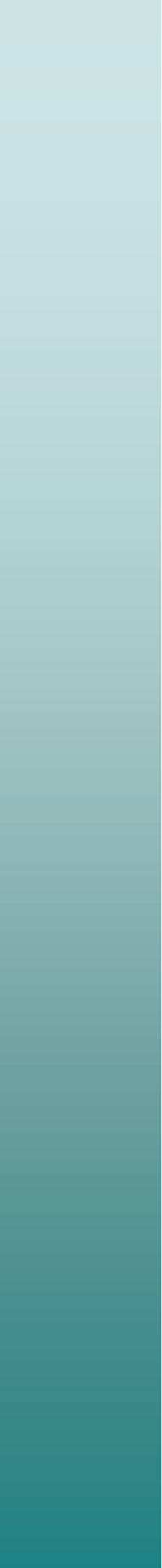
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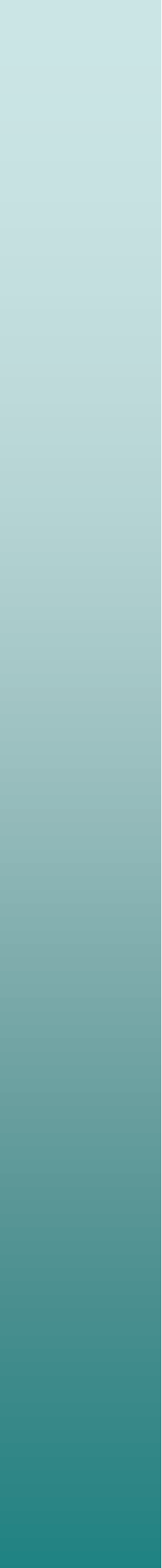
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Acknowledgements



Acknowledgements

The present thesis was performed at Instituto Biofisika (CSIC, UPV/EHU) under the supervision of Professor Alicia Alonso. The work was supported by FEDER/Spanish Ministry of Economy (BFU 2011-28566, BFU 2015-66306-P), and the Basque Government (IT838-13, IT849-13). The author was a recipient of a predoctoral fellowship from the University of the Basque Country.

The author is grateful to Dr. Guillermo Velasco and colleagues (Madrid) for their collaboration in the project, and to Dr. Jon Lane (Bristol) for his training in cell biology techniques and useful discussions. She is also indebted to Professor F.M. Goñi, Dr. Ane Landajuela and Dr. Javier Hervás for their help throughout the thesis, and to Ms Araceli Marcos for her skillful technical assistance.

