eman ta zabal zazu

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Proteomic study of sperm-specific molecular mechanisms in human spermatozoa underlying sperm fertility

Giza espermatozoideen ahalmen ugalkorra erregulatzen duten mekanismo molekular espezifikoen azterketa proteomikoa

Doctoral Thesis

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Laburpena
Summary

SUMMARY

Ejaculated human spermatozoa are immature and infertile and must undergo many biochemical and physiological modifications in the female reproductive tract to become fertile. Sequentially, human spermatozoa go through different processes such as motility capacitation, hyperactivation and acrosome reaction, being all these considered key functions in sperm fertility. Due to the fact that mature spermatozoa express a sperm-specific pool of proteins (around 30%), they could present unique features in their molecular mechanisms, which are important for the regulation of sperm fertility.

Sperm fertility is mainly regulated by ionotropic mechanisms through ionic channels and fast responses. However, the presence of numerous G-protein coupled receptors (GPCR) in the plasma membrane of human spermatozoa suggests that they also could be important regulators of the sperm fertility. However, the signaling pathways downstream these receptors in human spermatozoa remain uncharacterized. Using the opioid receptors as study model, phosphoproteomic and functional approaches revealed sperm-specific molecular mechanisms underlying GPCR in humans. Specifically, opioid receptors regulated the sperm fertility through the modulation of calcium channels and phosphorylation changes in sperm-specific proteins suggesting that could be considered as potential targets for reproductive management.

SPANX-A/D protein family belongs to the sperm-specific proteome in humans and its implication in fertility is largely unknown. Specifically, this family is part of a superfamily known as “Cancer Testis Antigens” (CTA), whose expression is limited to the testis and spermatozoa and to a wide variety of tumors. Thus, a better comprehension of the physiological function of SPANX-A/D in human spermatozoa could help us to understand its physiological role in cancer. By combining different approaches involving molecular biology and functional studies we provided a functional characterization of the physiological and physiological role of SPANX-A/D protein family. Specifically, SPANX-A/D protein family plays a multifunctional role in human spermatozoa. Moreover, it may act as a scaffold protein forming complexes into the nuclear envelope, promoting its physiological and physiopathological function by regulating nuclear processes in human spermatozoa and melanoma.

The results obtained in the following PhD thesis, could provide with valuable tools for a better comprehension of the sperm-specific molecular mechanisms underlying sperm fertility. This information could be essential for the identification of therapeutic targets for male infertility and skin tumors and to develop safer male contraceptives and tumor-directed pharmacological drugs.

LABURPENA

Eiakulatu berri diren giza espermatozoideak heldugabeak dira eta ugalkortasun ahalmena lortzeko emakumezkoaren ugaltze-traktuan zenbat aldaketa biokimiko eta fisiologiko pairatu behar dituzte. Sekuentzialki, espermatozoideek mugitzeko ahalmena, kapazitazioa, hiperaktibazioa eta erreakzio akrosomikoa bezalako prozesuak aurrera eraman behar dituzte obozito bat ernaldu ahal izateko. Jakina denez, gameto arrek espermatozoide-espezifikoak diren proteinak adierazten dituzte, proteoma guztiaren %30a osatzen dutelarik. Hori dela eta, giza espermatozoideen emankortasuna espermatozoide-espezifikoak diren mekanismo molekularren bidez erregula liteke.

Ezaguna denez, mekanismo ionotropikoek giza espermatozoideen emankortasuna erregulatzen dute kanale ionikoen eta erantzun azkarren bitartez. Hala ere, gaur egun, giza espermatozoideen gainazalean adierazten diren G-proteinetara loturiko hartzaileek (GPCR) emankortasunaren erregulazioan eginkizun garrantzitsua izan lezaketela uste da. Behin estimulua jasota GPCR hartzaileek G proteinen bidez seinaliztapen bidezidor intrazelular desberdinak pizten dituzte erantzun zelular espezifikoetan bukatu arte. Hartzaile hauek prozesu fisiologiko anitzetan parte hartzen dute eta gaur egun, industria farmakologikoan itu terapeutiko garrantzitsuak kontsideratzen dira. Giza espermatozoideetan, ordea, GPCR hartzaileen azpitik induzitzen diren seinaliztapen bidezidorrak aski ezezagunak dira. Hartzaile opioideak ikerketa eredu bezala erabiliz, azterketa fosfoproteomiko eta funtzionalek erakutsi dute giza espermatozoideek mekanismo molekular espezifiko eta bakarrak aurkezten dituztela GPCR hartzaileen azpitik. Bereziki, hartzaile opioideek giza espermatozoideen ahalmen ugalkorra erregulatzen dute kaltzio kanaleak modulatuz eta fosforilazio aldaketak sortuz espermatozoideek soilik adierazten dituzten proteina espezifikoetan.

Beste alde batetik, SPANX-A/D proteina familia espermatozoide-espezifikoa den proteomaren parte da eta emankortasunean betetzen duen funtzioa oraindik ez da argitu. Bereziki, ingelesezko “Cancer Testis Antigen” (CTA) taldearen barruan sartzen da, izan ere, bere adierazpena espermatozoideetara mugaturik egoteaz gain zenbait minbizietan ere agertu egiten da. Hori dela eta, SPANX-A/D proteina familiak giza espermatozoideetan betetzen duen funtzio fisiologikoa baliogarria litzateke minbizian duen funtzio fisiopatologikoa ulertzeko. Biologia molekularra aztergai duten teknikak eta azterketa funtzionalak erabilita, SPANX-A/D proteina familiaren karakterizazio funtzional fisiologikoa eta fisiopatologikoa egin ziren. Horien arabera, SPANX-A/D proteina familia funtzio nuklearraren erregulazioan dihardu giza espermatozoideetan eta melanoman.

Ondorengo doktore tesitik eratorritako emaitzek beraz, espermatozoide-espezifikoak diren proteinei eta horien mekanismo molekularrei buruzko informazio baliogarriaz hornitzen digute. Ezagutza hori gizonezkoaren antzutasuna zein azaleko minbizia tratatzeko itu terapeutikoen garapenerako erabilgarria izateaz gain, antisorgailuen eta tumoreetara zuzenduriko farmakoen sorkuntzan ezinbestekoa litzateke etorkizunean.

RESUMEN

Los espermatozoides liberados durante la eyaculación son inmaduros y deben sufrir una serie de cambios bioquímicos y morfológicos en el tracto reproductor femenino. Secuencialmente, los espermatozoides humanos deben adquirir la capacidad de movimiento para después sufrir la capacitación, hiperactivación y reacción acrosómica, y así poder fecundar un ovocito. Debido a que los espermatozoides presentan un proteoma único con un 30% de proteínas espermatozoide-específicas, los mecanismos moleculares por los que regulan la fertilidad podrían a su vez presentar características especiales.

Tal y como otros autores han descrito previamente, la fertilidad del espermatozoide está principalmente regulada por mecanismos ionotrópicos a través de canales iónicos y respuestas fisiológicas rápidas. Sin embargo, la presencia de numerosos receptores acoplados a proteínas G (GPCR) en la membrana plasmática de los espermatozoides humanos sugiere que estos podrían ser importantes reguladores de su fisiología. En respuesta a estímulos externos, los GPCR transducen señales a través de proteínas G para iniciar vías de señalización intracelulares que terminan en respuestas celulares específicas. Estos receptores participan en una gran variedad de procesos fisiológicos y a día de hoy se consideran una de las mejores dianas terapéuticas en la industria farmacológica. A pesar de ello, las vías de señalización subyacentes a los GPCR en espermatozoides humanos son desconocidas. Utilizando los receptores opioides como modelo de estudio, análisis fosfoproteómicos y funcionales revelaron mecanismos espermatozoide-específicos inducidos por los GPCR. Específicamente, los receptores opioides regulan la fertilidad del espermatozoide humano a través de la modulación de los canales de calcio y cambios de fosforilación en proteínas específicas de espermatozoides.

Por otra parte, la familia de proteínas SPANX-A/D pertenece al grupo de proteínas específicas de espermatozoides y su implicación en la fertilidad todavía no se ha elucidado. En concreto, es parte de una super familia de proteinas conocidas como “Cancer Testis Antigens” (CTA), expresadas unicamente en testículos y espermatozoides y en una gran variedad de tumores. Es por ello que una mayor comprensión de la función fisiológica de SPANX-A/D en los espermatozoides humanos podría ayudarnos a entender su función fisiopatológica en el cáncer. Combinando diferentes técnicas de biología molecular y estudios funcionales, presentamos la caracterización fisiológica y fisiopatológica de la familia SPANX-A/D. Concretamente, la familia de proteínas SPANX-A/D está involucrada en la regulación de funciones nucleares en los espermatozoides humanos y en el melanoma.

Los resultados obtenidos de esta tesis doctoral, podrían ser herramientas valiosas para tener un mayor entendimiento sobre las proteínas especificas de espermatozoides humanos y los mecanismos moleculares en los que éstas participan. Esta información podría ser de gran utilidad en la identificación de dianas terapéuticas en la infertilidad masculina y cáncer de piel, así como para desarrollar anticonceptivos masculinos y drogas farmacológicas dirigidas al tumor.

AURKIBIDEA
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AKRONIMOAK ETA LABURDURAK
ACRONYMS AND CONTRACTIONS

2n: Diploid
4n: Tetraploid
ABP: Androgen Binding Protein
ACN: Acetonitrile
ACR: Acrosin
ACRBP: Acrosin Binding Protein
ACTB: Actin, cytoplasmic
AKAP: A protein Kinase Anchoring Protein
ALH: Amplitude of Lateral Head Displacement
APN: Aminopeptidase N
AR: Acrosome Reaction
ATP: Adenosine Triphosphate
BCA: Bicinchoninic Acid
BSA: Bovine Serum Albumin
Ca^{+2}: Calcium
CAA: ChloroAcetamide
CABYR: Calcium-binding Tyrosine phosphorylation-Regulated protein
CAGE: Cancer Associated Gene
CaMK: Ca^{+2}/Calmodulin-dependent Protein kinase
cAMP: cyclic Adenosine Monophosphate
CBY: Chiby
CCDC: Coiled Coil Domain Containing
CGH: Chorionic Gonadotrophin Hormone
CNG: Cyclic Nucleotide Gated
COX: C Cytochrome Oxidase
CRH: Corticotropin Releasing Hormone
CTA: Cancer Testis Antigen
CTCF: Corrected Total Cell Fluorescence
DAG: Diacylcgycerol
DHT: Dihydrotestosterone
DNA: Deoxyribonucleic Acid
DNAH: Axonemal Dynein Heavy Chain
DOR: Delta Opioid Receptor
DPDPE: [D-Pen2,5]-Enkephalin hydrate
DTT: Dithiothreitol
ECL: Enhanced Chemoluminiscence
EGTA: Ethylene Glycol Tetra-acetic Acid

EIF4A3: Eukaryotic Initiation Factor 4A-III
EJC: Exon Junction protein Complex
EOP: Endogenous Opioid Peptide
EPAC: Exchange protein activated by cAMP
ERK: Extracellular-Signal- Regulated Kinase
FDR: False discovery rate
FH: Fumarate Hydratase
FS: Fibrous Sheath
FSH: Follicle-Stimulating Hormone
FSIP: Fibrous Sheath Interacting Protein
GAGE: G Antigen Gene
GDP: Guanosine Diphosphate
GnRH: Gonadotropin Releasing Hormone
GPCR: G protein coupled receptors
GRK: G protein Receptor Kinase
GTP: Guanosine Triphosphate
HA: Hyperactivation
HCO_3^-: Bicarbonate
HIST1H4A: Histone H4
HSPA8: Heat Shock Cognate 71 kDa Protein
IP_3: Inositol Triphosphate
IP_3R: Inositol Triphosphate Receptors
JNK: c-Jun N-terminal Kinase
K^+: Potasium
KOR: Kappa Opioid Receptor
LC-MS/MS: Liquid chromatography tandem mass spectrometry
LDH: Lactate Deshydrogenase
LH: Luteinizing Hormone
LIN: Linearity Progession
LMNA: Lamin A/C
MARCKS: Myristoylated Alanine-Rich C Kinase Substrates
MAGE: Melanoma Antigen Gene
MAGOH: Protein Mago Nashi Homolog
MAPK: Mitogen Activated Protein Kinase
MOR: Mu Opioid Receptor
MS: Mass Spectrometry
n: Haploid
NADH: Nicotinamide AdenosineDinucleotide Hydride
NDUFAF: Ubiquinone Oxidoreductase Complex Assembly Factor
NEP: Neutral Endopeptidase
NLS: Nuclear Localization Signal

NO: Nitric Oxide
NUP: Nucleoporin
ODF: Outer Dense Fiber
ON: Over Night
ORL: Opioid Receptor Like
PA: Phosphatidic Acid
PC: Phosphatidylcholine
PBS: Phosphate Buffered Saline
PDYN: Prodynorphin
PENK: Proencephalin
PFA: Paraformaldehide
PGK: Phosphoglycerate Kinase
PI4P5K: Phosphatidiylinositol 4-Phosphate-5-Kinase
PIP2: Phosphatidylinositol 4,5-bisphosphate
PKA: Protein Kinase A
PKC: Protein Kinase C
PLA: Phospholipase A
PLC: Phospholipase C
POMC: Propiomelanocortin
PRKAR1A: Protein Kinase cAMP-dependent type I Regulatory Subunit Alpha
PSA: Prostate Specific Antigen
pTyr: Tyrosine phosphorylated
PVDF: Polyvinylidene Fluoride
RhoGEF: Rho Guanine Nucleotide Exchange Factor
RIIAD: Regulatory Subunit of Typer PKA R-subunit
RIPA: Radioimmunoprecipitation Assay Buffer
RNA: Ribonucleic Acid
ROPN: Ropporin
RPL38: 60S Ribosomal Protein L38
RT-PCR: Reverse Transcriptase- Polymerase Chain Reaction
RT: Room Temperature
RyR: Ryanodine Receptors
SACY: Soluble form of Adenylyl cyclase
SDS: Sodium Dodecyl Sulfate
Ser: Serine
shRNA: short hairpin RNA
SLCO: Solute carrier organic anion Transporter
SNARE: Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptors
SOC: Store-Operated Channels
SPA: Sperm Autoantigenic Protein
SPANX: Sperm Protein Associated with the Nucleus mapped to the X chromosome

SPATA: Spermatogenesis Associated Protein
SSX: Sinovial Sarcoma X breakpoint
STR: Straightness
TCGA: The Cancer Genome Atlas
TEKT: Tektin
TFA: Trifluoroacetic Acid
TGF: Transforming Growth Factor
Thr: Threonine
TM: Transmembrane
tmAC: transmembrane Adenylyl cyclase
TMEM190: Transmembrane 190 protein
TMT: Tandem Mass-Tag
tRNA: transfer Ribonucleic Acid
TRP: Transient Receptor Potential
U50488H: (−)-trans-(1S,2S)-U-50488 hydrochloride hydrate
VAP: Average-path Velocity
VCL: Curvilinear Velocity
VCP: Valosin Containing Protein
WHO: World Health Organization
ZP: Zona Pellucida

1. Sarrera

Introduction

1. SARRERA

1.1. GIZONEZKOAREN UGAL-APARATUA

1.1.1. Gizonezkoaren ugal-aparatuaren anatomia fisiologikoa

Gizonezkoen ugal-aparatua testikuluek, epididimoak, hodi deferenteak, hodi isurleak, guruin besikularrak, prostatak, guruin-bulbo uretralak, uretrak eta zakilak osatzen dute (1.1 Irudia). Ondoren osagai bakoitzaren deskribapen anatomikoa eta funtzionamendu biologikoa azalduko dira.

Testikuluak

Testikuluak gonada maskulinoak dira eta espermatozoideak sortzeaz eta metatzeaz gain, hormona sexualak (testosterona) ere jariatzen dituzte. Forma obalatua duten organo espezializatu hauek eskroto deituriko azal egitura batean biltzen dira zakilaren azpian (1.2 Irudia). Eskrotoa, testikuluak barrunbe abdominaletik at zintzilikatzeaz arduratzen da, hauek gorputzaren tenperatura ohikoena baino 2-3°C baxuagoan manten daitezen. Izan ere, testikuluen tenperatura behar bezalakoa baino altuagoa denean, espermatogenesian akatsak sor daitezke (Munkelwitz and Gilbert, 1998; Zorgniotti et al., 1980). Testikuluak tubulu seminiferoez eta sare testikularrez osaturik daude.

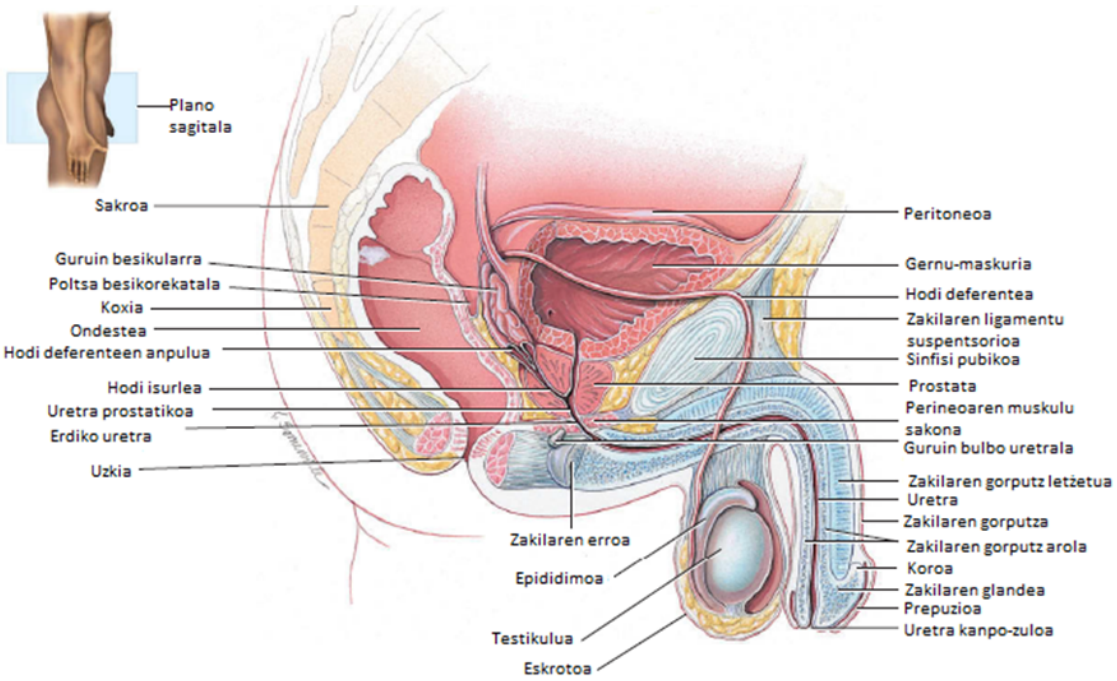
Tubulu seminiferoak testikuluen barneko ehunka hodixka bihurri eta era oso trinkoan paketaturik dauden egiturak dira. Tubuluetan espermatogenesia izeneko prozesua burutzen da, espermatozoidearen formakuntza helburu duena. Tubuluak 200-300 lobuluez osaturik daude, guztiek sare testikularra eratuz (Rhoades and Tanner, 1997; Tortora and Derrickson, 2013). Hain zuzen ere, tubulu seminiferoek euren edukia sare testikularra izeneko gune biltzailera jariatzen dute eta ondoren testikuluetako hodi eferenteetatik epididimora eramaten dituzte (Guyton and Hall, 1997).

Epididimoa

Epididimoa testikuluen atzean kokatzen den organoa da eta hodi deferentea eta testikulua lotzeaz arduratzen da. Testikulu bakoitzak epididimo bat dauka, beraz gizonezkoek bi epididimo dauzkate. Organo honek gizakian 5-6 metro neurtzen ditu luzeran eta espermatozoideek 3 egunetik gora behar dituzte bide hori egiteko (Wen and Yang, 2000). Epididimoa, hiru zatitan banatzen da: epididimoaren burua, gorputza eta isatsa. Ondoren, ikusiko dugun bezala, funtzionalki epididimoa espermatozoideen heltzeaz arduratzen da hauek mugitzeko ahalmena gara dezaten. Testikuluen eta epididimoaren jariakina hazi-likidoaren %5a bada ere, gametoen ugal-prozesurako ezinbestekoak diren propietateak berenganatzen laguntzen dute (Jequier and Crich, 1986).

Hodi deferenteak

Hodi deferenteak 45cm inguru duen hodia da eta epididimoaren edukia jasotzeaz arduratzen da, ondoren hodi isurlera eramateko. Funtzionalki hodi deferenteek epididimotik espermatozoideak helarazten dituzte uretrarantz mugimendu peristaltikoen bitartez. Bide horretan zehar dagoen anpulua hazia biltegiratzeaz arduratzen da. Hilabetetan biltegiratuta dagoen eta eiakulatzen ez den hazia birxurgatu egiten da (Tortora and Derrickson, 2013).



1.1 irudia. Gizonezkoen ugal-aparatuaren atalak. Azalpena testuan. (Tortora and Derrickson, 2013-tik moldatua).

Guruin osagarriak

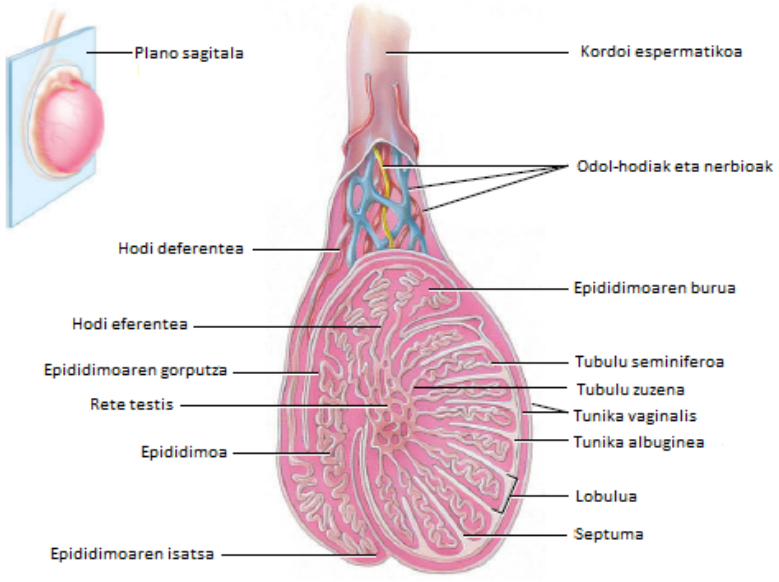
Arestian aipatutako organo eta hodiek espermatozoideak biltegiratu eta garraiatzen dituzte baina guruin osagarriek giza haziaren likidoaren zatirik handiena jariatzeaz arduratzen dira. Hauen funtziorik nagusiena espermatozoidearen biziraupena eta ugalkortasuna mantentzea da (Jequier and Crich, 1986). Izan ere, espermatozoideak epididimotik uretraraino garraiatzeko prozesuan zehar guruin osagarrien jariakin guztiekin nahasten dira. Hain zuzen ere, ekintza horri emisio deritzo eta epididimoaren, hodi deferenteen eta hodi isurleen muskuluen uzkurketari esker gertatzen da (Rhoades and Tanner, 1997; Tortora and Derrickson, 2013). Guruin besikularrak, prostatak eta guruin bulbo uretralak guruin osagarriak dira.

Guruin besikularra

Guruin besikularrak gernu-maskuriaren azpian kokatuta daude, hodi deferenteen anpuluaren ondoan hain zuzen ere. Hauek, jariakin horixka eta gelatinotsua sortzen dute hazi-likidoaren %60-80a osatuz. Guruin besikularrek ez dute espermatozoiderik biltegiratzen eta ejakulazioan zehar gertatzen den muskulu leunaren uzkurketak jariakina uretrarantz bidaltzen du. Jariakin hori fruktosan, zitratoan, amino azidotan eta prostaglandinetan bereziki aberatsa da. Gainera bere izaera alkalinoak gizonezkoaren uretraren eta emakumezkoaren ugal-aparatuaren ingurune azidikoa indargetzeaz arduratzen da espermatozoideak bizirik iraun dezaten. Fruktosa espermatozoidearen ATParen sintesirako erabiltzen da eta prostaglandinek espermatozoideen mugikortasunean funtzio garrantzitsua betetzen dute, besteak beste (Tortora and Derrickson, 2013; Vaamonde et al., 2016).

Prostata

Prostata gernu-maskuriaren azpiko aldean dagoen guruina da eta uretrarekin konektatuta dago (Rhoades and Tanner, 1997). Prostatak izaera esnetsua eta zertxobait azidikoa (pH 6.5) duen jariakina sortzen du. Honek likido seminalaren %15-30a osatzen du eta zenbait sustantziaz konposaturik dago: (1) Azido zitrikoak espermatozoidearentzat baliogarria den ATPa sortzen du Krebs zikloaren bidez, (2) prostata-antigeno espezifikoa (PSA), pepsinogena, lisozima, amilasa eta hialuronidasa bezalako entzima proteolitikoek guruin besikularretik eratorriak diren koagulazio proteinak apurtzen dituzte, (3) funtzio ezezaguna duen fosfatasa azidoa eta (4) emakumezkoaren ugal-aparatuan dauden bakterioak suntsitu ditzazkeen seminalplasmina (Tortora and Derrickson, 2013; Vaamonde et al., 2016).



1.2. Irudia. Testikuluaren barne anatomia. Azalpena testuan. (Tortora and Derrickson, 2013-tik moldatua).

Guruin bulbo-uretrala

Guruin bulbo-uretralak "Cowper"-en glandulak deitzen dira eta hazi-likidoari azken bi jariakinak gehitzen dizkiote. Kitzikapen sexualean zehar guruin bulbo-uretralek jariakin alkalinoa isurtzen dute uretrara. Likido honek hazi-likidoaren %2-5a osatzen du, glukoproteinetan aberatsa da eta izaera gelatinotsua eta urtsua ditu. Jariakinaren edukiak ez du espermatozoiderik eta bere funtzio nagusienak uretraren lubrikazioa eta espermatozoideen babespena dira (Jequier and Crich, 1986; Tortora and Derrickson, 2013; Vaamonde et al., 2016).

Uretra

Uretra gernu-maskuritik gernua hustutzeaz arduratzen bada ere, gizonezkoen ugal-aparatuaren parte ere bada semena gorputzetik kanpo jariatzen baitu (Vaamonde et al., 2016). Izan ere, testikuluaren eta kanpo-ingurunearen arteko azken egitura da. Uretra zati prostatikoaz, zati menbranosoaz eta zati arolaz osaturik dago. Uretraren zati arola zakilaren barruan dago eta gorputz arolak inguratzen du. Haziaren-isurtzea hazia uretratik kanporatzean ematen da (Guyton and Hall, 1997).

Zakila

Zakila gizonezkoaren kopulaziorako organoa da. Pubisaren aurreko aldean eta eskrotoaren gainean kokaturik dago. Zakil bigunak bataz beste 10cm-ko luzera dauka eta forma zilindrikoa dauka. Zakil tentetuak ordea, bataz beste 16cm-ko luzera hartzen du eta prisma triangeluarraren forma dauka ertz borobilduekin. Zakila gernua eta hazia jariatzeaz arduratzen da.

Hazia espermatozoideen eta likido seminalaren arteko nahasketa da (tubulu seminiferoetatik, guruin besikularretatik, prostatatik eta guruin bulbo uretraletatik eratorritako jariakinez osaturik). Ohiko eiakulazio batean isuritako semenaren bolumena 2.5-5 ml-takoa da 50-150 milioi espermatozoide/ml-ko dituelarik. Jariakin prostatikoak izaera azidikoa badu ere, semenak pH alkalinoa dauka (pH 7.2-7.7) guruin besikularrek isurtzen duten jariakinaren pH eta bolumen altuari esker. Jariakin prostatikoak semenari izaera esnetsua ematen badio ere guruin besikularrek eta bulbo-uretralek isuritako konposatuek izaera likina ematen diote. Gainera, semenaren likido seminala espermatozoideentzat beharrezkoak diren nutrienteez osaturik dago eta zelulen garraioan eta babesean funtzio garrantzitsua dauka (Tortora and Derrickson, 2013; Vaamonde et al., 2016).

1.2. ESPERMATOGENESIA

Espermatogenesia testikuluetako tubulu seminiferoetan gertatzen den prozesua da. Prozesu hau hozi zelula ametatik espermatozoidera arteko desberdintzapenean oinarritzen da. Espezifikoki, espermatogenesian zehar espermatogonia diploideetatik gameto haploideak sortzen dira (Vaamonde et al., 2016). Prozesu biologiko konplexu hau pubertaroan hasten da eta gizonezkoaren bizitza guztian zehar mantentzen da, zahartzaroan urritze txikia nabarituz (Sadler, 2004). Espermatogenesiak testikuluen zeluletan gertatzen diren adierazpen genikoaren eta zatiketa zelularraren arteko sikronizazio konplexua behar du. Prozesuaren hasiera hormona gonadotropikoen (FSH eta LH) kontzentrazioaren igoerarekin gertatzen da eta ondorioz testikuluetako Leydig zelulak testosterona hormona jariatzen hasten dira.

Espermatogenesiak 64-74 egun inguru behar ditu espermatozoide helduak (zelula haploideak) espermatogonietatik (zelula diploideak) sortu arte. Hala ere, ziklo bakoitza bukatu baino lehen modu paraleloan 2-3 asteko erregulartasunez ziklo berriak hasten dira. Hori dela eta, tubulu seminifero bakoitzak garapen-egoera desberdinean dauden zelulak izango ditu garatuenak tubuluaren lumen aldera kokatuz. Sertoli zelulak tubulu horien alde basalean kokatzen dira eta espermatogenesia erregulatzen dute, odolaren eta tubuluaren arteko hesia edo muga hematotestikularra eratuz (Ogawa et al., 2005).

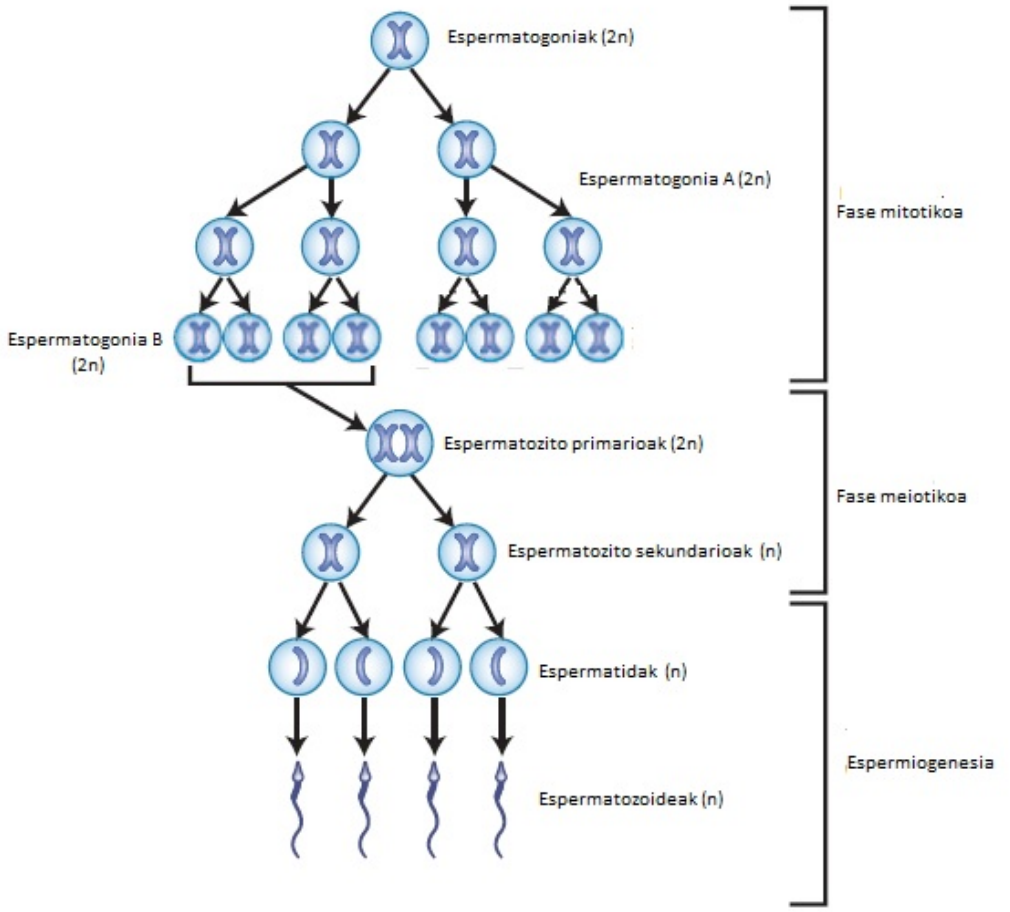
Espermatogenesiaren prozesua hiru fase desberdinetan bana daiteke (1.3 eta 1.4 Irudiak):

1.- ***Fase mitotikoa:***

Enbrioaren garapenean zehar, hozi zelula primordialak testikuluetara heltzen dira eta espermatogonia deituriko zelula germinatibo heldugabeetan bihurtzen dira. Lehenengo fase hau pubertaroarekin hasten da eta espermatogoniak mitosiaren bidez zatitzen hasten dira. Heldugabeak diren zelula germinatiboak edo espermatogoniek informazio genetikoa errepikaturik daukate (diploideak edo 2n). Giza testikuluan bi motako espermatogoniak aurki daitezke: A motako espermatogoniak (46, 2n), zatiketa mitotiko mugatua burutzeko eta populazioa mantentzeko gai direnak; eta B motako espermatogoniak (46,2n), aurrekoen zatiketa mitotikoetatik eratorriak direnak eta meiosiaren prozesua hasten dutenak (Clermont, 1972; Kierszenbaum and Tres, 2012).

2.- Fase meiotikoa:

Fase honetan zehar zelulen eduki genetikoa erdira murrizten da. Izan ere, zelulak diploide izatetik (2n) haploide (n) izatera pasatzen dira. Gainera, denbora tarte honetan zehar kromosoma homologoen errekonbinazioa gertatzen da gizonezkoak sortu ditzazkeen espermatozoide guztiek konbinazio geniko desberdinak izanik eta aldakortasun genetikoa handituz (Clermont, 1972; Kierszenbaum and Tres, 2012).



1.3. Irudia. Espermatogenesiaren faseak. Zelula diploideek (2n) 46 kromosoma dituzte eta zelula haploideek (n) 23. Zelula ama germinaletatik eratorritako A motako espermatogoniak, espermatogenesi prozesuaren lehenengo zelulak dira. Zelulen zatiketaren bidez zelula-klonak eratzen dira eta zelula berri horiek guztiak, zubi zitoplasmatikoen bidez, lotuak geratzen dira espermatozoideak hondakin-gorputzetatik banatzen diren arte. (Vaamonde et al., 2016-tik moldatua).

Etapa hau B motako espermatogonioen lehen zatiketa mitotikoarekin hasten da. Bertan, DNA bikoizten da eta espermatozito primario bihurtzen dira (46, 4n). Espermatozito hauek meiosiarekin jarraitzen dute lehen zatiketa meiotikoaren ostean bi espermatozito sekundario (23, 2n) sortuz.

Lehen zatiketa meiotikoak profase luzea dauka eta leptoteno, zigoteno, pakiteno, diploteno eta diakinesia izeneko azpifaseak ditu. Hauek oinarrizko lau gertakariek bereitzen dituzte: 1) konplexu sinaptonemalaren sorkuntza kromosoma homologoen arteko sinapsia errazteko, 2) sinapsia edo kromosoma homologoen arteko elkarketa, 3) kromosoma homologoen kromatida ez-ahizpen arteko geneen informazio-trukea eta 4) gurutzatutako kromosoma homologoen banaketa (Kierszenbaum and Tres, 2012).

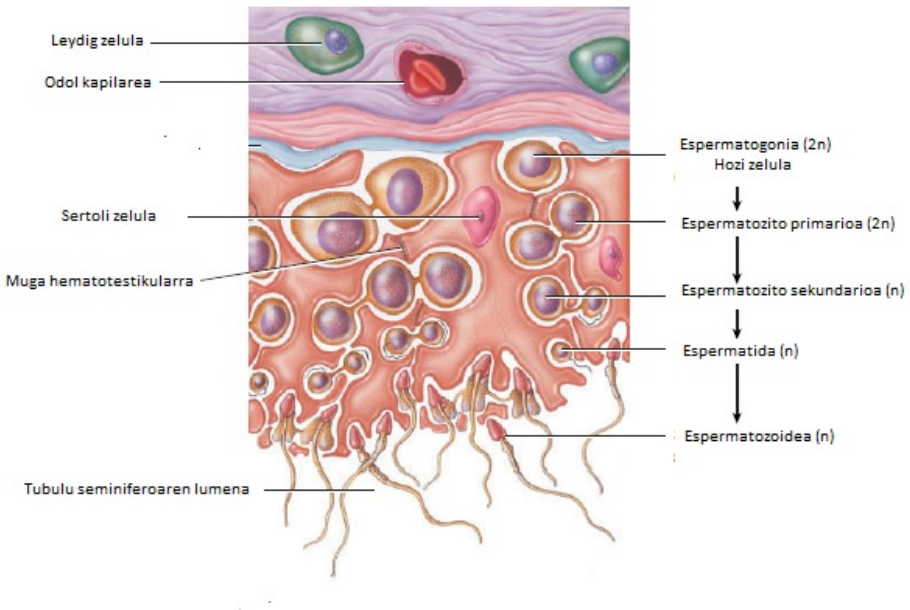
Espermatozito sekundarioek bigarren meiosia egiterakoan karga genetikoa erdira murrizten dute bakoitzak 2 espermatida haploide sortuz (23, 1n). Beraz, fase honetan sartzen den

espermatogonia bakoitzak, lau espermatida sortuko ditu. Aipatzekoa da Sertoli zelulak garatzen ari diren espermatozoideak elikatzeaz eta babesteaz arduratzen direla (Clermont, 1972; Kierszenbaum and Tres, 2012).

3.- Espermiogenesia:

Fase honetan zehar espermatidak espermatozoide heldu bihurtzen dira. Hain zuzen ere, espermatidak ziklo mitotiko berrian sartzen dira, bakoitzak bi espermatida berri sortuz. Espezifikoki, fase horretan espermatozoidearen gorputzaren eta materia nuklearraren heltzea gertatzen da. Espermatogonietatik hasita eta espermatiden sorrerarekin bukatzen den prozesuan zehar ez da zitozinesi osoa gertatzen, eta horregatik zelula batetik eratorritako zelula-klon luzeak zubi zitoplasmatikoen bidez lotuak agertzen dira. Puntu horretan, axonemaren garapena gertatzen da erdiko piezaren eta mikrotubuluen lodierarekin. Gainera flageloa eratzen da zelula baten zentrioloetatik abiatuta. Nukleoaren heltzea DNA-ren kondentsatzio eta paketatzearekin hasten da, lehenengo proteina basikoekin eta ondoren protaminekin. Fase honetara heldu eta gero kromatina ez da inoiz gehiago transkripzionalki aktiboa izango. Ondoren akrosoma garatzen da Golgi aparatutik abiatuz eta zitoplasmaren zati nagusia desagertuko da (Kierszenbaum and Tres, 2012; Tortora and Derrickson, 2013; Vaamonde et al., 2016).

Behin espermatozoideak eraturik daudenean, espermazioaren bidez tubuluaren argira askatzen dira. Ezaguna da, egunero eta egoera normalean, gizonezko batek 50-150 milioi espermatozoide inguru jariatzen dituela tubulu seminiferoetara (Clermont, 1972).



1.4.Irudia. Tubulu seminiferoen anatomia mikroskopikoa eta espermatogenesiaren faseak. Espermatozitoen garapena tubulu seminifero baten zehar-ebakian. Xafla basalean, zelula ama germinalen populazioa dago (espermatogoniak). Zelula horiek zatitzean sortzen diren zelula alabak lumen aldera migratuko dute eta 64 egun eta gero, espermatozoideetara desberdintzen dira (Tortora and Derrickson, 2013-tik moldatua).

1.2.1. Espermatozoideen heltzea

Espermatogenesia bukatu ondoren espermatozoideak tubulu seminiferoko argira askatzen dira eta epididimoaren lehen ataletan ez dira gai obozitoa ernaltzeko. Aitzitik, epdidimoan 18 eta 24 ordu bitartean egon eta gero, mugikortasun gaitasuna garatzen dute (Amann and Howards, 1980). Hala ere, epididimoaren jariakinean dauden proteina inhibitzaileek eiakulazioa gertatu arte benetako mugikortasuna inhibitzen dute (Yoshida et al., 2008).

Espermatozoideek zenbait egun behar dituzte epididimoa zeharkatzeko baina aipatu bezala, hauek ez dira dira "heldutzat" hartzen eta ez dira obozito bat *in vivo* edota *in vitro* ernaltzeko gai. Hori dela eta, aldaketa morfologikoez gain, aldaketa fisiologiko eta biokimikoak jasan behar dituzte emakumezkoaren ugaltze traktuan. Aldaketa horiei guztiei espermatozoidearen heltze-prozesu deritze (De Jonde, 2005; Suarez, 2008a; Yanagimachi, 1994).

Mugimendu peristaltikoen bidez, espermatozoideak sare testikularra igaro eta hodi efereentatik epididimora heltzen dira. Alde batetik, epididimoaren burutik isatserainoko bidean, espermatozoideek mugikortasun ahalmena berenganatzen dute zitosoleko AMP ziklikoa (cAMP) eta ATParen mailak igoz eta epididimoaren isatsean metatuz. Beste alde batetik, espermatozoideek zenbait molekula trukatzen dituzte ingurunearekin, izan ere, gluzido, proteina eta lipido ugari mintzetik erauzi, eraldatu eta berenganatzen dituzte (Yanagimachi, 1994). Heltze-prozesuaren helburu nagusiak, beraz, mugikortasuna lortzea eta obozitoa ezagutu eta berarekin fusionatzeko beharrezkoak diren konposatuak berenganatzea dira (Gatti et al., 2004). Hala ere, geroago sakonduko dugun bezala, ernalketarako gaitasun osoa emakumezkoaren ugaltze traktuan bukatzen da, espermatozoidearen kapazitazioarekin, erreakzio akrosomikoarekin eta gametoen fusioarekin (Sadler, 2004).

1.2.2. Espermatozoideen isurtzea

Behin espermatozoideak "heldu" bilakatu direnean, gizonezkoaren ugal aparatutik kanporatuak izan arte, hodi deferentean eta haren anpuluan denbora luzez egon daitezke metaturik. Gizonezkoaren kitzikatze eta erekzioarekin batera, emisio izeneko prozesuari hasiera ematen zaio. Horretan, espermatozoideak likido seminala osatzen duten zenbait jariakinekin elkartzen dira. Behin uretra giza haziarekin bete denean, zentzumen-seinaleak sortu egiten dira eta hazia zakiletik kanpo isuri egiten da. Aipatzekoa da isurtzen den hazia osagai zelularrez (espermatozoideak, linfozitoak, Sertoli eta Leydig zelulak) eta osagai jariatuez (likido seminala) osaturik dagoela.

Likido seminala espermatozoideak emakumezkoaren ugal aparatuan barrena garraiatzeaz arduratzen da. Behin giza hazia emakumezkoaren ugaltze traktuan dagoenean, koagulazioa gertatzen da fibrinogeno eta semenogelinari esker, haziak baginatik alde egin ez dezan eta horren alde sakonetan manten dadin. Ondoren, fibriolisinari esker, koagulua 15 minutuan disolbatzen da espermatozoideen mugikortasuna areagotuz. Gainera, likido seminalean

dauden faktore inhibitzaileek kapazitazioa eta erreakzio akrosomiko espotaneoa sahiestu egiten dute, haien bideragarritasuna mantenduz (Guyton and Hall, 1997).

1.2.3.Espermatogenesiaren erregulazioa

Espermatogenesia hasten duten hasierako faktoreak ezezagunak badira ere, pubertaroan zehar hipotalamoan aurkitzen diren zelula neurojariakorrek gonadotropinaren hormona jariatzailea (GnRH) askatzen dute. Hormona honek aurreko pituitarian dauden gonadotrofoak estimulatzen ditu bi gonadotropinen jariaketa areagotzeko: hormona luteinizatzailea (LH) eta folikuluen estimulazioarako hormona (FSH). 1.5 irudian testosterona jariaketa eta espermatogenesia kontrolatzen dituen feedback negatiboa azaltzen da. Bereziki, LHak tubulu seminiferoetan agertzen diren zelula interstizialak edo Leydigen zelulak estimulatzen ditu testosterona hormona jariatzen hasteko. Hormona esteroide hau androgenorik nagusiena da eta testikuluetan sintetizatzen da kolesteroletik abiatuta. Gainera, bere izaera lipidiko solugarriak zelula interstizialetatik likido interstizialera igarotzeko ahalmena ematen dio ondoren odolera isurtzeko. Feedback negatiboaren bidez, testosteronak LHaren jariaketa inhibitzen du aurreko pituitarian agertzen diren gonadotrofoen bidez eta ondorioz hipotalamoaren zelula neurojariakorrek GnRHaren jariaketa inhibituko dute. Zenbait zelula ituetan (prostatan eta kanpoko genitaletan, esaterako), 5 alfa-redukatasa izeneko entzimak testosterona dihidrotestosteronan (DHT) eraldatzen du (Tortora and Derrickson, 2013).

FSHak zeharka espermatogenesia estimulatzen du eta testosteronak sinergikoki jokatzen du Sertoli zelulengan androgenoei-loturiko proteina (ABP) tubulu seminiferoen lumenera eta zelula espermatogenikoen likido interstizialera jariatzeko. Horrela, ABPa testosteronarekin lotzen da horren kontzentrazioa altu mantentzeko. Testosteronak tubulu seminiferoetan gertatzen den espermatogenesiaren azken faseak estimulatzen ditu.
Behin gizakiaren ugalketarako funtzioak asetzeko beharrezkoa den espermatogenesi maila egokia lortu denean, Setoli zelulek inhibina izeneko hormona jariatzen dute aurreko pituitarian FSHren jariaketa inhibituz. Espermatogenesia astiroegi gertatzen bada eta honen areagotzea beharrezkoa bada, inhibina maila gutxitzen da FSHaren jariaketa ahalbidetuz eta espermatogenesia bultzatuz.

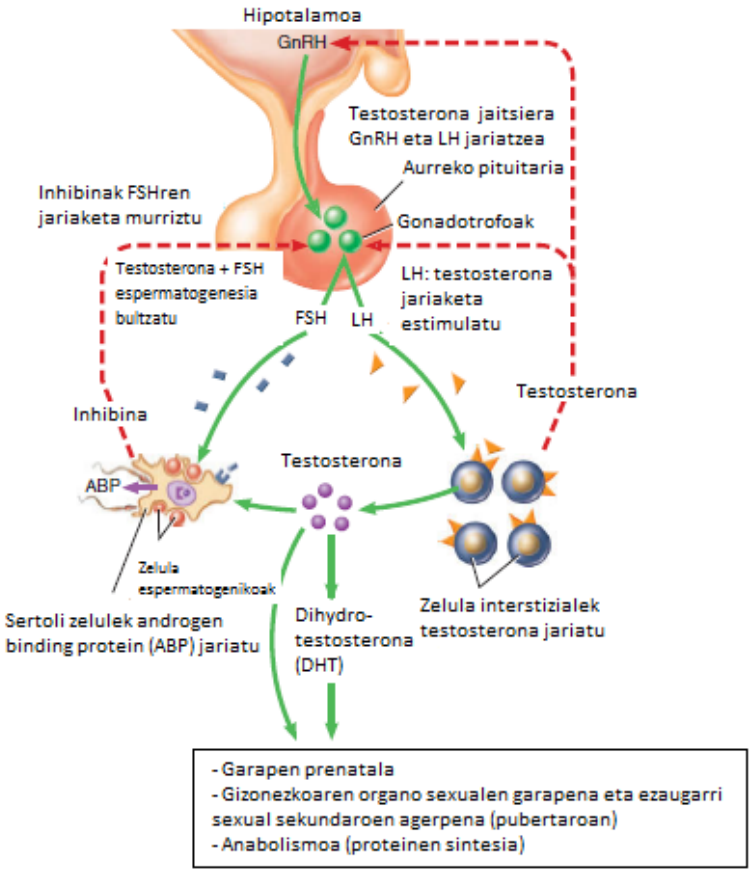
Beste alde batetik, testosterona eta dihidrotestosterona androgenoa hartzaile berdinetara lotzen dira, zeinak itu zelulen nukleoetan aurkitzen diren. Hormona-hartzaile konplexu horrek adierazpen genikoa erregulatzen du, gene batzuk aktibatuz edota inhibituz. Ondorioz, androgenoek zenbait efektu sortuko dituzte (Tortora and Derrickson, 2013):

- **Garapen prenatala**: Jaiotza baino lehenago, testosteronak gizonezkoaren ugal aparatuaren eraketaren aurrekariak diren zenbait organoren eraketa estimulatzen du. DHTak kanpo genitalen garapena bultzatzen du eta testosterona estrogeno (hormona femeninoa) bihurtzen da gizonezkoaren garunaren garapenean parte hartu dezan.
- **Gizonezkoaren sexu-ezaugarrien garapena:** Pubertaroan, testosteronak eta DHTak sexu-organoen handipena eta ezaugarri sexual sekundarioen garapena bultzatzen

dituzte. Sexu-ezaugarri sekundarioek gizonezkoak desberdintzatzen dituzte eta laringearen handitzea suposatzen du ahotsaren sakontzearekin.

- **Funtzio sexualen garapena:** Androgenoek espermatogenesia eta gizakiaren jarrera sexuala kontrolatzen dute emakumezko zein gizonezkoetan. Aipatzekoa da, emakumezkoetan giltzurrun kortexa dela andorgenoen iturririk nagusiena.
- **Anabolismoaren estimulazioa**: Androgenoak hormona anabolikoak dira eta proteinen sintesia bultzatzen dute. Efektu hau argia da gizonezkoen muskuluen tamainan eta hezurren masan emakumezkoekin alderatuta.

Beraz, feedback negatiboak testosteronaren ekoizpena erregulatzen du. Odoleko testosterona kontzentrazioa altua denean, hipotalamoko zelulen GnRH jariaketa inhibitzen da. Ondorioz, hipotalamotik aurreko pituitara doan odol emarian GnRH maila txikitzen da. Hori dela eta, aurreko pituitariako gonadotrofoek LH gutxiago jariatzen dute, odol sistemikoan mailak jaitsiz eta ondorioz, Leydig zelulek testosterona gutxiago jariatuz. Aitzitik, odoleko testosterona maila oso baxua bada, hipotalamoak berriz ere GnRH jariatzen du aurreko pituitariaren bidezko LH jarioa areagotuz. LHak era berean testosterona sintesia aktibatzen du testikuluetan (Tortora and Derrickson, 2013).



1.5. Irudia.Espermatogenesiaren erregulazioa eta testosterona eta dihidrotestosteronaren (DHT) eragina. Azalpena testuan. Lerro gorriak feedback negatiboaren inhibizioari egiten dio erreferentzia. (Tortora and Derrickson, 2013-tik moldatua).

1.3. ESPERMATOZOIDEAREN MORFOLOGIA

Espermatozoidea gorputzean aurki daitekeen zelularik txikiena eta espezializatuena da. Egunero 300 milioi espermatozoide inguru sortzen dira espermatogenesi prozesuan zehar. Espermatozoideak 60 µm-ko luzera (5 µm buruak) du eta obozitoa ernaltzeko egitura bereziak behar ditu. Hain zuzen ere, espermatozoidea bi zati nagusitan banatzen da: burua eta isatsa.

1.3.1. Burua

Espermatozoidearen burua konikoa da formaz eta akrosomaz, nukleoaz eta zitoplasmaz osaturik dago:

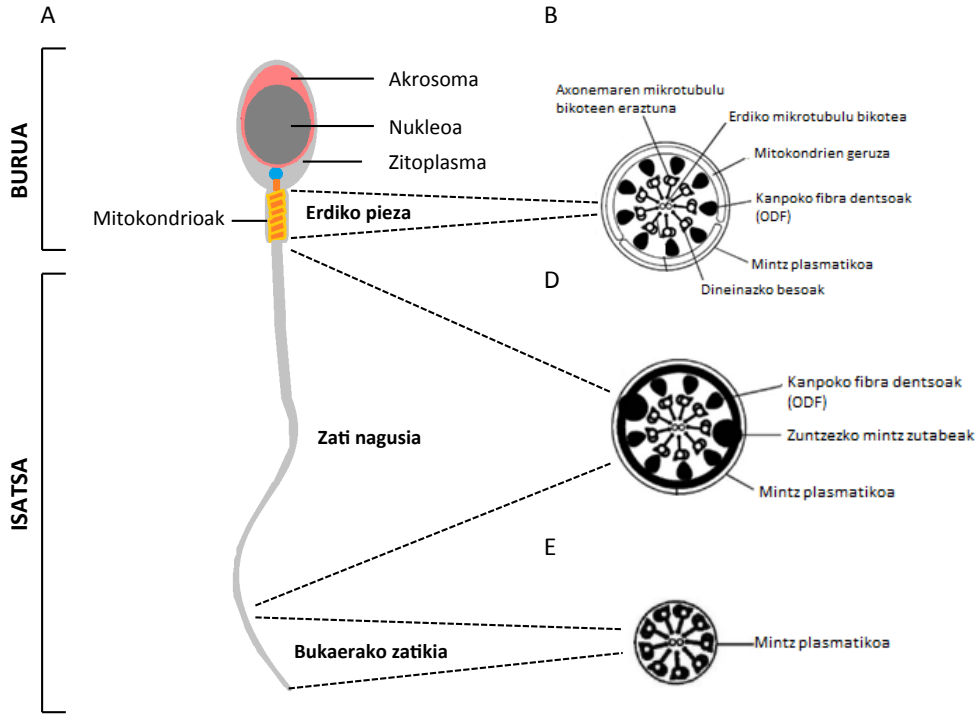
- ***Akrosoma:*** Espermatozoidearen buruaren alde apikalean kokatzen den besikula da. Espezifikoki nukleoaren eta mintz plasmatikoaren artean kokatzen da eta espermatogenesian zehar eratzen da, Golgi aparatutik hain zuzen ere. Akrosoma mintz batez inguraturik dago eta barruan fosfatasa azidoa edota hialuronidasa bezalako entzima hidrolitiko desberdinak gordetzen ditu. Entzima hauek, espermatozoideak obozitoaren matrize estrazelularra (mintz peluzidoa) zeharkatzeko beharrezkoak dira. Izan ere, izaera proteolitikoa daukate eta mintz plasmatikoa disolbatzen laguntzen dute. Akrosomaren barneko entzimen askapenari erreakzio akrosomikoa deritzo eta aurrerago azalduko dugu.

- ***Nukleoa:*** Nukleoak espermatozoidearen buruan dagoen espazioaren zatirik handiena okupatzen du eta honen formak espermatozoidearen buruaren forma baldintzatzen du. Egitura hau mintz nuklear batez inguraturik dago eta normalean honen ondoan zentriolo proximala aurkitzen da. Espermatozoideak erreakzio akrosomikoa jasan eta gero, nukleoa obozitora sartzen du. Nukleoak DNA eta proteina basikoak gordetzen ditu eta ez du nukleolorik honen barnean (Alberts et al., 2002). DNA oso paketatutarik dago espermatogenesian zehar protaminek histonak ordezkatu baitituzte eta ondorioz, transkripzioaren prozesua inaktibo mantentzen da proteina berriak sortzeko makinaria badute ere (Balhorn, 2007).

  Kromatinaren kondentsazio handiagatik, ez da ARN mezulari (mRNA) berririk *de novo* sortuko, eta zitoplasmaren tamaina murritza dela eta, mRNA hori ez da proteinetara itzuliko. Espermatozoide helduek beraz proteinen adierazpena inaktibaturik izango dute (Miller and Ostermeier, 2006). Honek esan nahi du espermatozoideak ez direla proteina berriak eratzeko gai eta espermatogenesian zehar sortutako bildumarekin heldu beharko direla obozitora. Hala ere, ikusi izan da epididimoan zeharreko garraioan, likido seminalean kontaktuan dauden bitartean edota emakumezkoaren ugal-aparatuan barrena daudenean, proteina eta lipidoen trukea gertatzen dela euren fisiologia modulatu ahal izateko (Schuel and Burkman, 2005). Bestalde, badirudi orain dela hamarkada bat sortutako hipotesia mantendu egiten dela. Izan ere, zenbait ikerlarik proposatu izan dute proteina jakin batzuen mRNA-ren itzulpena

mitokondrioetan egiten dela (Miller and Ostermeier, 2006). Honez gain mRNA hau, badirudi baliogarria litzakeela zigotoaren garapenerako lehenengo faseetan.

- ***Zitoplasma:*** Espermatozoideek zitoplasma oso murritza dute, izan ere, espermatogenesiaren azken fasean edo espermiogenesian galtzen dute. Espezifikoki, espermatozoideak epididimoan heltzen direnean eta tubulu seminiferoen epiteliotik askatzera doazenean gertatzen da. Askatzen den zitoplasma zatiari "hondakin gorputza" deritzo. Testikuluen lumen tubularrean, Sertoli zelulek hondakin gorputzen zatirik handiena fagozitatzen dute eta geratzen den zitoplasma zatiari "tanta zitoplasmatikoa" deritzo (Huszar et al., 1998). Giza espermatozoideek tanta zitoplasmatikoaren zati txikia erdiko piezan atxikiturik dute (Rengan et al., 2012).



1.6. Irudia. Espermatozoidearen eta isatsaren ultraegituraren irudi eskematikoa. **A)** Gizakiaren espermatozoidea buruaz (akrosoma, nukleoa eta zitoplasma) eta isatsaz (lepoa, tarteko zatikia, zatiki nagusia eta bukaerako zatikia) osaturik dago. **B)** Tarteko zatiaren zehar-ebakiaren eskema. Bertan, kanpotik barrura, mintz plasmatikoa, mitokondrien geruza, 9 kanpo-zuntz dentso eta axonema eratzen duten egiturak [mikortubulu-bikoteen eraztuna, dineinazko besoak eta erdiko mikrotubulu-bikotea] daude. **D)** Zatiki nagusiaren zehar-ebakiaren eskema. Bertan, mintz plasmatikoa, 7 kanpo-zuntz dentso eta 2 zuntzezko mintz zutabe daude. Axonemako osagaiak ez dira aldatzen. **E)** Bukaerako zatiaren zehar-ebakiaren eskema. Bertan, mintz plamatikoa eta axonema agertzen dira. Azalpena testuan. (Turner, 2006-tik moldatua).

1.3.2. Isatsa edo flageloa

Espermatozoidearen isatsa edo flageloak gameto arrari mugitzeko ahalmena ematen dio. Zelula eukariotikoen flageloetan, mugimendua sortzeko motorrari axonema deritzo. Axonemaren egitura espermatozoideen isatsaren zatiki guztietan mantentzen den egitura komuna da eta mikrotubuluz eta proteinez osaturik dago. Kanpoko aldean, tubulinaz eraturiko 9 mikrotubulu bikotea agertzen dira eraztun bat osatuz eta erdian bi mikrotubulu azaltzen dira. Mugimendu flagelarra dineina izeneko proteina motore batzuen bidez gertatzen da eta hauek mikrotubulu bikoteetara loturik daude. Proteina horrek, ATP hidrolisiaren bidez, energia kimikoa energia mekaniko bihurtzeko ahalmena dauka mikrotubuluen arteko "irristadura" ahalbidetuz, eta ondorioz mugimendua sortuz (Alberts et al., 2002; Marigomez and Cajaraville, 1999). Isatsa lau zatitan banatzen da: lepoa, tarteko zatikia, zatiki nagusia eta bukaerako zatikia (Tortora and Derrickson, 2013):

-***Lepoa:*** Buruaren eta isatsaren arteko giltzadura da. Lepoa bi zentriolo motaz osaturik dago: zentriolo proximala eta distala. Bi zentrioloak oso hurbil kokaturik daude bata bestearengatik eta ernalkuntza prozesuan zehar nukleoarekin batera obozitora sartzen dira. Izan ere, bi zentriolo mota hauek beharrezkoak dira zigotoaren zatiketa zelularra hasteko. Ezaguna denez, zentrioloek ardatz mitotikoa sortzen dute. Aipatzekoa da zentriolo distalak espermatozoidearen isatsaren mikrotubuluen eraketan parte hartzen duela.

-Tarteko zatikia: Axonemaren inguruan, 9 kanpo-zuntz dentso daude eta haien inguruan mitokondrioz osaturiko geruza bat dago. Askotan, mitokondrio ugari agertzen dira fusionaturik zuntzen inguruan espiral bat sortuz. Mitokondrioek ATPa sortzen dute, zelularen mugikortasuna lortzeko. Dena inguratzen mikrotubuluz osatutako mintz plasmatikoa dago. Mintz horri "*manchette*" deitzen zaio.

-Zatiki nagusia: Espermatozoidearen flageloaren zatirik luzeena osatzen du eta axonema inguratzen duten kanpo zuntz-dentsoak daude, baina egitura honetako 3. eta 8. posizioetan zuntzezko bi zutabe agertzen dira. Antolaketa honek, bi konpartimentutan banatzen du zatiki nagusia: batek 3 zuntz izanik eta besteak 4, isatsaren bultzada areagotuz norabide baterantz. Zatiki honetan ere ATPa sortzen da.

-***Bukaerako zatikia:*** Axonema eta mintz plasmatikoaz osaturik dago.

1.4. ERNALKETA

Espermatozoidearen eta obozitoaren arteko loturari ernalketa deritzo eta umetoki tronparen anpuluan gertatzen da. Hori dela eta, lehenengo eta behin espermatozoideak emakumezkoaren umetoki tronpara heldu behar dira. Bertan, obariotik askatu den eta tronpara migratu duen obulua egongo da zain ernalketa gerta dadin (Mortimer, 1997).
Espermatozoideek ernaltzeko gaitasuna lortzeko eraldaketa morfologikoak jasateaz gain, emakumezkoaren ugaltze traktuan zenbait aldaketa fisiologiko eta biokimiko pairatu behar dituzte. Modu sekuentzialean, espermatozoideek mugitzeko ahalmena, kapazitazioa eta hiperaktibazioa garatu behar dituzte, eta behin oboziotoarekin kontaktuan daudenean, erreakzio akrosomikoa jasan behar dute honen barrura sartu ahal izateko eta zigotoa eratzeko (Flesch and Gadella, 2000).

1.4.1. Mugikortasun Espermatikoa

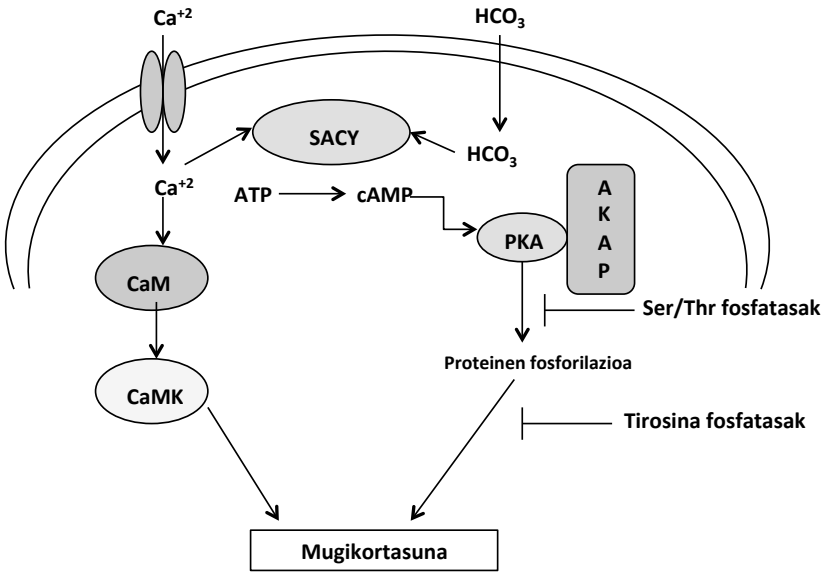
Lehenik eta behin espermatozoideek mugitzeko ahalmena garatu behar dute. Nahitaezko ezaugarria da ernalkuntza gertatzeko. Izan ere, testikuluetatik eratorriak diren espermatozoideak zelula oso espezializatuak izan arren, oraindik ez baitira obulua ernaltzeko gai. Honen arrazoia dakartzaten mugikortasun nulua edo oso ahula izan daiteke eta hori mintz plasmatikoaren heltze faltari dagokio. Horregatik, espermatozoideek epipidimotik bidaiatzen doazen heinean mugikortasun progesiboa eta gameto emearekin elkarrekintza izateko ahalmena garatuko dituzte (Yanagimachi, 1994). Hala ere espermatozoideek ez dute beti mugikortasun patroi berbera aurkezten, izan ere, emakumezkoaren ugaltze traktuan barrena egoera askori aurre egin beharko dizkiete.

Alde batetik, mugikortasun aktiboa daukaten espermatozoideak daude eta horiek bide simetrikoak eta progresiboak egiten dituzte espermatozoidea zuzenki bideratuz oso biskosoak ez diren medioetan barrena (Turner, 2006). Beste aldetik, hiperaktibazioa pairatzen duten espermatozoideak daude. Horiek mugikortasun hiperaktiboa daukate. Prozesu hori kapazitazioarekin batera gertatzen da eta ernalketarako prozesu kritikoa dela onartzen da. Mugikortasunaren patroi aldaketa horiek fekundazioaren momentuan agertzen dira eta Yanagimachik (Yanagimachi, 1970) deskribatu izan zituen lehenengoz. Mugimendu horretan, flageloaren irabiapenaren anplitudearen gorakada bat gertatzen da, mugimendu asimetrikoez gain (Suarez, 2008a; Yanagimachi, 1994). Hori dela eta, espermatozoideak obulura arte egin behar duen bidea zein ernalketarako matrize extrazelularrean egin behar duen barneraketa, erabat menpekoak dira espermatozoidearen mugikortasun ahalmenarekiko.

Espezifikoki, espermatozoidearen isatsaren mugimendua axonemak sorturiko astinduran datza; mikrotubuluen arteko irristaduran, hain zuzen ere (Marigomez and Cajaraville, 1999). Axonema 250 bat proteina inguruz osaturik dago eta hauen artean tubulina bezalako proteina zitoeskeletikoak, dineina bezalako proteina motoreak, proteina txaperonak, kaltzioa lotzen duten proteinak zein proteina kinasak aurkitu daitezke besteak beste (Inaba, 2003). ATPa axonemaren dineina besoetara lotzen denean, dineinaren ATPasa aktibatzen da eta honen hidrolisiak eraginda dineinak konformazio aldaketa jasaten du, mikrotubuluen gainean

mugitzeko ahalmena garatuz. Aipatzekoa da axonemaren dineina molekula guztiak ez direla batera aktibatzen eta oinaldeko molekulak aktibatzen direla lehenik. Aktibazio hori ondoren axonemaren puntetarantz hedatu egiten da proteinaz proteina motako elkarrekintzen bidez (Marigomez and Cajaraville, 1999; Turner, 2006). Espermatozoideek ATP iturria mitokondrioetan gertatzen den fosforilazio oxidatibotik eskuratzen dute baina flageloan organulu hau ez daukatenez, garrantzitsua da zatiki nagusiaren zuntzezko mintzetan gertatzen den glikolisia (Turner, 2006).

Edozein zelulak bezala, espermatozoideek seinalizazio-bide asko aktibatu edo inhibitzen dituzte erantzun espezifikoak sortzeko. Mugikortasunari dagokionez, cAMP/ proteina kinasa A (PKA) eta kaltzioari loturikoa dira nagusi (Ho et al., 2002; Turner, 2006; Yanagimachi, 1994). Espezifikoki, behin kaltzio ioia zelula barnera sartzean edota espermatozoidearen barneko kaltzio biltegiak hustutzean, kaltzioak, kalmodulina (CaM) proteinara lotu eta kalmodulina kinasa (CaMK) aktibatzen du mugikortasuna aktibatuz (Ignotz and Suarez, 2005; Turner, 2006). Beste alde batetik, ezaguna da kaltzioak espermatozoideetan espresatzen den ezohiko adenilato ziklasa solugarria (SACY) aktibatzeko gai dela (bikarbonatoak ere aktibatzen du) cAMP sortzen du (1.7 Irudia). Ondoren, ATP-tik sortzen den cAMP-ak, cAMPren menpeko PKA aktibatzen du eta proteina kinasa honek flageloan aurkitzen diren tirosina kinasak estimulatuko ditu. Esaterako, dineina bezalako zenbait proteína fosforilatuko ditu mugikortasuna sortuz (Tash, 1989; Turner, 2006).



1.7. Irudia. Giza espermatozoideen mugikortasunaren erregulazioan parte hartzen duten hainbat seinaleztatze-bideen eskema: Kaltzioaren menpeko bideak eta SACY menpeko bideak bat egin dute mugikortasuna erregulatzeko. Azalpena testuan.

Kaltzioa eta bikarbonatoak estimulatzen dituzten seinaliztatze-bidezidor horiez gain, espermatozoideen pH-an gertatutako aldaketek, GTPa lotzen duen proteina eraentzaile (G proteina; ingelesezko GTP binding regulatory protein) txikiek edota heterotrimerikoez

gidaturiko seinaleztatzeak ere zerikusia dute mugikortasunaren erregulazioan (Turner, 2006; D. Wang et al., 2003; Yanagimachi, 1994). Hala ere, mekanismo horiek ez dira oraindik oso ondo ezagutzen.

1.4.2. Kapazitazioa

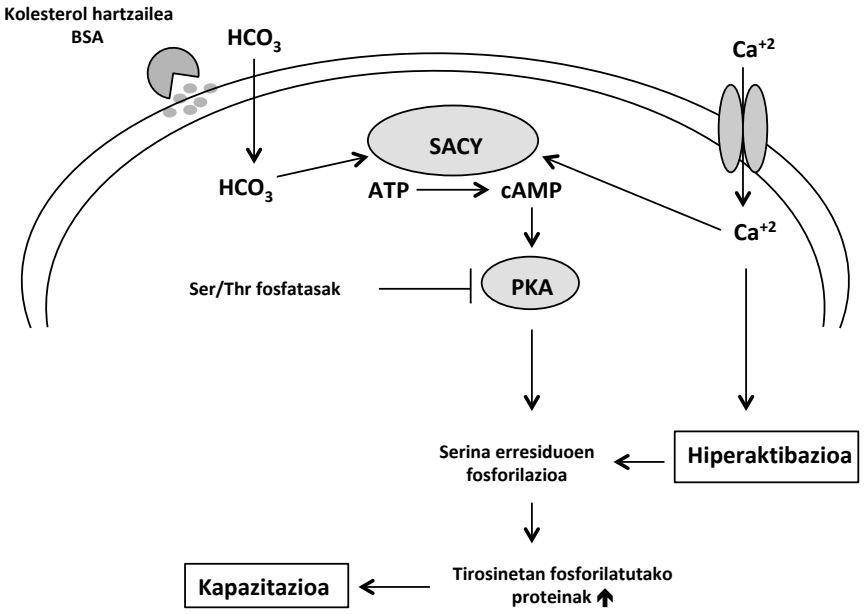
Behin, espermatozoideak mugitzeko ahalmena garatu duela, kapazitazioa izeneko prozesua gertatzen da. Lehen aldiz, era independientean, Austinek (Austin, 1952) eta Changek (Chang, 1951) deskribatu zuten. Kapazitazioa prozesu itzulgarria da zeina espermatozoideari lotuta dauden faktoreen desagerpenarekin hasten den. Faktore horiek plasma seminaletik eratorriak dira eta kapazitazioa bukatutzat ematen da espermatozoidea, obuluaren Zona Pelluzida-ren estimuluei erantzuteko gai denean. Hori gertatu ondoren, espermatozoidea prest dago erreakzio akrosomikoa jasateko. Kapazitazioan zehar espermatozoidearen mintz plasmatikoan aldaketa biokimikoak daude (Yanagimachi, 1994) zeinak, gainazal zelularrean dauden molekulen berrantolatzea sortzeaz gain, mintz plasmatikoa desantolatzen duen eta hiperaktibazio espermatikoa sorrarazten duen. Era horretan, espermatozoidea erreakzio akrosomikoa jasateko prestatzen da (Visconti et al., 2002).

Kapazitazioan zehar espermatozoideak jasaten dituen aldaketa biokimikoei dagokienez, espermatozoideek euren mintz plasmatikotik kolesterola askatu behar dute (Visconti et al., 2002), mintz plasmatikoaren fluidotasuna eta proteinen antolaketa aldatzeko. Horren ondorioz, espermatozoidearen matrize extrazelularrak zenbait aldaketa jasaten ditu ioi extrazelularren kontzentrazioan eta osmolaritatean, pH-aren alkalinizazioa bideratuz. Dakigunez, kapazitazioa HCO_3^- -ren menpoekoa da (Visconti et al., 2002) zeinak cAMP-ren metabolismoa erregulatzen duen espermatozoideen espezifikoa den SACY aktibatuko duen. Horren bitartez, espermatozoidearen ahalmen ugalkorra garatzeko beharrezkoak diren hainbat prozesu bideratzen dira, mugikortasuna eta kapazitazioa esaterako (Hess et al., 2005). Adenilato ziklasaren estimulazioaren ondorioz, cAMP-ren sintesia gertatzen da (Hess et al., 2005). cAMP-ari dagokionez, mugikortasun, kapazitazio eta erreakzio akrosomikoaren erregulatzaile nagusia dela dirudi.

Zenbait ikerketek erakutsi duten bezala kapazitazioa ere kaltzioaren menpekoa da (Jimenez-Gonzalez et al., 2006). Kaltzioaren bidez gertatzen den kapazitazioaren erregulazioa zenbait bideren bidez gertatzen da eta horietako zenbait cAMP-ren metabolismoarekin lotuta daude. Izan ere, Ca^{+2}-ak ere adenilato ziklasaren estimulazioan paper garrantzitsua betetzen du (HCO_3^- -rekin batera). Espermatozoideetan Ca^{+2}/kalmodulina konplexuak adenilato ziklasaren bitarteko cAMP-ren sintesia (Lasko et al., 2012) eta fosfodiesterasen bidezko cAMP-ren degradazioa (Galantino-Homer et al., 2004) aktibatu ditzazke.

Bikarbonatoak adenilato ziklasa solugarria aktibatzean sortzen den cAMP-ak, proteina kinasa A (PKA) aktibatzen du eta horrek era berean kapazitazioa gertatzeko beharrezkoak diren beste proteina batzuk fosforilatuko ditu. Fase horretan zehar, PKA-k zenbait proteina aktibatuko ditu serina eta treonina hondarretan, eta hauek, zuzenean edo zeharka, beste proteina kinasa

batzuk aktibatuz edota proteina fosfatasak inhibituz tirosina hondarren fosforilazioa handituko dute (1.8 Irudia).
Laburbilduz, kapazitazioaren prozesuan 1) mintz plasmatikoaren fluidotasunean aldaketak 2) pH intrazelularraren igoera 3) espermatozoidearen mintz plasmatikoaren potentzialaren hiperpolarizazioa 4) cAMP-ren seinalizazio bidearen aktibazioa, 5) kaltzio intrazelularraren kontzentrazioaren igoera eta 6) tirosina hondarren fosforilazioen mailen handipena, bezalako gertaera molekular eta fisiologikoak gertatzen dira.



1.8. Irudia. Kapazitazioarekin asoziaturiko prozesu molekularrak. Azalpena testuan.

1.4.3. Erreakzio Akrosomikoa

In vivo kapazitazioa eta gero gertatzen den prozesu bat da eta espermatozoidearen mintz plasmatikoaren eta kanpo-mintz akrosomalaren arteko fusioaren ondorioz gertatzen den exozitosian datza. Azkenik barne -mintz akrosomala agerian geratzen da (Patrat et al., 2000). Akrosoman dauden entzimak askatzen dira eta horiek ahalbidetzen dute espermatozoidea granulosaren zeluletatik zeharkatzea. Espermatozoideak obuluaren *Zona Pelluzida*ra elkartzen direnean (ZP3 proteinei esker), akrosomaren kanpo-mintza erabat disolbatu, akrosomaren barneko osagaiak askatu eta espermatozoidea *Zona Pelluzida* zeharkatzen ahalbidetzen du.
Progesterona, ZP3, prostaglandinak, esterolaren sulfatoak, glukosaminglikanoak… erreakzio akrosomikoaren induktoreak izan daitezke eta hauek folikuluaren fluidoetan zein *cumulus oophurus* zeluletan aurkitu daitezke. Aipatzekoa da, badaudela erreakzio akrosomikoaren induktore sintetiko edo artifizialak kaltzioaren ionoforoak, lektinak edota neoglikoproteinak bezalakoak (Abou-haila and Tulsiani, 2009).

Erreakzio akrosomikoa seinalizazio zelular konplexuek bideratzen dute: proteina kinasen aktibazioa eta euren ondoriozko proteina intrazelularren fosforilazioa eta kanal ionikoen

aktibazioa (Breitbart, 2002; Herrick et al., 2005). Izan ere, zitosolean dagoen kaltzioaren kontzentrazioaren igoera bat nahitaezkoa eta beharrezkoa da akrosomaren exozitosia eta erreakzio akrosomikoa gertatzeko (Patrat et al., 2000). Kaltzioaren igoera hori zenbait bidez gerta daiteke: kanpo ingurutik sartuta, organuluetan gordeta zegoen kaltzioaren mobilizazioak (akrosomatik edota mitokondriatik)… etab. Beraz, esan daiteke kaltzioa exozitosi akrosomalaren erregulatzaile nagusiena dela (Darszon et al., 2001; Lishko et al., 2011; Publicover and Barratt, 1999).

Dirudienez, kaltzio horren sarrera kaltzio-espezifikoak diren kanaleek bideratzen dute. Kaltzio kanale hauek espermatozoideen mintz plasmatikoan aurkitzen dira eta mota askotakoak izan daitezke. Hala ere, gaur egun, CatSper izeneko kaltzio kanale espermatozoide-espezifiko batzuk aurkitu izan dira eta badirudi horiek direla progesteronak sortutako kaltzio intrazelularraren igoeraren arduradun nagusienak. Kanale hauek, boltai menpekoak eta progesteronarekiko sentikorrak ez ezik, pH-arekiko sentikorrak ere badira eta espermatozoidearen flageloaren zati nagusian aurkitzen dira. CatSper bidezko barruranzko kaltzio fluxuak espermatozoidearen hiperaktibazioa, kimiotaxia eta erreakzio akrosomikoa kontrolatzen ditu (Lishko et al., 2011).

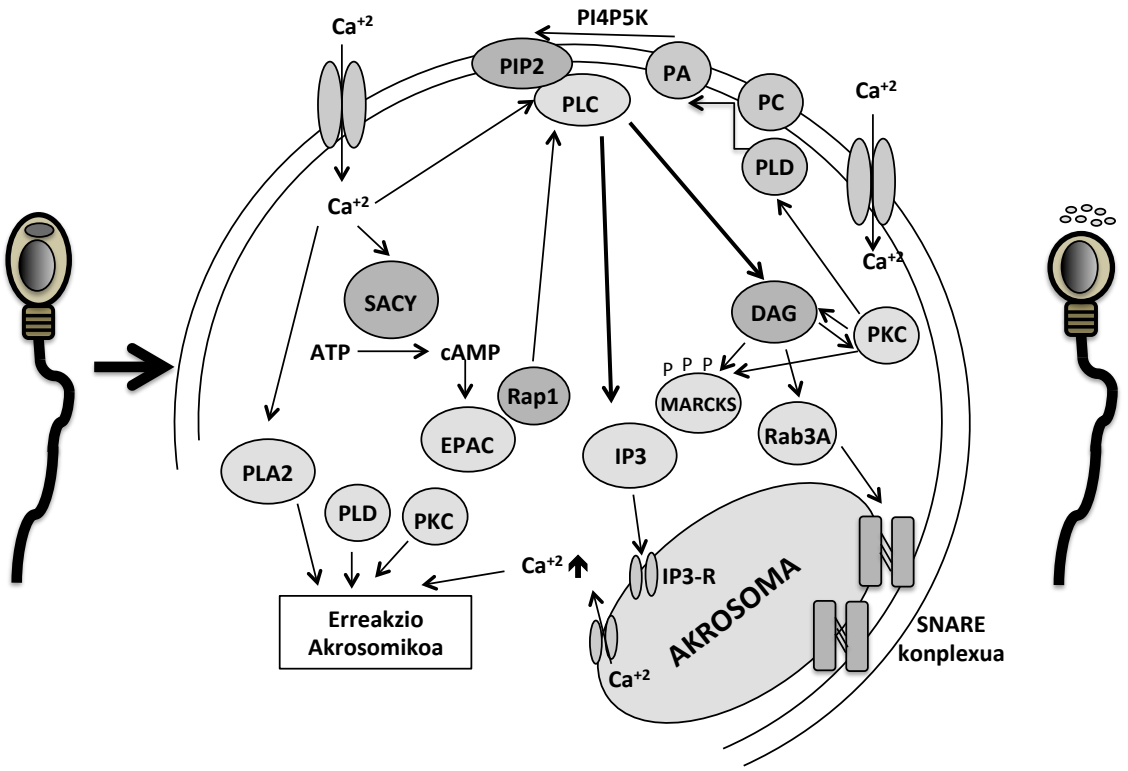
Kaltzioaren sarrerak, proteina kinasa C (PKC), fosfolipasa A2 (PLA2), SACY, fosfolipasa C (PLC) edo inositol trifosfato (IP3) bezalako entzimen aktibitatea erregula ditzake zuzenean espermatozoidearen fisiologian eraginez (Allegrucci et al., 2001; Gerwins and Fredholm, 1995; Lopez et al., 2012; Reinhardt et al., 1999; Stojilković et al., 1992; Suhaiman et al., 2010; Vargaftiq and Braquet, 1987).

Alde batetik, kaltzioak PKC proteina aktibatzen du, erreakzio akrosomikoaren seinaliztapen bidezidor intrazelularrean paper garrantzitsua jokatzen duena (Rodriguez Peña et al., 2013). PKC proteinak giza espermatoideetan erregulatzen dituen prozesuak oso ezagunak ez badira ere, fosfolipasa D (PLD), diazilglizerola (DAG) edota *myristoylated alanine-rich C kinasak* (MARCKS) deritzen sustratuak aktibatu ditzazke (Lopez et al., 2012; Rodriguez Peña et al., 2013). PLD entzimak funtzio garrantzitsua dauka exozitosiko mintzen arteko fusioa erregulatzen, izan ere fosfatidilkolina (PC) azido fosfatidikoan (PA) hidrolizatzen du (Hu et al., 2011).

Ondoren PA-k, fosfatidilinositol 4-fosfato-5-kinasa (PIP4P5K) izeneko entzima aktibatzen du fosfatidilinositol 4,5-bisfosfatoa (PIP2) sortuz. Era berean, PKC entzimak MARCKS-en fosforilazioa induzitzen du, PIP2-tik askapena sustatuz eta kaltzioaren mobilizazioa bultzatuz (Rodriguez Peña et al, 2013). PLC entzimak PIP2-ren hidrolisia areagotuko du DAG eta IP3 sortuko du (Rodriguez Peña et al, 2013). Bigarren mezulari hauek seinaliztapen bidezidor anitzak induzituko dituzte.

Bereziki, IP3-a espermatozoideen akrosoman eta lepoan aurkitzen diren kaltzio erreserben IP3 hartzaileetara lotuko da eta kaltzioaren askapena sustatuz, erreakzio akrosomikoa bultzatuko du. Bestalde, DAG-ak PIP2-aren ekoizpena eta PKC-ren aktibazioa erregulatzeaz gain, mintzaren fusioaren makinariaren muntaian inplikaturiko seinaliztapen bidezidorrarekin elkartzeko gai da (Lopez et al., 2012). Izan ere, DAG-ak Rab3A aktibatzen du, SNARE

konplexuen eraketan parte hartzen duena. Ondorioz, mintz plasmatikoan kokatzen den SNARE-ak, kanpo mintz akrosomalean aurkitzen diren SNARE-ekin elkarrekingo dute eta kaltzioak erreakzio akrosomikoan askatzen diren besikulen eraketan parte hartuko du (Lopez et al., 2012). Era berean zelula kanpoko kaltzioaren barneraketak SACY entzima aktibatzen du cAMP mailaren gorakada eraginez. Horrek, Epac izeneko proteina aktibatzen du zeinak Rap1 proteinaren estimulazioaren bitartez, PLC entzima pizten du (Lopez et al., 2012; Lucchesi et al., 2016). Horrez gain, kaltzioaren fluxuak PLA2 ere estimula dezake, erreakzio akrosomikoa bultzatzen duena (Patrat et al., 2000) (1.9 Irudia).



1.9. Irudia. Giza espermatozoideen erreakzio akrosomikoarekin loturiko prozesu molekularrak. Ezkerrean erreakzio akrosomikoa jasan ez duen espermatozoidea akrosoma duelarik. Erdian, erreakzio akrosomikoarekin erlazionaturiko prozesu molekularrak. Eskuman, erreakzio akrosomikoa pairatu duen espermatozoidea, akrosoma kanporatu duelarik (Azalpena testuan).

1.4.4. Gametoen mintzen fusioa

Behin espermatozoideak erreakzio akrosomikoa jasan eta *Zona Pelluzida* zeharkatu duela, bi mintz plasmatikoen arteko elkarrekintza gertatzen da. Erreakzio akrosomikoaren ostean espermatozoidearen gainazalaren beste berrantolaketa bat gertatzen da, barne mintz akrosomala agerian geratuz. Ondoren, obuluaren eta espermatozoidearen mintz plasmatikoaren elkartze eta fusioa gertatzen da segmentu ekuatorialean eta horrela fusioa gameto bakoitzaren alde espezifiko batera mugatzen da (de Lamirande et al., 1997). Gametoen fusioa gertatu eta gero, espermatozoidearen material genetikoa obuluarenean barneratzen da zigoto totipotente bat sortzeko.

Arestian aipatutako prozesu biokimiko eta fisiologikoak beharrezkoak dira espermatozoideek ahalmen ugalkorra garatzeko. Prozesu hauek mekanismo ionotropikoen bitartez erregulaturik badaude ere, gaur egun uste da, espermatozoideen mintz plasmatikoan dauden G proteinetara loturiko hartzaileak (GPCR) modulatzaile garrantzitsuak izan daitezkeela. Ondorengo, *review*-ak GPCR hartzaileen funtzioari eta hauen azpitik induzitzen diren mekanismo molekularrei buruzko informazioa jasotzen du. Aipatzekoa da, lan hau ingelesez egin dela, aldizkari zientifiko batera bidali izan delako.

1.5. SPERM-SPECIFIC MOLECULAR MECHANISMS UNDERLYING G PROTEIN COUPLED RECEPTORS IN HUMAN SPERMATOZOA

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Abstract

Background: G-protein coupled receptors (GPCRs) belong to the seven transmembrane receptor superfamily encoded by approximately 1% of the human genome and are considered to be one of the best therapeutic targets. In response to external stimuli, they transduce signals via G proteins to initiate numerous different intracellular signaling pathways, which culminate in specific cellular responses. While in somatic cells GPCRs participate in a broad variety of physiological processes, the expression of diverse GPCRs on the surface of human spermatozoa suggests their involvement in the regulation of sperm fertility.

Objective and rationale: Recent accomplishments encourage a review of the literature on the role and molecular mechanisms downstream of GPCRs in human spermatozoa due to the fact that they possess sperm-specific proteins.

Search methods: We performed a systematic search using PubMed up to November 2018. We included human studies that examined the effect of GPCR ligands on the signaling pathways of human spermatozoa and sperm fertility.

Outcomes: Our search identified the most remarkable peculiarities and differences that make the molecular mechanisms underlying GPCR distinct and unique in human spermatozoa. Moreover, we provide the longest list of GPCRs described in human male germ cells to date with 36 types of receptors, as well as the description of their role in sperm physiology.

Wider implications: A better understanding of the specific features of the intracellular signaling pathways in human spermatozoa could be helpful for different researchers in fully comprehending the etiology of many cases of infertility as well as developing new therapeutic strategies.

Introduction

Ejaculated mammalian sperm cells are immature and infertile and must undergo many biochemical and physiological modifications in the female oviduct to become fertile (Suarez, 2008a). Sequentially, human spermatozoa go through processes including motility, capacitation, hyperactivation and acrosome reaction, all these being considered key functions in sperm fertility (Baldi *et al.*, 1996).

It is well established that ion channels and transporters are the main regulators of sperm fertility via rapid signaling events. In fact, they control the membrane potential, cytoplasmic Ca^{+2} and intracellular pH, crucial for successful fertilization (Babcock *et al.*, 1983; Dan, 1954; Hille, 1992; Yanagimachi and Usui, 1974). Specifically, selective ion channels such as Hv1 and Nav1.8 expressed in human spermatozoa (Lishko and Kirichok, 2010) are considered essential for cell alkalization and depolarization (Ramsey *et al.*, 2006) and control sperm motility (Cejudo-Roman *et al.*, 2013; Lishko and Kirichok, 2010).

On the other hand, the KSper channel, which is permeable to K^{+}, is activated through an increase in pH (Chávez *et al.*, 2013) inducing membrane hyperpolarization and being essential for capacitation and hyperactivation. In addition, human sperm hyperactive motility and acrosome reaction are mainly regulated by Ca^{+2} (Roldan and Shi, 2007; Yanagimachi, 1994). Various different calcium channel families have been described on the surface of human spermatozoa including CatSper, CNG, voltage-activated Cav, and TRP channels (Brenker *et al.*, 2012; Darszon *et al.*, 2011; Publicover *et al.*, 2007). Specifically, CatSper channels are located in the flagellum of spermatozoa and are voltage- and progesterone-dependent, sensitive to pH (Lishko *et al.*, 2011) and responsible for hyperactive motility and the progesterone-dependent acrosome reaction. In fact, CatSper has been shown to be required for sperm fertilization, knockout mice being infertile due to impaired calcium signaling (Qi *et al.*, 2007). In addition, mutations in CatSper genes have been correlated with human infertility (Hildebrand *et al.*, 2010).
Despite of the importance of the ion channels in the regulation of sperm fertility, the presence of a large number of G-protein coupled receptors (GPCRs) in human spermatozoa suggests that not only ionic mechanisms but also metabotropic mechanisms could play an important role in sperm fertilization. Specifically, it is believed that the signals originated in the oviduct as well as the substances released by the egg chemotactically attract sperm by metabotropic mechanisms (Flesch and Gadella, 2000; Suarez, 2008a).

Over the last 20 years, numerous papers have reported the presence of diverse GPCRs in the plasma membrane of human spermatozoa as well as their involvement in the regulation and modulation of sperm motility, capacitation, hyperactivation and acrosome reaction (See Supplementary Table S1, See CD). Nevertheless, although various authors have tried to decipher the signaling pathways underlying this receptor superfamily, the truth is that little is known about the features and peculiarities of GPCR-induced signaling pathways.

While the signaling pathways of mouse spermatozoa have been studied for several decades (See reviews Buffone *et al.*, 2014; Visconti *et al.*, 2002, 2011), this review focuses on human spermatozoa, and in particular on the differences identified to date in the molecular mechanisms induced by GPCRs as well as the roles of different GPCRs. In fact, it has previously been suggested that there are distinguishing dissimilarities in the signaling pathways downstream of GPCRs and sperm fertility in human spermatozoa compared to other sperm species (Kaupp and Strünker, 2017). Therefore, a better understanding of the human sperm-specific molecular mechanisms could help us to explain cases of male infertility and identify new therapeutic targets.

G protein coupled receptors

G protein coupled receptors are seven transmembrane receptors (7TM) that transduce signals via G proteins to initiate various different intracellular signaling pathways and produce specific cellular responses. This type of receptors are found in certain prokaryotic and all eukaryotic cells from yeast to man, being the largest and most diverse membrane bound protein family, encoded by more than 800 genes (1%) in the human genome (Foord *et al.*, 2005; Fredriksson, 2003). Moreover, they are involved in many physiological and pathophysiological processes such as sensory perception, chemotaxis, neurotransmission, cell communication, and the senses of sight, smell and taste, as well as neurological disorders, inflammatory diseases, cancer and metabolic imbalances (Tuteja, 2009). Due to their involvement in human pathophysiology, GPCRs have been studied as targets for drug discovery for more than a decade, this research taking advantage of improvements in high-throughput procedures (Salon *et al.*, 2011).

GPCRs are activated through a wide variety of molecules that act as ligands or primary stimuli such as organic odorants, amines, peptides, proteins, lipids and nucleotides (Tuteja, 2009). The receptor activation proceeds with the stimulation of signal transducers involving the heterotrimeric guanine nucleotide binding proteins (G proteins), and finally achieving certain cellular responses (Tuteja, 2009). Briefly, once the GPCRs perceive the stimulus, the G proteins transduce the signal to produce cellular responses. The G proteins are heterotrimeric polypeptide chains that form two independent subunits: the Gα and Gβγ. In the inactive state, the G protein is trimeric and the Gα and Gβγ subunits are bound by a GDP (Birnbaumer, 2007; Waelbroeck, 2012). When the GPCR interacts with the specific ligand, the GDP is released and the binding of the GTP to the Gα subunit induces dissociation from the Gβγ. In this way, both subunits will recognize and regulate their respective effectors. When the Gα-GTP hydrolyses the GTP to GDP, the resting trimeric complex reforms immediately (Waelbroeck, 2012).

Specifically, heterotrimeric G proteins are responsible for the activation of membrane receptors via the modulation of intracellular effectors (Oldham and Hamm, 2008) such as the calcium and potassium channels, adenylyl cyclase (AC), phospholipase C (PLC), phospholipase A2 (PLA), PLD, phosphoinositide 3-kinase (PI3-kinase) and protein kinases (Tuteja, 2009). The intracellular pathways induced by GPCRs differ depending on the complexity of the cellular response. Classically, two major signaling pathways have been

described as important in the governing of basic cellular activities and coordination of cell actions downstream of GPCRs: the canonical or the G protein-dependent signaling pathway, that leads the activation of AC/cAMP/PKA and/or PLC/DAG-IP_3/ Ca^{+2} signaling pathways; and the non-canonical or the G protein-independent signaling pathway involving GRK/β-arrestin/MAPK activation and receptor desensitization. Although the signaling pathways downstream of GPCRs are largely established, the propagation of the signaling underlying the GPCR can involve a cross-regulation of different and specific pathways at the same time, as well as crosstalk between the G protein-dependent and -independent intracellular mechanisms (Hur and Kim, 2002). In addition, the activation of different signaling pathways is even more complex due to the fact that GPCRs are also able to dimerize forming homodimers or heterodimers to accomplish diverse functions such as recruitment of different protein complexes or receptor desensitization (Gurevich and Gurevich, 2008; Waelbroeck, 2012), among others.

GPCR signaling pathways in human spermatozoa

Over the last decade, several different researchers have reported the presence of a wide variety of GPCRs on the surface of human spermatozoa, mainly involved in the acquisition of sperm fertility through metabotropic mechanisms (See Supplementary Table S1, See CD). Despite the importance of these processes in reproductive health, the molecular mechanisms underlying these receptors are largely unknown. In fact, it has been suggested that the mechanisms of human spermatozoa could present different features to those of the signaling found in somatic and sensory cells (Kaupp and Strünker, 2017). This idea is based on the fact that at least 30% of the proteins in human spermatozoa are unique and sperm-specific, leading to the suggestion that the signaling pathways in which they are involved present specific characteristics (Amaral *et al.*, 2014; Kaupp and Strünker, 2017).

For these reasons, a better knowledge of the molecular cascades downstream of GPCRs in human sperm may be crucial to understanding the etiology of many cases of infertility, and in turn, developing new therapeutic targets and strategies.

G proteins in human spermatozoa

The heterotrimeric guanine nucleotide binding proteins (G proteins) are responsible for the activation of membrane receptors via the modulation of intracellular effectors such as adenylyl cyclase, phospholipases C and A2, and ion channels (Merlet *et al.*, 1999). Nowadays, the functional roles of some G proteins are well understood in somatic cells. For example, $G\alpha_s$ stimulate adenylyl cyclase; $G\alpha_q$ and $G\alpha_{11}$ stimulate phospholipase Cβ and increase RhoGEF protein activity (linked to RhoA activation), and pertussis toxin (PTX)-sensitive G proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$ and $G\alpha_{o2}$) inhibit adenylyl cyclase and participate in the regulation of ion channels and membrane trafficking (Lowry *et al.*, 2002; Lutz *et al.*, 2007). Depending on the $G\alpha_{12/13}$ isoform, they are associated with the p115-RhoGEF or RhoA signaling pathways (Siehler, 2009), whereas the function of $G\alpha_z$ remains unclear. On the other hand, the Gβ subunit together with the Gγ subunit may regulate PLCβ, K^+ channels, adenylyl cyclase and PI3-kinase

activity in somatic cells (Digby *et al.*, 2006; Neves *et al.*, 2002; Sprang *et al.*, 2007; Syrovatkina *et al.*, 2016).

Regarding spermatozoa, although several studies have provided information about the existence of the different G proteins in the sperm of various different species (Adeoya-Osiguwa *et al.*, 2006; Adeoya-Osiguwa and Fraser, 2007; Baxendale and Fraser, 2003; Watling *et al.*, 2001), little is known about the subtypes found in humans (Table 1). In the first study to mention the presence of G proteins in human spermatozoa, published back in 1992, Lee et al. (Lee *et al.*, 1992) described the $G\alpha_i$ subunit in the head region. Since then, various authors have reported the expression of $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{q/11}$, $G\alpha_{olf}$, $G\alpha_o$, $G\alpha_{12/13}$ and $G\alpha_t$ in human spermatozoa, showing different localization patterns within the cell (Allegrucci *et al.*, 2001; Hinsch *et al.*, 1995; Merlet *et al.*, 1999; Pérez-Cerezales *et al.*, 2015; Spehr *et al.*, 2004; Urizar-Arenaza *et al.*,2018, under revision). Although no clear function has been attributed to most of the Gα subunits, photolabeling experiments using human spermatozoa showed that GPCR ligands trigger the activation of $G\alpha_{q/11}$ (Schaefer *et al.*, 1998) to further regulate PLC activity (Allegrucci *et al.*, 2001; Flegel *et al.*, 2016). Furthermore, $G\alpha_{olf}$ and $G_{\alpha t}$ subunits may be involved in sperm chemotaxis and thermotaxis, respectively (Pérez-Cerezales *et al.*, 2015; Spehr *et al.*, 2004).

Concerning the Gβγ subunit, only the presence of Gβ (1,2,4) has been described by different approaches in human spermatozoa. Although it has been localized by immunofluorescence in the midpiece, head, tail and the equatorial segment of the acrosome, to date there are no studies reporting Gβ subunit activation by GPCRs in human spermatozoa (Hinsch *et al.*, 1995; Liu *et al.*, 2006; Merlet *et al.*, 1999; Urizar-Arenaza *et al.*,2018, under revision). Therefore, the molecular mechanisms involved have yet to be identified. Regarding the Gγ subunit, it usually appears bound to Gβ forming the Gβγ complex in somatic cells, but so far no studies have identified this subunit in human spermatozoa.

Nonetheless, considering that human spermatozoa express different isoforms of Gα and Gβ, we suggest that these cells may be able to regulate sperm fertility through G protein-dependent signaling pathways. On the other hand, the absence of Gγ in human spermatozoa leads us to wonder about the existence of heterotrimeric G proteins in male germ cells suggesting that the activation through GPCRs could involve different molecular mechanisms. Given the limited evidence of the direct activation of G proteins through GPCRs in human spermatozoa, further studies are needed to understand the singularities of the underlying molecular mechanisms.

3.2. G protein-dependent signaling pathways

3.2.1 AKAP/PKA signaling pathway in human spermatozoa

The AC/cAMP/PKA signaling pathway is one of the most well studied transduction pathways in somatic cells. Specifically, $G_{\alpha s}$ stimulates the transmembrane adenylyl cyclase (tmAC) and once activated, it catalyzes the synthesis of the second messenger cAMP (Lodish *et al.*, 2000). The effects of cAMP are mediated through protein kinase A (PKA), whose activity intensifies when cAMP production increases (Syrovatkina *et al.*, 2016;

Wettschureck and Offermanns, 2005). This protein kinase is a tetramer composed of two regulatory and two catalytic subunits. Each regulatory subunit has two distinct binding sites and the binding of cAMP to both sites leads to the release of the catalytic subunit, which activates its kinase activity. Then, PKA phosphorylates different target proteins in serine or threonine residues, finally enhancing the cell response (Lodish *et al.*, 2000). The $G_{\alpha i}$ isoform, however, has the opposite effect inhibiting adenylyl cyclase and consequently decreasing cAMP synthesis and PKA activity (Turnham and Scott, 2016). In human spermatozoa, the cAMP/PKA-dependent signaling pathway plays a central role in various aspects of sperm function required for the acquisition of fertilizing capacity. It is well established that cAMP is involved in sperm capacitation, acrosome reaction, membrane lipid remodeling, hyperpolarization of the sperm membrane, increases in pH_i and Ca^{2+}, and protein tyrosine phosphorylation (Buffone *et al.*, 2014). Nevertheless, the activation of this signaling pathway through the GPCR is largely unknown in human spermatozoa.

In these cells, cAMP is mainly synthetized by the specific testicular soluble form of AC (SACY), which is activated by HCO_3^- (Kaupp and Strunker, 2017; Wang et al. 2007) and seems to be insensitive to G-proteins (Buffone *et al.*, 2014). In fact, the presence of tmAC in human spermatozoa is still under doubt since forskolin activation does not involve an increase in cAMP levels (Aitken *et al.*, 1986; Hess *et al.*, 2005; Rojas *et al.*, 1992; Strünker *et al.*, 2011). Although several studies have described the presence of tmAC in mouse spermatozoa (Baxendale and Fraser, 2005; Chien *et al.*, 2010; Livera *et al.*, 2005), its presence in human spermatozoa has yet to be observed (Aitken *et al.*, 1986; Rojas *et al.*, 1992; Strünker *et al.*, 2011; Urizar-Arenaza *et al*., 2018, under revision)(Table 2). In fact, several papers have demonstrated that different GPCR ligands that raise cAMP levels in mouse spermatozoa (Adeoya-Osiguwa *et al.*, 1998; Adeoya-Osiguwa and Fraser, 2003; Fraser and Duncan, 1993), fail to increase the levels of this second messenger in humans (Brenker *et al.*, 2012; Calogero *et al.*, 1996) (Supplementary Table S1, See CD). This is also consistent with the fact that in human spermatozoa GPCR activation does not activate SACY (Brenker *et al.*, 2012; Urizar-Arenaza *et al*., 2018, under revision), which is responsible for cAMP production.

On the other hand, there are a few studies describing PKA activation and tyrosine phosphorylation in human spermatozoa via GPCRs that culminate with the regulation of fertility (Fénichel *et al.*, 1996; Jiménez-Trejo *et al.*, 2012; Neuhaus, 2006; Zhou *et al.*, 2015)(Supplementary Table S1, See CD). Although several different authors have reported the presence of different regulatory and catalytic subunits of PKA in human spermatozoa (Table 3), it is known that they also express a sperm-specific catalytic subunit known as $C\alpha_2$, which could be a central player in the regulation of sperm motility and capacitation (Table 3) (Hereng *et al.*, 2012; Nolan *et al.*, 2004). Nevertheless, to date, no studies have reported activation of the $C\alpha_2$ catalytic subunit of PKA via GPCRs in human spermatozoa.

Considering all the aforementioned findings, there are enough data to conclude that there are differences in the AC/cAMP/PKA signaling pathway downstream of GPCRs in human

spermatozoa. In fact, how can GPCRs activate the PKA signaling pathway in human spermatozoa without SACY activation or cAMP production? We have recently reported a link between GPCR activation and phosphorylation changes in the sperm-specific A kinase anchoring protein 3 and 4 (AKAP3 and AKAP4) in human spermatozoa (Urizar-Arenaza et al., 2018, under revision). Specifically, AKAP phosphorylation results in the selective recruitment and activation of PKA through the direct interaction of the $R\beta_{II}$ PKA regulatory subunit (Luconi, 2004). This is consistent with the fact that GPCRs regulate PKA/AKAP signaling in other cell types (Malbon *et al.*, 2004; West and Hanyaloglu, 2015). In addition, Niu et al. (2001) previously described the physical interaction between the sperm-specific AKAP3 protein and the Gα subunit, suggesting that AKAP3 could be the effector of this G protein in human spermatozoa. In conclusion, AKAPs could provide a link between G proteins and cAMP-independent activation of PKA in human spermatozoa to regulate sperm fertility.

3.2.2 Calcium signaling pathway in human spermatozoa

The Ca^{2+}/PLC/PKC signaling pathway represents another canonical signaling pathway in somatic cells and it has been widely studied. Classically, when $G\alpha_q$, $G\alpha_o$ or $G\beta\gamma$ are stimulated, PLC is activated to transduce the diacylglycerol-triphosphate (DAG-IP_3) dependent-pathway. Specifically, it begins with the activation of the β isoform of phospholipase C (PLCβ) and continues with the cleavage of the phosphoinositide PIP_2 (phosphatidylinositol 4,5-bisphosphate) that is bound to the plasma membrane. This generates two important second messengers: 1,2-diacylglycerol (DAG), a lipophilic molecule that remains linked to the membrane, and inositol 1,4,5-trisphosphate (IP_3), which moves directly to the cytosol (Gilman, 1987; Lodish *et al.*, 2000).

Then, IP_3 binds to the IP_3-gated Ca^{+2} receptors (IP_3 receptors) that are located in the lumen of the endoplasmic reticulum (ER) and other vesicles. This channel is an ATPase that pumps the Ca^{+2} stored in these organelles to the cytosol inducing various cellular responses. Among others, it activates protein kinases that, in turn, phosphorylate transcription factors modifying their activity and regulating gene expression (Lodish *et al.*, 2000; Rhee and Bae, 1997). The other second messenger, DAG, remains bound to the plasma membrane and activates a family of protein kinases collectively called protein kinase C (PKC) (Syrovatkina *et al.*, 2016), responsible for many aspects of cellular growth and metabolism. When the receptor is in a resting state, PKC remains an inactive cytosolic protein. A rise in the cytosolic Ca^{+2} provokes the binding of PKC to the plasma membrane where DAG activates it. Then, PKC phosphorylates various transcription factors resulting in the induction of a range of cellular responses (Cullen, 2003; Mellor and Parker, 1998).

In human spermatozoa, hyperactive motility and acrosome reaction are mainly regulated by calcium (Roldan and Shi, 2007; Suarez, 2008b; Yanagimachi, 1994). Specifically, an increase in intracellular calcium levels induces acrosomal exocytosis, which involves the fusion of the outer acrosomal and plasma membranes of spermatozoa (Kirkman-Brown *et al.*, 2004;

Lopez *et al.*, 2012). Regarding GPCRs, it has been reported that a considerable number of different ligands are able to regulate the human sperm acrosome reaction (See Supplementary Table S1, See CD). Although these molecular mechanisms are not perfectly defined in human spermatozoa, it is known that GPCRs are able to modulate calcium channels regulating intracellular Ca^{+2} levels, presumably through voltage-operated and store-operated channels (Aitken *et al.*, 1986; Kawabata *et al.*, 1996; Morales *et al.*, 2000; Pinto *et al.*, 2012; Serhan *et al.*, 1982; Suhaiman *et al.*, 2010; Urizar-Arenaza *et al.*, 2018, under revision).

To date, several calcium channels have been described in human spermatozoa. In particular, CatSper, a voltage-dependent channel activated by progesterone, is considered the most important calcium channel involved in sperm hyperactivation, acrosome reaction and chemotaxis (Lishko *et al.*, 2011; Strünker *et al.*, 2011). Although GPCRs seem to regulate calcium influx mainly through voltage-operated channels, no association has been described between GPCR and CatSper, the main regulator of the acrosome reaction (Brenker *et al.*, 2012; Urizar-Arenaza *et al*., 2018, under revision). This suggests that GPCRs could modulate sperm fertility by regulating the calcium influx via other voltage-operated channels in human spermatozoa.

On the other hand, GPCRs are also able to regulate the intracellular calcium levels modulating the store-operated channels of human spermatozoa. In these cells, the acrosomal vesicle and other vesicular membranous structures seem to be functionally important calcium stores located in the head and midpiece (Correia et al., 2015, Costello et al., 2009), as during the later stages of the spermatogenesis, male gametes shed much of their cytoplasm including the intracellular organelles (Correia et al., 2015).

Specifically, the mobilization of calcium stores can be brought about by the activation of PLC in human spermatozoa. In fact, GPCR stimulation triggers the activation of the $G\alpha_{q/11}$ subunit (Schaefer *et al.*, 1998) to further stimulate PLC (Allegrucci *et al.*, 2001; Flegel *et al.*, 2016). To date, various PLC isoforms have been described in human spermatozoa including the sperm-specific PLCζ (Table 4), which is important for the first stages of embryo development. Specifically, PLC hydrolyzes PIP2 into DAG and IP_3 second messengers (Rodriguez Peña *et al.*, 2013).

As Allegrucci *et al.* (2001) reported, some GPCR ligands are able to increase IP_3 levels in human spermatozoa (Allegrucci *et al.*, 2001). This second messenger is responsible for the opening of the calcium reservoirs located in the acrosome and other vesicular structures through binding to IP_3 receptors (Correia *et al.*, 2015), inducing a rise in intracellular calcium levels and leading to acrosomal exocytosis (Lopez *et al.*, 2012). On the other hand, both DAG and calcium usually activate PKC, which actively participates in the acrosome reaction (Table 5). As has been reported, GPCRs are also able to induce changes in the phosphorylated substrates of PKC (Urizar-Arenaza *et al.*, 2016; Urizar-Arenaza *et al*., 2018, under revision). Nevertheless, there are no studies reporting the regulation of DAG levels via GPCRs in human spermatozoa. In spite of that, we suggest that GPCRs may be able to regulate sperm fertility through the canonical Ca^{2+}/PLC/PKC signaling pathway.

In addition, in previous research, we demonstrated that GPCRs are also able to regulate sperm physiology through other sperm-specific proteins such as calcium binding tyrosine phosphorylated-regulated protein (CABYR), which is involved in the calcium signaling pathway (Urizar-Arenaza *et al*., 2018, under revision). Specifically, we have reported that the kappa opioid receptor (a typical GPCR) regulates human sperm motility and acrosome reaction inducing phosphorylation changes in CABYR (Urizar-Arenaza *et al*., 2018, under revision). Notably, we identified phosphorylation changes not only in CABYR but also in other sperm-specific proteins involved in the PKA signaling pathway, such as AKAPs. In relation to this, it has been demonstrated that CABYR and AKAPs interact physically in human spermatozoa (Li *et al.*, 2011) suggesting that these proteins could be important scaffold proteins for the *cross talk* between the AKAP/PKA and calcium signaling pathways downstream of GPCRs to regulate sperm fertility.

3.3. G protein-independent signaling pathways

Although classically we have related GPCRs to the regulation of signaling pathways involving G proteins, this receptor superfamily also transduces signals simultaneously via G-protein-independent mechanisms (Heuss and Gerber, 2000). The signal transduction begins with the recruitment of the G-protein-coupled receptor kinases (GRKs) to further phosphorylate ligand-bound GPCRs (Hao and Tatonetti, 2016). This process involves phosphorylation of the receptor by GRKs, PKA or PKC and the subsequent binding of β-arrestin to them. This complex of kinases- β -arrestin blocks the binding between the receptor and the G protein, further desensitizing the receptor and its signaling (Barki-Harrington and Rockman, 2008; Luttrell and Lefkowitz, 2002). Recently, it has been discovered that β-arrestin also regulates another mechanism for the interruption of the signaling in which there is a degradation of second messengers, such as cAMP, by scaffolding phosphodiesterases to the vicinity of the effector (Perry, 2002).

On the other hand, G-protein receptors are able to activate signaling pathways independent of G-protein activation (Pierce *et al.*, 2002; Seta *et al.*, 2002; Wei *et al.*, 2003) through β-arrestins. In fact, these proteins serve as adaptors, scaffolds, and/or signaling transducers, and connect the activated receptors with the diverse signaling pathways (Shenoy and Lefkowitz, 2003), such as the ERK1/2 and JNK3 mitogen-activated protein kinase (MAPK) cascade (Barki-Harrington and Rockman, 2008; Lefkowitz and Shenoy, 2005; Luttrell and Luttrell, 2003) via the recruitment of c-Src to the receptor (Lefkowitz and Shenoy, 2005). This pathway has been widely studied and has also led to the discovery of a new role of β-arrestin as an adaptor molecule in the formation of complexes with ERK and non-receptor tyrosine kinases (Rocca *et al.*, 1999; Shah and Catt, 2004). Specifically, MAPKs translocate from the cytosol to the nucleus phosphorylating and activating transcription factors, finally regulating programs of transcription that lead to proliferation, differentiation, and many other cellular processes (Morrison and Davis, 2003). Moreover, MAPKs can also phosphorylate various cytosolic substrates, leading to changes in cell shape and motility (Ge *et al.*, 2002).

Regarding non-canonical signaling pathways in human spermatozoa, several

studies have reported the presence of proteins like the GRKs, β-arrestin-2, ERK1/2 and p38 in male germ cells, suggesting their involvement in the regulation of sperm fertility (Almog *et al.*, 2008; Almog and Naor, 2010; Luconi *et al.*, 1998; Minelli *et al.*, 2008; Storto *et al.*, 2001). Nevertheless, there are few studies which depict the G-protein independent molecular mechanisms downstream of GPCRs in human spermatozoa. Specifically, Neuhaus et al. (2006) reported that the stimulation of the hOR17 (OR1D2) odorant receptor induces the PKA-dependent desensitization. In fact, PKA is involved in the phosphorylation of the receptor and the following translocation of β-arrestin-2 to the nucleus for further phosphorylation of ERK1/2 and p38. Due to the fact that human spermatozoa are considered transcriptionally and translationally inactive cells, it has been suggested that the activation of these signaling pathways may be involved in the gene expression of a limited and specific set of genes implicated in embryo development beyond the immediate post-fertilization period (Neuhaus, 2006).

Although the non-canonical signaling pathways downstream of GPCRs need to be further examined in human spermatozoa, it seems that these cells could also have specific features underlying their molecular mechanisms. In fact, Ben-Navi et al. (2016) have recently reported the role of sperm-specific AKAPs as scaffold proteins for *crosstalk* with different signaling pathways, including PKA, PKC and ERK1/2, involved in the regulation of human sperm fertility.

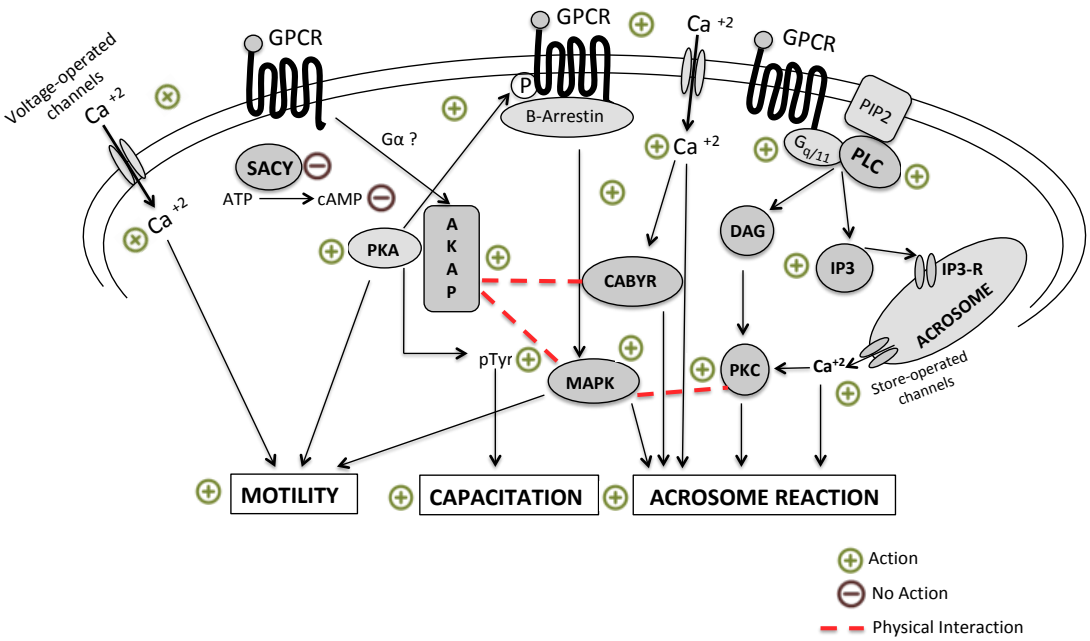

Figure 1. Schematic representation of the signaling pathways induced by GPCRs in human spermatozoa. On the one hand, GPCRs induce phosphorylation changes in AKAPs, PKA and tyrosine residues (pTyr) of different proteins, to further regulate sperm motility or capacitation; however, to date, there are no studies describing the regulation of SACY activity and cAMP production by GPCRs in human spermatozoa. At the same time, PKA is able to cause receptor desensitization by phosphorylation. This implies the recruiting of β -arrestin and the subsequent phosphorylation of MAPK to culminate in the regulation of sperm fertility. On the other hand, the $G\alpha_{q/11}$ subunit activates the PLC, which catalyzes the

hydrolysis of PIP2 into DAG and IP_3. IP_3 binds to IP_3-R located in the calcium reservoirs of the acrosome and neck of spermatozoa to further liberate intracellular calcium. At the same time, GPCRs act on voltage-operated calcium channels to regulate calcium influx into the cell and sperm fertility. The increase in calcium levels activates various proteins, such as CABYR and PKC, involved in the regulation of sperm motility and acrosome reaction. Based on several different interaction studies (discontinuous red line) in human spermatozoa, we propose the AKAPs as scaffold proteins for the *crosstalk* between different signaling pathways such as PKA, CABYR and ERK1/2

Conclusions

In this review, we have gather for the first time evidence that demonstrates differences in the molecular mechanisms underlying somatic and human sperm GPCRs. Specifically, we demonstrate that human spermatozoa present unique features in their G-protein dependent and independent signaling pathways underlying GPCRs, by which they regulate sperm fertility. Moreover, we complement this analysis with a summary of the GPCRs expressed in human spermatozoa as well as a description of their role in fertilizing capacity.

Although further studies are needed to improve our understanding of the exact molecular mechanisms induced by GPCRs, we suggest that the sperm-specific AKAP proteins may act as important scaffold proteins for *crosstalk* between diverse signaling pathways involving PKA, CABYR and ERK1/2 (Figure 1) to regulate sperm fertility. Improved knowledge of these sperm-specific processes may contribute to the development of useful biochemical tools for the diagnosis and treatment of male infertility. Moreover, it could also represent an innovative opportunity for reproductive management, for either enhancing the probability of fertilization or reducing it through the identification of novel targets for contraceptives.

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Table 1. Heterotrimeric G proteins in somatic cells and human spermatozoa (Wettschureck and Offermanns, 2005 Modified).

Name	***Somatic Cells***	***Human spermatozoa***
α- subunits		
$G\alpha_s$ class		
$G\alpha_s$	Ubiquitous	**Described** (Urizar-Arenaza et al, 2018; under revision)
$G\alpha_{s\ XL}$	Neuroendocrine	
$G\alpha_{olf}$	Olfactory Epithelium	**Described** (Spehr et al, 2004)
$G\alpha_{i/o}$ class		
$G\alpha_{i1}$	Widely Distributed	**Described** (Lee, Check and Kopf, 1992)
$G\alpha_{i2}$	Ubiquitous	**Described** (Lee, Check and Kopf, 1992; Urizar-Arenaza et al, 2018, under revision)
$G\alpha_{i3}$	Widely Distributed	**Described** (Merlet et al, 1999)
$G\alpha_o$	Neuronal, neuroendocrine	**Described** (Urizar-Arenaza et al, 2018, under revision)
$G\alpha_z$	Neuronal, platelets	
$G\alpha_{gust}$	Taste cells, brush cells	
$G\alpha_{t\text{-}R}$	Retinal Rods, taste cells	$G\alpha_{t1}$ **Described** (Perez-Cerezales et al, 2015)
$G\alpha_{t\text{-}C}$	Retinal cones	
$G\alpha_{q/11}$ class		
$G\alpha_q$	Ubiquitous	**Described** (Merlet et al, 1999; Schaeffer et al, 1998; Urizar-Arenaza et al, 2018, under revision)
$G\alpha_{11}$	Almost ubiquitous	**Described** (Merlet et al, 1999; Schaeffer et al, 1998; Urizar-Arenaza et al, 2018, under revision)
$G\alpha_{14}$	Kidney, lung, spleen	
$G\alpha_{15/16}$	Hematopoietic cells	
$G\alpha_{12/13}$ class		
$G\alpha_{12}$	Ubiquitous	
$G\alpha_{13}$	Ubiquitous	**Described** (Urizar-Arenaza et al, 2018, under revision)
β subunits		
β_1	Widely, retinal rods	**Described** (Liu et al, 2006)
β_2	Widely distributed	**Described** (Hinsch et al, 1995; Merlet et al, 1999; Urizar-Arenaza et al, 2018, under revision)
β_3	Widely, retinal cones	

β_4	Widely distributed	**Described** (Urizar-Arenaza et al, 2018, under revision)
β_5	Mainly brain	
γ subunits		
γ_1, γ_{rod}	Retinal rods, brain	
$\gamma_{14}, \gamma_{cone}$	Retinal cones, brain	
$\gamma_2; \gamma_6$	Widely	
γ_3	Brain, blood	
γ_4	Brain and other tissues	
γ_5	Widely	
γ_7	Widely	
$\gamma_8; \gamma_9$	Olfactory/vomeronasal epithelium	
γ_{10}	Widely	
γ_{11}	Widely	
γ_{12}	Widely	
γ_{13}	Brain, taste buds	

Table 2. Adenylate ciclase isoforms in somatic cells and human spermatozoa.

AC isoform	Somatic cells	Human Spermatozoa
I	**Described** (Feinstein et al, 1991; Mons et al, 1993)	-
II	**Described** (Feinstein et al, 1991; Mons et al, 1993)	-
III	**Described** (Bakalyar et al, 1990)	-
IV	**Described** (Gao et al,1991)	-
V	**Described** (Glatt and Snyder, 1993; Pieroni et al, 1993)	-
VI	**Described** (Kuprinski et al, 1992)	-
VII	**Described** (Watson et al, 1994; Hellevuo et al, 1995)	-
VIII	**Described** (Feinstein et al, 1991; Mons et al, 1993)	-

IX	-	-
X	-	**Described** (Rojas et al,1992, Brenker et al, 2012; Urizar-Arenaza et al, 2018, under revision)

Table 3. Regulatory and Catalitic isoforms of Protein Kinase A in somatic cells and human spermatozoa

Protein kinase A	***Somatic cells***	***Human spermatozoa***
Regulatory subunits		
$R\alpha_I$	Taylor et al, 2012	**Described** (Urizar-Arenaza et al, 2018, under revision)
$R\beta_I$	Taylor et al, 2012	**Described** (Urizar-Arenaza et al, 2018, under revision)
$R\alpha_{II}$	Taylor et al, 2012	**Described** (Pariset and Weinman, 1994; Urizar-Arenaza et al, 2018, under revision)
$R\beta_{II}$	Taylor et al, 2012	
Catalitic subunits		
Cα	Taylor et al, 2012	**Described** (Urizar-Arenaza et al, 2018, under revision)
$C\alpha_s$	----	**Described** (Reinton et al, 2000)
$C\alpha_1$	Hereng et al, 2012	----
$C\alpha_2$	----	**Described** (Hereng et al, 2012)
Cβ	Taylor et al, 2012	**Described** (Urizar-Arenaza et al, 2018, under revision)
$C\beta_1$	Skalhegg and Tasken, 2000	
$C\beta_2$	Skalhegg and Tasken, 2000	
$C\beta_3$	Skalhegg and Tasken, 2000	
$C\beta_4$	Skalhegg and Tasken, 2000	
Cγ	Taylor et al, 2012	**Described** (Urizar-Arenaza et al, 2018, under revision)

Table 4. Phospholipase C isoforms in somatic cells and human spermatozoa.

PLC isoform	Somatic cells	Spermatozoa
β	**Described** (Kawakami and Xiao, 2013)	-
γ	**Described** (Hwang et al, 1996)	**Described** (Flegel et al, 2016)
δ	**Described** (Hwang et al, 1996)	**Described** (Urizar-Arenaza et al, 2018, under revision)
ε	**Described** (Sorli et al, 2005)	**Described** (Lucchesi et al, 2016)
ζ	**Described** (Monick et al, 2000; Yu et al, 2001)	**Described** (Yoon et al, 2008; Ferrer-Vaquer, 2016; Urizar-Arenaza et al, 2018, under revision)
η	**Described** (Hwang et al, 2005)	-

Table 5. Protein Kinase C isoforms in somatic cells and human spermatozoa.

	PKC isoform	Somatic cells	Human Spermatozoa
Conventional PKCs	α	**Described** (Govekar and Zingde, 2001; Grange et al, 1998)	**Described** (Rotem et al, 1992: Urizar-Arenaza et al, 2018, under revision)
	β	**Described** (Grange et al, 1998)	**Described** (Rotem et al, 1992)
Novel PKCs	δ/θ	**Described** (Grange et al, 1993; Steinberg, 2008)	**Described** (Urizar-Arenaza et al, 2018, under revision)
	ε/η	**Described** (Grange et al, 1998)	**Described** (Rotem et al, 1992)
	μ	**Described** (Govekar and Zingde, 2001)	-
Atypical PKCs	ζ	**Described** (Govekar and Zingde, 2001)	**Described** (Urizar-Arenaza et al, 2018, under revision)

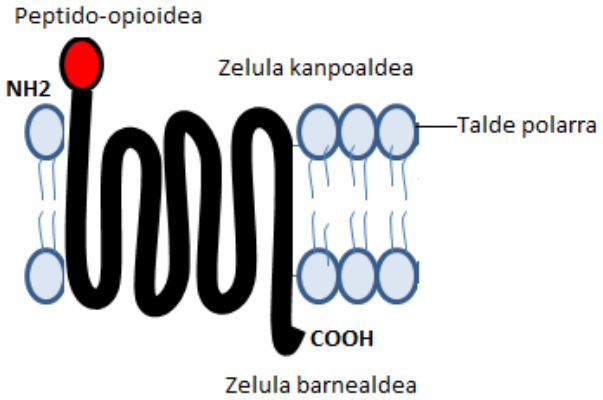
	ι/λ	**Described** (Steinberg, 2008)	**Described** Urizar-Arenaza et al, 2018, under revision)

1.6. SISTEMA OPIOIDEA

Aurretiaz esan bezala espermatozoideek modu sekuentzialean, mugitzeko ahalmena, kapazitazioa eta hiperaktibazioa garatu behar dituzte, eta behin obozitoarekin kontaktuan daudenean, erreakzio akrosomikoa jasaten dute, horren barrura sartu ahal izateko eta zigotoa eratzeko. Komunikazio sistema askok erregulatzen dituzte prozesu hauek. Gaur egun arte, komunikazio sistema anitzak deskribatu izan dira espermatozoidearen fisiologian eragina dutenak: serotonina (Meizel, 1997), purinak (Allegrucci et al., 2000; Meizel, 1997), azetilkolina (Avellar et al., 2010), glizina (Meizel, 2004), neurokinak (Candenas et al., 2005), angiotensinak (Leung and Sernia, 2003), kanabinoideak edota, gure ikerkuntza taldearen ikerketek erakutsi duten bezala, opioideak (Agirregoitia et al., 2006; Fernández et al., 2002; Irazusta et al., 2004; Subirán et al., 2012, 2011, 2010, 2008).

Sistema opioidea, fisiologian garrantzi handia duten prozesu zelular asko erregulatzen dituen komunikazio zelularreko sistema bat da. Tradizionalki, minaren eta plazerraren erregulazioarekin erlazionatua izan da Nerbio Sistema Zentralean, eta pixkanaka-pixkanaka, ugalkortasuna eta garapena bezalako beste prozesu fisiologikoekin erlazionatzen joan da (Subirán et al., 2011).

Hartzaile opioideak, $G\alpha_i/G\alpha_o$ proteinetara akoplaturiko hartzaile familiaren barruan kokatzen dira (GPCR) eta 7TM egitura daukate. Espezifikoki, domeinu N- extrazelular bat daukate, 7 mintzarteko domeinu eta bukaerako buztan C-terminala (1.10 Irudia). Geroago hobeto azalduko den bezala, hartzaile opioideek aurrera eramaten dituzten seinaliztapen bidezidor intrazelularren artean adenilato ziklasaren inhibizioa, boltai menpeko kaltzio kanalen inaktibazioa, potasio kanalen aktibazioa, kaltzio erreserben mobilizazioa edota MAPK-en transdukzio bideen estimulazioa daude (Waldhoer et al., 2004).



1.10. Irudia. **Hartzaile opioideen ezaugarri molekularrak**. Hartzaile metabotropiko hauek, izatez, monomerikoak dira eta mintz plasmatikoa zeharkatzen duten 7 α-helizez eratuta daude. Karboxilo muturra zelula barnean kokatzen da eta bertan palmitoiloa lotzen da, aldiz, zelularen kanpoan dagoen amino muturra glikosilatuta dago eta kanpoan dauden 2 eta 7 domeinuekin estekatzailearen lotura gunea osatzen dute. Zelularen barruan dagoen hirugarren domeinuak eta G proteina batek elkarri eragiten diote. Opioide-hartzaileen moten artean, mintzean zeharreko domeinu eta zelula barnean geratzen diren bukleen homologia handia izan arren (%73-76 eta %86-100, hurrenez hurren), N- zein C-

muturren eta zelulaz kanpoko 2. eta 3. bukleen homologia txikia da (%9-10, %14-72 eta %14-20, hurrenez hurren). Mintzean zeharreko hirugarren domeinu zitoplasmatikoa, 2. eta 3. barne-bukleak eta C-muturreko zatiren bat G proteina heterotrimerikora lotzen dira (Merkouris et al., 1996-tik moldatua).

Farmakologikoki, hartzaile opioideen hiru mota deskribatu izan dira: delta opioide-hartzailea (δ = delta, "deferens" hitzagatik, saguaren hodi deferentean identifikatu zelako; DOR), mu opioide-hartzailea (μ = mu, morfinagatik; MOR), morfinarekiko duen loturarengatik aurkitua, eta kappa opioide-hartzailea (κ = kappa, ketoziklazozinagatik; KOR) (Lord et al., 1997). Azken hamarkadan, opioide-hartzaileekin konparatuta oso antzekoa den nozizeptina lotzen duen opioide-hartzailea (nozizeptina dinorfinaren antzeko peptidoa da; NOR) deskribatu izan da (Mollereau et al., 1994; Wick et al., 1994). Hartzaile horien artean, aminoazido-sekuentzien antzekotasuna oso handia da. Izan ere %60ko antzekotasuna daukate sekuentzia osoa konparatuta eta %73-76koa mintzean zeharreko aldeetan (Minami and Satoh, 1995).

Jarraian opioide-hartzaile bakoitzaren ezaugarrien azalpena emango da:

- **δ opioide-hartzailea (DOR)**

  Gizakiari dagokionez, hartzaile honen dentsitate handia deskribatu izan da nerbio-sistema zentralaren kortex eta kaudatu-putamenean (Pilapil et al., 1987). Espezifikoki, DOR hartzailea axoietan kokatzen da gehienetan (Elde et al., 1995), euren funtzioa gune presinaptikoetara esleituz. Ehun periferikoei dagokienez, delta-opioide hartzailea linfozitoetan (Sharp et al., 1998), guruin adrenalean eta hestean deskribatu izan da (Wittert and Pyle., 1996). Horrez gain, hartzailearen RNAm, kortexean, hipokanpoan, kaudatu-putamenean eta amigdalan azaltzen da (Simonin et al., 1994).

- **μ opioide-hartzailea (MOR)**

  Mu-opioide hartzailea kortetxean, kaudatu-putamenean eta accumbens nukleoan azaltzen da nerbio sistema zentralean. Bereziki dendritetan eta gorputz zelularrean kokatzen da eta postsinaptikoki funtzionatzen duela iradokitu izan da (Elde et al., 1995). Ehun periferikoei dagokienez, saguaren hestean (Pol et al., 2001) eta arratoiaren hodi deferentean (Lemaire et al., 1978), barean, guruin adrenalean eta giltzurrenean (Wittert and Pyle., 1996) espresatzen da. mRNA-ren adierazpena ordea, kortexean eskasa da talamoan eta hipotalamoan ugaria den bitartean (Mansour et al., 1994).

- **κ opioide-hartzailea (KOR)**

  Kappa-opioide hartzailea kortexean, amigdalan eta klaustroan azaltzen da (Pilapil et al., 1987). Hartzailea dendritetan eta gorputz zelularrean kokatzen da eta MOR-ekin gertatzen den bezala badirudi postsinaptikoki funtzionatzen duela (Elde et al., 1995). Ehun periferikoei dagokienez, KOR hestean (Wittert and Pyle., 1996) eta zelula immuneetan azaltzen da (Rivière, 2004). Hartzailearen mRNA, ordea, kortexean,

hipotalamoan, talamoan, gorputz ildaskatuan eta accumbes nukleoan adierazten da (Peckys and Landwehrmeyer, 1999).

- **ORL1 opioide-hartzailea (NOR)**

  ORL1 hartzailea nerbio-sistema zentralean deskribatua izan bada ere, kaudatu-putamenean eta zerebeloan ez da bere arrastorik topatu. Ehun periferikoei dagokienez, muskulu leunetan, gongoil periferikoetan eta sistema inmunean zehar adierazten da eta uste da hartzaile honen jarduera biologikoa anitza dela (zentzumen pertzepzioan, memoria prozesuetan edota emozio portaeran, besteak beste) (Mollereau et al., 1994; Mollereau and Mouledous, 2000). Bestalde, hartzaile honen mRNA eta lotura guneen adierazpenak bat datozenez, pentsa daiteke hartzaile hau neuronen zirkuitu lokaletan ere adierazten dela.

1.6.1. Barne-sistema opioidea

Barne sistema opioidea arestian aipatutako hartzaile opioideek, barne-peptido opioideek (BPO) eta andeakuntza-prozesuetan parte hartzen duten entzimek osatzen dute. BPO-en eraketari erreparatuz, nerbio- sistema zentralean aurki daitezkeen peptido ia guztiak (karnosina dipeptidoa eta glutation tripeptidoa izan ezik) neuronetako erribosometan molekula aitzindari peptidiko edo pre-pro-proteina bezala sintetizatzen dira (Siegel et al., 1993).

Barne-sistema opioideari dagokionez, BPO-en aitzindariak sortzen dituzten hiru gene identifikatu dira: proentzefalina (PENK), propiomelanokortina (POMC) eta prodynorfina (PDYN).

- **Proentzefalina (PENK):** Nerbio-sistema zentralean eta muin adrenalean sintetizatzen da (Comb et al., 1982). 243 aminoazido dauzkan proteina horretatik, zazpi peptido opioide sor daitezke tripsina, endopeptidasa edota N-karboxipeptidasa motako entzimen eraginez. Aipagarrienak bost aminoazidoz eratutako entzefalinak dira, zeinak peptido opioide guztiek komunean duten sekuentzia bat gordetzen dutenak (Tyr-Gly-Gly-Phe-Met" edo "Tyr-Gly-Gly-Phe-Leu"; Met eta Leu-entzefalinak alegia).

- **Propiomelanokortina (POMC):** Barne peptido opioideez gain, beste peptido batzuk ere gordetzen ditu (Nakanishi et al., 1979). Adibidez, β-endorfina peptido opioidea sortzeaz gain, hormona adrenikortikotropikoa (ACTH), α eta β-lipotropina (LPH), α eta β-melanotropina (MSH) ere sortzen ditu.

- **Prodinorfina (PDYN):** Dinorfina A (1-17), dinorfina B (1-13) eta β-neo-endorfina bezalako peptido opioideak sortzen ditu (Kakidani et al, 1982).

Peptido opioideen jarduera hartzaile opioideekiko loturari esker gertatzen da eta bere ekintza zenbait entzimek inaktibatzen dute. Gaur egun, 20 barne-peptido opioide ezagutzen dira eta 5-32 aminoazido arteko luzera izan dezakete. Ugaztunen peptido opioide naturalak

Metentzefalina, Leu-entzefalina, β-endorfina, Dinorfina A, Dinorfina B, α-neoendorfina, Nozizeptina eta Endomorfina dira (Florez, 1998). 1.1 taulan, peptido opioideek hartzaile bakoitzerako dituzten afinitateak azaltzen dira. Barne peptido opioideek afinitate aldagarria erakusten dute euren hartzaileengatik. Esaterako, met eta leu-entzefalina delta opioide-hartzailearen barne lotugaitzat onartzen dira. Aipatutako hartzailearentzat afinitate altua aurkezten duten arren, mu opioide-hartzailearekin batzeko ere gai dira baina afinitate gutxiagorekin. Beta-endorfinek, mu eta delta opioide-hartzaileentzat antzeko afinitatea dute eta , dinorfinak kappa opiode-hartzaileetara lotzen dira.

1.1 Taula. Hartzaile opioide bakoitzak opioide lotugaiekiko afinitateen profilak lotura analisien arabera (Subirán et al., 2011).

	K_i (nmol/L)			
Ligands	δ	μ	κ	ORL_1
(Met^5)-enkephalin	0.9	9.5	4440	—
(Leu^5)-enkephalin	3	20	835	—
β-Endorphin	2.7	2	57	—
Dynorphin A	3.2	0.7	0.12	110
Dynorphin B	4.4	3.4	9	—
α-Neoendorphin	0.57	1.3	0.2	—
Endomorphin	>500	0.67	>500	—
Nociceptin	380	133	267	0.1

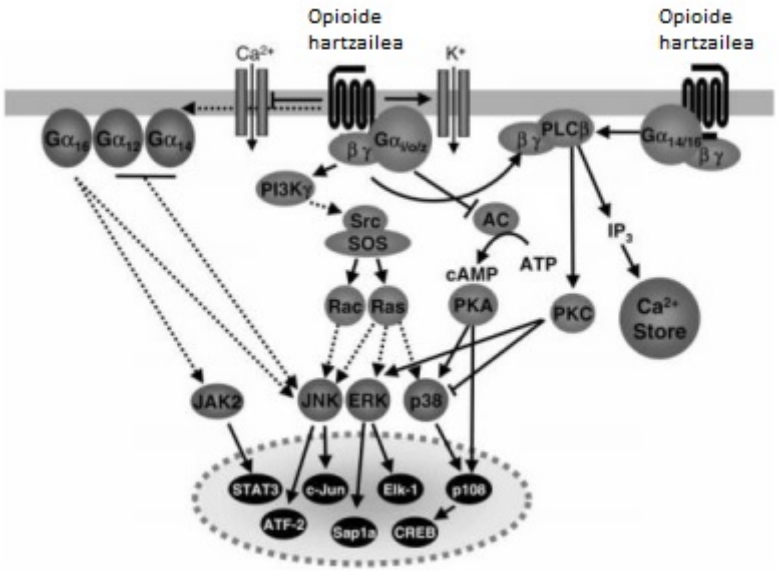
Peptido opioideen inakibazioari dagokionez, peptidasa izeneko entzimek andeakuntza metabolikoa burutzen dute eta hori biologikoki aktiboak diren peptidoen apurketan datza, zatiki inaktiboak sortuz (Iversen, 1987). Espezifikoki, N aminopeptidasa eta 24:11 endopeptidasa dira peptido opioideak *in vivo* degradatzen dituztenak, hurrenez hurren (Subirán et al., 2011).

1.6.2. Opioide hartzaileen aktibazioa eta seinaleztapen bidezidor intrazelularrak

Lehenago aipatu den bezala, hartzaile opioieak 7TM egitura duten G proteinetara loturiko hartzaileen familiaren parte dira. Izan ere, zenbait erantzun zelular induzitzen dituzte G proteinen inhibitzaileen $G\alpha_i/G\alpha_o$ azpiunitateen ekintzaren bitartez (Koch and Höllt, 2008) (1.11 Irudia).

Neuronetan, behin agonistak hartzailea aktibatzen duenean, mintzarteko adenilato ziklasaren (AC) inhibizioa gertatzen da, cAMParen mailak murriztu eta ondorioz, PKAren aktibazioa inhibitzen da (Sharma et al., 1977). Horrek, neuronetan, zelula barneko eta nukleoko proteina askoren fosforilazio-egoeran eragingo du. Horren eraginez, badirudi transkripzio-prozesu askoren aldaketak gerta daitezkeela, eta epe batean hauek, opiazeoekiko tolerantziaren edota abstinentzia-sindromearen prozesuen errudun bihurtzen dira (Lorenzo et al., 1999).

Horrez gain, $G\alpha_i$ azpiunitateak boltai-menpeko kaltzio kanaleak inaktibatu eta potasio kanaleak aktibatuko ditu (Law et al., 2000). MAPK bidezko seinaliztapen bidezidorrari dagokionez, opioideek opioide-hartzailearen fosforilazioa sorrarazi eta gero, ERK, p38 edota JNK bezalako proteinen aktibazioa sustatuko dute $G_{\beta\gamma}$ azpiunitateen bitartez (Al-Hasani and Bruchas, 2011; Law et al., 2000). ORL1-en aktibazioaren kasuan, berriz, ERK-ren aktibazioa, partzialki, PLC-ren aktibazioaren bitartez gertatzen da (Fukuda et al., 1997; Lou et al., 1998).



1.11. Irudia. Opioide-hartzaileek aktibatutako seinaliztapen bidezidor intrazelularrak. Opioideek adenilato ziklasa entzimaren inhibizioa, tentsioaren menpeko kaltzio-kanalen inaktibazioa, potasio-kanalen irekiera, zelula barneko kaltzioaren askapena eta MAP kinasek bideratutako transdukzioaren estimulazioa eragiten dute (New and Wong, 2002-tik moldatua).

Gainera, $G_{\beta\gamma}$ azpiunitateek C_β fosfolipasaren aktibazioa bultzatuko dute eta inositol trifosfato bidezko kaltzioaren mobilizazioa erregulatuz eta proteina kinasa C aktibatuko dute (Chan et al., 1995; Tsu et al., 2002). Hala ere, ez dago argi zein den Gβγ azpiunitatearen inplikazioa nozizeptinak bideratutako PLCβ-ren aktibazioan (New and Wong, 2002).

Erantzun molekularrari dagokionez, MOR eta DOR hartzaileen aktibazioak mintzaren hiperpolarizazioa eta jarduera bioelektriko neuronalaren inhibizioa dakar. Horrek potasio kanalen irekiera sortzeaz gain, agonistaren bidezko zelula barneko kaltzioaren askapena sustatuko du, potasio kanalen irekiera bultzatuz (Williams and Clouet, 1982). KOR hartzailea aktibatzean, ordea, tentsioaren menpeko kaltzio-kanalen inaktibazioa gerta daiteke. Honek, kaltzioa neurona barrura sartzea ekiditu eta neurotransmisoreen askapena inhibituko du (Lorenzo et al., 1999; Surprenant et al., 1990). Horrek guztiak, nerbio-ehunean aktibitate bioelektrikoaren inhibizioa eta neurotransmisoreen askapenaren inhibizioa eragingo du.

1.6.3. Peptido opioideen eta hartzaile opioideen ekintza fisiologikoa

Peptido opioideen funtzio ezagunen Nerbio Sistema Zentralean minaren ezabapenarekin edo analgesiarekin erlazionatu izan badira ere, gaur egun funtzio fisiologiko desberdinak betetzen dituztela aski ezaguna da. Horien artean 1) burmuinaren garapenean zein birsorkuntza eta plastikotasunean parte hartzea (memoria eta ikaskuntza bezalako funtzioetan); 2) funtzio sentsorialen erregulazioa; 3) aldaketen eragilea da elikadura portaeran; 4) depresioa edota antsietatea bezalako arazoen modulazioa; 5) heste, giltzurrun eta gibel funtzioetan parte hartzea; 6) odol presioaren eta erantzun kardiobaskularraren modulazioa; 7) arnasketaren modulazioan parte hartzea arnasketa urritasuna eta erantzun termoerregulatzailea eraginez; 8) erantzun immuneen modulazioa; 9) lokomozioaren eta aktibitate orokorraren erregulazioa eta 10) funtzio ugaltzailearen erregulazioa (Bodnar and Klein, 2004). Hala ere, aipatzekoa da eragin horien intentsitatea ezberdina dela opioide motaren eta aktibatutako hartzailearen arabera (Fabbri et al., 1989).

1.7. SISTEMA OPIOIDEA ETA GIZONEZKOAREN UGALKORTASUNA

Sistema opioidea, ugalketa funtzioa erregulatzeko gai da maila askotan eta jakina da peptido opioideak gizonezkoaren eta emakumezkoaren ugal aparatuen organo eta ehunetan aurkitzen direla (Fabbri et al., 1989). Horregatik, peptido opioideak zenbait lekutan arituz, ugaltze sisteman era anitzetan eragiten dute.

1.7. 1. Sistema opioideak hormonen bidez bideratutako ugaltze-funtzioaren kontrola

Lehenago azaldu bezala, zenbait hormonek espermatogenesiaren prozesua erregulatzen dute. Peptido opioideek, Nerbio Sistema Zentralaren bidez, ugal funtzioa ere kontrolatzen dute. Hau, hipotalamoko GnRHaren jariapena inhibituz egiten dute hipofisiko hormona gonadotropikoen jariapena inhibitzen dutelako (Kalra et al., 1988). Horrez gain, peptido opioideek guruin pituitarioa zuzenean inhibitzeko ahalmena dute. Ehun periferikoetan ordea, Leydig zelulek sortutako β-endorfinek tubulu seminiferoen funtzioa inhibitzen dute (Fabbri et al., 1989).

Esaterako, gizaki zein beste animalietan, morfinaren tratamenduak LHaren askapenaren inhibizioarekin lotuta dago hipotalamoan (Kalra et al., 1988), naloxona edota naltrexona bezalako antagonista opioideek LHaren askapena bultzatzen duten heinean (Cicero et al., 1975; Fraioli et al., 1985). Ikerketek erakutsi dutenez, peptido opioideek Nerbio Sistema Zentralaren bidez, ugaltze-funtzioa kontrolatzen dute GnRH jariapena ekidinez (de Gandarias et al., 1993; Delitala et al., 1981; Fraioli et al., 1984; Sirinathsinghji et al., 1983).

Ezaguna denez, peptido opioideak testikuluetan ere badaude eta bertan gertatzen diren prozesuak erregulatzen dituzte zelulen arteko komunikazioan paper garrantzitsua betez.

Aipatzekoa da, sistema opioideak espermatogenesiaren erregulazioan duen funtzioa aztertzeko egin diren ikerketa gehienak animalietan egin direla, saguan batez ere.

Aurreko lanek erakutsi duten bezala, peptido opioideak arren gonaden zeluletan topatzen dira espermatogenesia erregulatzen duten mekanismoetan parte hartuz (Estomba et al., 2016; Fraioli et al., 1985; Zhou et al., 1990). Espezifikoki, MOR, DOR eta KOR opioide hartzaileak sagu zein gizonezkoen hozi-zeluletan daudela ikusi izan da (Estomba et al., 2016). Horrez gain, peptido opioideak prostatan eta epididimoan ere detektatu izan dira (Gerendai, 1991).

Saguari dagokionez, hiru opioide-hartzaileak saguen espermatida, espermatogonia, espermatozito eta espermatozoideen alde periferikoan kokatzen dira eta ziklo espermatogenikoan efektu modulatzailea dute (Estomba et al., 2016).

Horrekin lotuta, giza testikuluetako zelula espermatogenikoetan ere baieztatu da MOR, DOR eta KOR-ren presentzia. Gainera badirudi aitzindari opioideak testikuluetako zelula somatiko eta germinaletan modu bereizgarrian adierazten direla eta *de novo* sintesi bidez, lokalki, funtzio testikularra erregulatzeko gaitasuna dute (Estomba et al., 2016). Zehazki, gonadotropinek eta kortikotropina hormona jariatzaileek, Leydig zeluletan, parakrinoki Sertoli zelulen funtzioa inhibitzen duten peptido opioideen ekoizpena estimulatzen dute.

1.7.2. Sistema opioidea eta funtzio espermatikoaren kontrola

Sistema opioidea, ugalketa funtzioa erregulatzeko gai da maila askotan eta azken ikerketek erakutsi dute fisiologia espermatikoan ere garrantzia duela (Subirán et al., 2011). Maila espermatikoari dagokionez, giza espermatozoideetan ikusi da sistema opioidearen osagaiak daudela (Agirregoitia et al., 2006; Albrizio et al., 2010; Fernández et al., 2002; Irazusta et al., 2004; O'Hara et al., 1994; Subirán et al., 2008). Sistema horrek espermatozoideetan eragin zuzena du eta horregatik ugalketa funtzioaren erregulazioan betekizun garrantzitsua jokatuko lukeela proposatzen da.

Jakina denez, likido seminalean ere, hainbat peptido opioideren kontzentrazio handiak deskribatu izan dira, β-endorfina (Davidson et al., 1989; Fraioli et al., 1984), metentzefalina (Fujisawa et al., 1996; Sastry et al., 1982) edota leu-entzefalina (Sastry et al., 1982), besteak beste. Aipatzekoa da peptido horien kontzentrazioa handiagoa dela plasma seminalean plasma periferikoan baino (Sharp et al., 1980). Horrez gain, aipatzekoa da entzefalina degradatzen duten entzimak gizonezkoen likido seminal osasuntsu eta subugalkorretan aurkitu direla (Irazusta et al., 2004).

Ebidentzia horiekin, ikertzen hasi zen ea opioideek espermatozoideetan efekturik zeukaten eta euren funtzioa Nerbio Sistema Zentralaren bidez edota ehun periferikoen bidez zeharka eragiteaz gain, zuzeneko efektua ere ote bazuen. Izan ere, orain dela 40 urte baino gehiago ikusi zen lehen aldiz sustantzia opioide exogenoen kontsumoak (heroina edo metadona), emakumezkoetan amenorrea sortzeaz gain (Santen et al., 1975), espermatozoideen mugikortasunean kalteak eragiten zituela (Cicero et al., 1975; Ragni et al., 1985).

Ez zen izan 2006.urtera arte izan ordea, noiz Agirregoitia eta lankideek (Agirregoitia et al., 2006), MOR, DOR eta KOR hartzaile opioideak deskribatu zituzten lehenengoz giza espermatozoideen mintz plasmatikoan. Bereziki, MOR giza espermatozoideen buruan adierazten da, isatsean, erdiko piezan eta atal postakrosomalean edo ekuatorialean intentsitate handiagoa erakutsiz. Bestetik, DOR giza espermatozoideen buruan, erdiko piezan eta isatsetik uniformeki banaturik agertzen da. DOR-ekin konparatuta KOR-ek banaketa nahiko antzekoa dauka. Izan ere, hartzaile hau ere giza espermatozoiden buruan, erdiko piezan eta isatsean adierazten da. Espermatozoideek hartzaile opioideak adierazteaz gain, era askotako peptido opioideak ere badituzte. Horien artean, proentzefalina (Kew et al., 1990) eta horretatik eratorritako meta-entzefalina, leu-entzefalina eta β-endorfina aurkitu dira (Sastry et al., 1982).

Sistema opioidearen lehen ikerketa funtzionalak mugikortasun espermatikoan egin dira. Hartzaile opioideen agonista/antagonista bidez egindako ikerketa farmakologikoek erakutsi izan dute sistema opioideak mugikortasun espermatikoarekiko efektu bibalentea duela (Agirregoitia et al., 2006; Albrizio et al., 2005). Horrelako eta beste motako opioideen kontzentrazio aldagarriek, espermatozoideen mugikortasunean eragina dutela frogatu da. Hala ere, ezaguna da, peptido motaren, kontzentrazioaren eta inkubazio-denboraren arabera, opioideek efektu bat baino gehiago sor ditzaketela.

Horren adibide metaentzefalinak dira. Izan ere, leu-entzefalina, edo met-entzefalinaren D-ala2 -Mephe4-Met-(O)-OIenkephalin (DAMME) analogoa erabiltzean, espermatozoideen mugikortasunaren inhibizioa gertatzen da (Foresta et al., 1985; Sastry et al., 1982). Met-entzefalina erabiltzean, berriz, espermatozoideen mugikortasunean ez da efektu nabaririk antzematen (Fraioli et al., 1984; Fujisawa et al., 1996). Orain dela urte gutxiko ikerketek, ordea, met-entzefalinak giza espermatozoideen mugimenduaren erregulazioa modu autokrinoan areagotzen duela erakutsi dute (Subirán et al., 2012).

Beste alde batetik, Agirregoitia eta lankideek (Agirregoitia et al., 2006), proposatu zuten opioideen jarduera aldakorra hartzaile desberdinen aktibazioaren ondorioz gertatzen zela. Hain zuzen ere, morfinak (MORen agonista espezifikoa) eta naltrindolak (DORen antagonista) giza espermatozoideen mugikortasuna inhibitzen badute ere, DPDPE-k (DOR-en agonista espezifikoa), ez du mugikortasunean eragin nabaririk sortzen. KOR opioide hartzaileari dagokionez, U50488H eta nor-binaltorfiminak (agonista eta antagonista espezifikoak, hurrenez hurren) ez dute efekturik sortzen espermatozoidearen mugikortasun progresiboan (Agirregoitia et al., 2006). β-endorfina peptido opioidea erabiliz espermatozoidearen mugikortasuna inhibitzen da baina hau naloxonarekin batera inkubatzean, bi efektu kontrajarri ikusi dira zenbait lanetan; mugikortasunaren inhibizioa ekiditzea eta ez ekiditzea hain zuzen ere (Fraioli et al., 1984; Mari et al., 2005).

Aldez aurreko ikerketek iradoki duten bezala, badirudi espermatozoideen mugikortasunaren efektu kontrajarri hauen azalpena opioide hartzaile desberdinen aktibazioari dagokiela (Subirán et al., 2011, 2008). Ildo beretik jarraituz, deskribatu izan da naloxonaren

kontzentrazio baxuek espermatozoidearen mugikortasuna mantentzen laguntzen dutela (Albrizio et al., 2005; Mari et al., 2005), kontzentrazio altuagoek mugikortasuna murrizten (Albrizio et al., 2005; Mari et al., 2005) edota areagotzen (Sastry et al., 1991) duten bitartean. Naltrindol antagonistari dagokionez, kontzentrazio baxuetan mugikortasuna areagotzen du, altuetan murriztu egiten duelarik (Albrizio et al., 2010). Aipatzekoa da, paradoxikoki hazi astenozoospermikoan topatutako met-entzefalina eta β- endorfinaren kantitatea normozoospermikoan topatutakoa baino txikiagoa dela (El-Haggar et al., 2006; Fujisawa et al., 1996).

Sistema opioideak giza espermatozoidearen kapazitazioan duen eraginari dagokionez, aipagarria da gure espeziean ez dela lan nahikoa egin. Hala ere, zaldien espermatozoideekin egindako *in vitro* esperimentuetan ikusi izan da naloxonak kapazitazioa induzitzen duela, erreakzio akrosomikoan eraginik aurkezten ez duen bitartean (Albrizio et al., 2005). Naltrindolari erreparatuta, ordea, DOR-en antagonistak kapazitazioa inhibitzen du.

Erreakzio akrosomikoari dagokionez, DAMME-ak dosiaren menpeko erreakzio akrosomikoa inhibitzeko gai da (Foresta et al., 1986) eta eragin berdina ikusten da naltrindol antagonistaren kontzentrazio baxuetan. Kontzentrazio altuetan berriz, kontrakoa gertatzen da (Albrizio et al., 2010).

Hartzaile opioideez eta BPOez aparte, barne-sistema opioidea eratuz, andeakuntza entzimak ere badaude. Entzimen-jarduerari dagokionez, APNa espermatozoideetan, likido seminalean eta prostasometan agertzen da, NEPa soilik prostasometan agertzen den bitartean (Fernández et al., 2002). APNa eta NEPa inhibitzean, espermatozoideen mugikortasuna mantentzen da denboran zehar, eta eragin hori naloxonak ekiditen du (Subirán et al., 2008). Bestalde, hazi astenozoospermikoek, normozoospermikoekin alderatuta, APN jarduera txikiagoa dute (Irazusta et al., 2004).

Aipatutako guztiari erreparatuta argi dago sistema opioideak espermatogenesian zein espermatozoideen ahalmen ugalkorrean eragin zuzena duela. Gaur egun bibliografian dauden lanek ikuspuntu orokorra ematen badigute ere hartzaile opioideek espermatozoidearen ugalkortasunean betetzen duten funtzioan sakontzea beharrezkoa da. Bereziki, hartzaile hauek espermatozoideen mugikortasunean, kapazitazioan eta erreakzio akrosomikoan jokatzen duten funtzioa ikertzea interesgarria litzateke, efektu hori lortzeko aktibatu/inhibitu behar diren seinaliztatze bidezidor intrazelularrak sakonki deskribatuz.

1.8. TESTIKULUETAKO PROTEINA ESPEZIFIKOAK

Azken hamarkadan egindako ikerketen arabera, giza espermatozoideen proteomaren ikuspuntu orokorra lortzerako bidean aurrerapen garrantzitsuak egin dira. Hori horrela izanda, seinaliztapen bidezidor anitzetan parte hartzen duten 6198 proteina identifikatu dira gaur egun arte, giza espermatozoidearen proteoma 7500 proteina inguruz konposaturik egon litekeela uste den arren. Identifikatutako proteinen % 78a beste sistema batzuetan identifikatu bada ere, gainerako % 22a espezifikoki espermatozoidearen proteomari dagokiola uste da (Amaral et al., 2014). Seinaleztapen molekula berriak identifikatu ahala, badirudi espermatozoideek, bakarrak, espermatozoide- eta espezie-espezifikoak diren seinaliztapen bidezidor intrazelularrak induzitzen dituztela, zelula somatikoengandik desberdinak kontsideratzen direlarik (Kaupp and Strünker, 2017).

Testikuluetan eta espermatozoideetan soilik adierazten diren proteinei erreparatuta, badaude ingelesezko "Cancer/Testis Antigen" (CTA) deritzen proteina talde bat. Bereziki, CTAk karraskari zein gizakien testikuluetako zelula germinaletan (zelula germinal primordialetan, espermatogonietan, espermatozitoetan, espermatidetan eta espermatozoideetan) adierazten dira baina zelula somatiko normaletan, orokorrean, ez dago horien arrastorik (Mirandola et al., 2011; Scanlan et al., 2004; Simpson et al., 2005). Aitzitik, CTAk minbizien %40an atipikoki aktibatu eta adierazten dira (Chen et al., 1997; Mirandola et al., 2011; Scanlan et al., 2004).

Teknika molekular desberdinak erabilita, gaur egun, CTA bezala sailkatzen diren 225 gene inguru identifikatu dira baina badirudi horien bilduma handiagoa izan daitekeela (Almeida et al., 2009; Hofmann et al., 2008). Aipatzekoa da, gaur egungo klasifikazio horrek ez dituela gene bakoitzaren ezaugarri antigenikoak frogatzen, izan ere, testikulu zein tumorean daukaten adierazpenaren arabera egin da (Whitehurst, 2014).

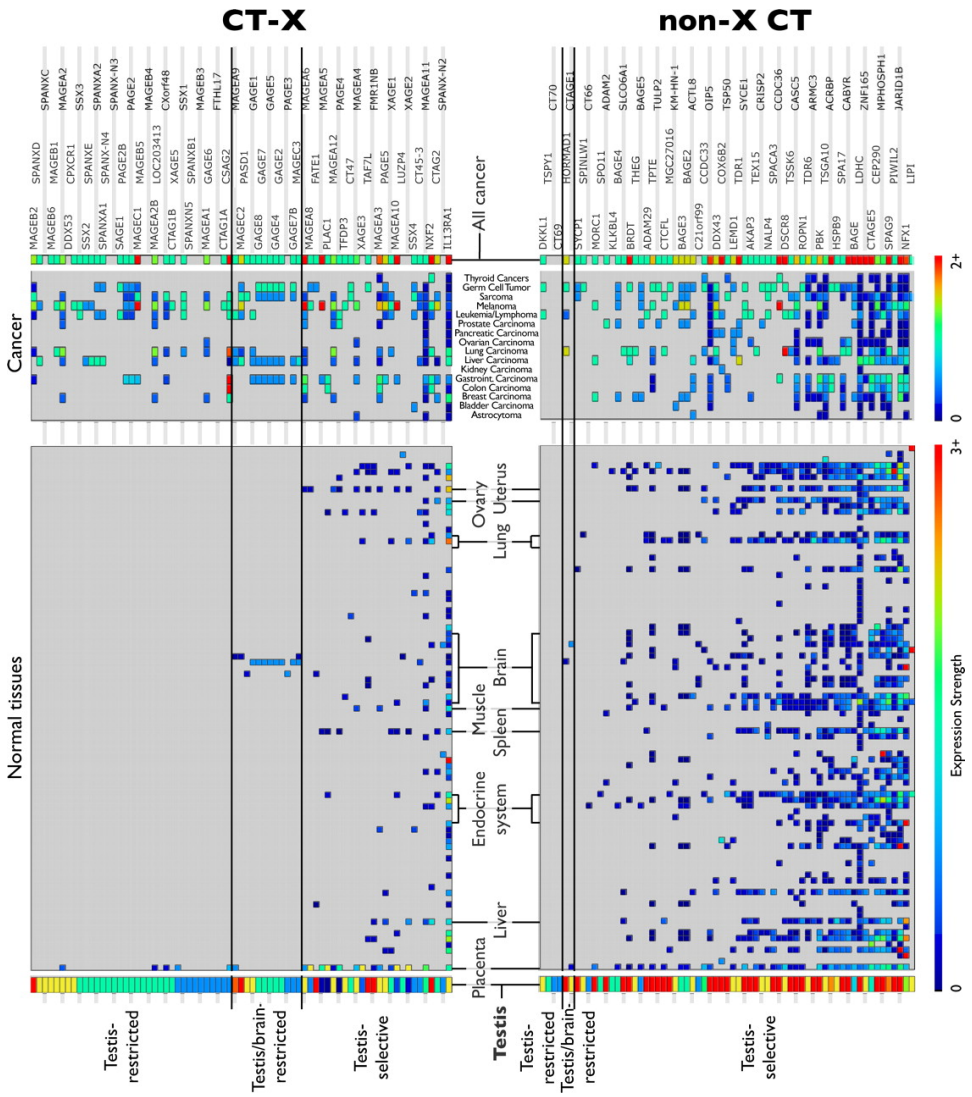
Tumoreetan egindako adierazpen azterketen arabera, CTAk birika, gernu-maskuri, melanoma zein ugatz minbizian maila altuan agertzen dira. Hala ere, maila baxuagoan adierazten dira leuzemian, linfometan, giltzurruneko, koloneko eta pankreako minbizietan (Old, 2001; Simpson et al., 2005). Horrez gain, badirudi CTA espezifikoak tumore jakin batzuetan adieratzen direla. Adibidez, melanoma metastatikoaren kasuen %70ak gutxienez MAGEA1-4 geneetako bat adierazita dauka (Brasseur et al., 1995). Era berean, ACRBP (acrosin binding protein) genearen maila altuak ikusi dira obarioko tumoreetan (%70) eta CTAG1B genearenak ugatz minbizian (%46) (Grigoriadis et al., 2009; Tammela et al., 2006).

Arestian aipatutako kasuak, adibide soilak badira ere "The Cancer Genome Atlas (TCGA) (http://tcga-data.nci.nih.gov/tcga/) izeneko datu basean CTA-en adirazpena biltzen dituen eta eskala handian egindako datu multzoak aurki daitezke.

1.8.1. CTA-en sailkapena

Gaur egun arte egindako ikerketen arabera CTAk maila baxuan adierazten dira ehun somatikoetan edota burmuina bezalako leku immuno-pribilegiatuetan (Whitehurst, 2014). Arestian aipatu bezala, CTA-ak existitzen diren arren, Old eta lankideek (Old, 2001), gene horien adierazpen diferentzialen profiletan oinarritzen den sailkapen sistema proposatu zuten (1.12 Irudia):

- Adierazpena testikuluetara mugatua: CTA hauek soilik testikuluetan adierazten dira.
- Adierazpena testikuluetara zein burmuinera mugatua: CTA hauek testikuluetan zein Nerbio Sistema Zentralean adierazten dira.
- Adierazpena testikuluetarako selektiboa: CTA hauek testikuluetan adierazten dira eta maila baxuago batean, gehienez bi ehun osagarritan agertzen dira.



1.12. Irudia. X-CT eta non-X-CT antigenoek minbizian duten adierazpen diferentziala. Gene bakoitzak testikuluetan, ehun normaletan eta zenbait minbizietan duen adierazpena deskribaturik agertzen da. Koloreek adierazpen mailari egiten diote erreferentzia, urdinez adierazpen maila baxua, berdez erdiko adierazpen maila eta gorriz adierazpen maila oso altua (Hofmann et al., 2008).

Horrez gain, kromosoman zeharreko CTAren kokapena ere sailkapenerako kontuan hartzen da:

- X kromosoman kodifikatutako CTAk: X-CT antigeno ere deritze eta gaur egun arte identifikatutako CTA guztien %50a dira. Normalean, X kromosoman zehar kluster antolatuetan agertzen dira familia multigenikoak osatuz (Akers et al., 2010; Zendman et al., 2003a).
- X kromosoma ez den kromosometan kodifikatutako CTAk: non-X-CT antigeno deritze, genoma osoan zehar banaturik agertzen dira eta kopia bakarra duten geneak dira (Simpson et al., 2005).

1.8.2. Funtzio fisiologikoa: gametoetan

CTA-ek gametoan duten funtzioa gaur egun oso ezaguna ez bada ere, adierazpen genikoan eta *knockout*-etan oinarritutako lanek argia daramate talde honetako proteinen funtzioa aztertzerako bidera. Esaterako MAGEA1 eta NY-ESO-1 bezalako X-CT antigenoak, espermatogenesiaren lehen faseetan beharrezkoak direla deskribatu da (Jungbluth et al., 2000). Bestalde, badirudi GAGE geneen adierazpena hozi zelulen funtziorako garrantzitsua dela (Gjerstorff et al., 2007). Aipatutakoa kontuan izanda, badirudi CTA-ek arraren ugalkortasunean funtzio garrantzitsua betetzen dutela.

Esaterako, jakina da CTA espezifikoek espermatogenesia kontrolatzen dutela. Espezifikoki meiosian zehar gertatzen den errekonbinazio homologoan parte hartzen duten antigenoen presentzia ezaguna da eta kromosomen parekatzea eta material genetikoaren trukaketa egokia ahalbidetzen dute. Gene horien ezabapenak beraz, errekonbinazio prozesuaren ahalmena mugatzen du eta saguen antzutasuna sortzen du (Schramm et al., 2011; Vries et al., 2005). Horrez gain, badirudi CTAk RNAren erregulazioan funtzio garrantzitsua betetzen dutela. Izan ere, genomaren osotasuna mantentzen zein mRNA-ren adierazpenaren erregulazioan parte hartzen dute (Whitehurst, 2014). Bestalde, badirudi CTA asko aurkitu direla gorputz kromatoideetan (Chromatoid body ingelesez), hau konpaktazio handia duen gunea da eta mRNA-z eta proteinez eraturik dago espermiogenesian zeharreko gene adierazpenaren erregulaziorako (Meikar et al., 2014).

Espermatozoideei dagokienez, hauek mugikortasuna berenganatzen dute flageloari esker. Arestian aipatu bezala flageloak axonema izeneko motore batez eraturik dago, zeina gutxienez 13 proteinez osaturik dagoen. Egitura hau osatzen duen proteina edo CTA nagusiena AKAP4 da. Izan ere, flageloaren %50a betetzen du. Horrez gain, AKAP3, CABYR, SPA17 edota ROPN1 izeneko antigenoak ere espermatozoidearen flageloaren osagai dira (Brown et al., 2003; Fiedler et al., 2008; Mirandola et al., 2011). Ikusi izan denez, AKAP4 eta ROPN-rik ez daukaten saguak ugalkortasun murriztua daukate flageloan ematen diren akatsengatik (Brown et al., 2003; Fiedler et al., 2008). Flageloaren mugikortasuna erregulatzen duten mekanismo molekularrak argiak ez diren arren, AKAP4 eta Rho proteinara elkartzen diren rophilin-1 eta ropporin proteinak zenbait seinaliztatze bidezidor erregulatzen dihardutela uste da. Horrekin erlazionatuta, ezaguna da PKA eta Rho, tumoreetan aberrantikoki aktibatzen direla. Hori dela eta, posible izango litzateke AKAP4 eta ROPN1 zitoeskeletoaren dinamika erregulatzen egotea

zelularen mugimendua, inbasioa edota mitosia aurrera ermateko (Bossis and Stratakis, 2004; Vega and Ridley, 2008).

Horrekin lotuta, jakina da CTA askok espermatozoideen energiaren produkzioa erregulatzen dutela. COX6B2, esaterako, espermatozoidearen espezifikoa den elektroi garraiatzailearen katearen IV.konplexua osatzen duen proteinetako bat da eta fosforilazio oxidatiboaren funtzionamendu egokian parte hartzen du (Hüttemann et al., 2003). SPATA19, mitokondrioetan agertzen den proteina bat da eta hauen paketatzea erdiko piezaren inguruan egokia izateaz arduratzen da (Suzuki-Toyota et al., 2007). SPATA19 zein COXB2 ezinbestekoak izan litezke espermatozoideak energia lortzeko. Beste alde batetik, laktato deshidrogenasa C (LDHC) espermatozoide-espezifikoa den entzima bat da eta laktatoa pirubatoan bihurtzeaz arduratzen da (Goldberg et al., 2010). Honen ezabaketak ugalkortasun murriztua sortzen du.

Hau guztia, kontuan hartuta eta CTA-k espermatozoidearen funtzionamendu egokia lortzeko beharrezkoak direla badakigu ere, horien mekanismo molekularrak sakonago ulertzea guztiz beharrezkoa da.

1.8.3. Funtzio fisiopatologikoa: minbizian

Gaur egun, tumoreek testikuluetako CTA espezifikoak aktibaturik dituztela jakina da. Hipotesi hau XX.mendean sortu zen 1904.urtean gizakiaren hormona koriogonadotropikoa (hCGH) gernu-maskuriko minbizian aurkitu zenean. Geroztik aurrera, CTA hau ugatzeko, giltzurruneko, obarioko, prostatako, pankreako eta koloneko minbizian agertzen zela deskribatu izan zuten (Acevedo et al., 1995) eta minbiziaren tratamedurako markatzaile pronostiko bezala erabiltzen hasi zen (Stenman et al., 2004). hCGH-ren kasua adibidetzat izanda, ikerlariek CTA espezifikoen mekanismo molekularrak ikasten saiatu izan dira, gutxi baitakigu tumoregenesian aktibatzen diren geneei buruz (Whitehurst, 2014). Gene hauek tumore zelulen proliferazioa eta biziraupena sustatzen dute eta minbiziaren aurkako terapiak sortzeko baliogarriak izan litezke.

CTA espezifikoen funtzio fisiopatologikoak guztiz ezagunak ez badira ere, azken aldian egindako ikerketek erakutsi dute gene hauek ziklo zelularraren erregulazioan, itzulpenaren kontrolean, zelularen biziraupenean edota apoptosian paper garrantzitsua joka lezaketela. Horrekin lotuta, ikerketek erakutsi dute CTA askok tumorearen migrazioan, inbasioan zein metastasian funtzio garrantzitsua betetzen dutela (Garg et al, 2008; Ghafouri-Fard, 2015; Whitehurst, 2014; Dang et al, 2018; Song et al, 2017).

CTA-en antigenizitatea dela eta, ikerketa asko minbiziaren aurkako txertoen diseinuan oinarritzen dira (Gjerstorff et al., 2007). Tradizionalki, antigenizitate hau testikuluen kokapen immuno-pribilegiaturekin erlazionatzen da (Whitehurst, 2014). Hau muga hematotestikularrari esker lortzen da. Izan ere, zelula immuneak epitelio seminiferotik (bertan espermatozoideen heltzea gertatzen da) banatzen dituen muga da. Hau, Sertoli zelulez eraturik dago eta zelula immuneen infiltrazioa sahiesten du. Izan ere, zelula hauek TGF- β, aktibina A, granzima B edota FAS ligandoa bezalako konposatuak jariatzen ditu epitelio interstizialean dauden zelula immuneen hazkundea eta biziraupena inhibitzeko. Hori dela eta, CTA-ek aurkezten dituzten

zelula tumoregenikoak txertoen diseinurako antigeno perfektuak lirateke, zelula immuneen itua tumorea izanez.

Arestian aipatutakoa kontuan hartuta eta gaur egun CTA jakinak tumoreen prozesuekin erlazionatu badira ere, proteina horiek induzitzen dituzten mekanismo molekularrak aski ezezagunak dira. Horri buruzko ezagutza zehatzagoa baliogarria litzateke minbizia tratatzeko immunoterapia zein beste farmakoen garapenerako.

1.9. SPERM PROTEIN ASSOCIATED WITH THE NUCLEUS MAPPED TO THE X CHROMOSOME (SPANX)

SPANX (Sperm Protein Associated with the Nucleus mapped to the X chromosome) familia multigenikoa X kromosoman kodeaturik dago eta bere adierazpena testikulu eta zelula espermatikoetara mugaturik dago. Proteinen superfamilia honek bi familia kodeatzen ditu gizakietan: SPANX-N azpifamilia, homologia handia erakusten duten 5 proteinez eratua (SPANX N1-N5) (Kouprina et al., 2007), eta SPANX-A/D azpifamilia, sekuentzian %90-98ko homologia erakusten duten SPANX-A1, -A2, -C eta –D (97 aminoazido) isomeroez eraturik eta hauekin %75-80ko antzekotasuna duen SPANX-B (103 aminoazido) isoforma adierazten dueña (Kouprina et al., 2004; Zendman et al., 2003b). Bi azpifamiliek adierazpen postmeiotikoa azaltzen dute espermatogenesian, lehenengo aldiz espermatida haploideetan eta espermatozoide helduetan adierazten dira (Kouprina et al., 2004; Salemi et al., 2004; Westbrook et al., 2001).

SPANX-N, SPANX-A/D azpifamiliako proteinen aitzindari filogenetikoa da. Ugaztun guztietan adierazten bada ere, SPANX-A/D familiaren sorkuntza hominidoen leinuan gertatu zen (Kouprina et al., 2004). SPANX-N familiari buruz gutxi badakigu ere, ikerketek erakutsi dute espermatozoide heldu guztietan akrosoman adierazten dela, ugalkortasunean paper garrantzitsua joka lezakeela iradokituz (Kouprina et al., 2004). Horrez gain, SPANX-N2 eta SPANX-N3 testikuluetan adierazteaz gain, prostata, ondestea, uteroa, birikiak edota urdaila bezalako ehun ez-gametogenikoetan aurkitu daitezke maila askoz baxuagoetan (Kouprina et al., 2007).

Bestalde, eta beste CTA batzuekin gertatzen den bezala, SPANX-N familia melanoma bezalako zenbait minbizi motetan agertzen da eta hauetan funtzio garrantzitsua bete lezake. Gaur egun, funtzio fisiopatologikoa egokitu ez bazaio ere, badirudi gene hauek minbizia tratatzeko immunoterapia itu garrantzitsuak izan litezkeela (Kouprina et al., 2004).

1.9.1. SPANX-A/D proteina familia

SPANX-A/D proteina azpifamiliari dagokionez, 15-20 kDa pisatzen dituen proteina txiki hauek, akrosomaren biogenesiaren Golgi fasean dauden espermatida gozitiarren nukleoaren azalean identifikatu ziren lehenengoz. Espezifikoki, nukleoaren kondentsazioa eta elongazioa hastean, SPANX-A/D proteinak nukleoaren gainazaleko domeinu postakrosomalera migratzen dute. Espermatida helduetan, SPANX-A/D azpifamilia tanta zitoplasmatikoarekin asoziaturik agertzen da. Espermatozoide helduei dagokienez, SPANX-A/D krater nuklearretan eta tanta zitoplasmatikoan adierazten da eta, zelulen %50an soilik agertzen da (Westbrook et al., 2001). SPANX-A/D familiak espermatida batzuen azpipopulazioan eta espermatozoideetan erakusten duen kokapenak espermatogenesian zeharreko proteinaren banaketa zehatza eta tenporala isladatzen du (Kouprina et al., 2004).

SPANX superfamiliaren funtzio biologikoa ezezaguna bada ere, espermatogenesi prozesuan eta giza espermatozoidearen ernaltzeko gaitasunean funtzio garrantzitsua bete dezakeela uste da (Kouprina et al., 2004; Westbrook et al., 2001). Hala ere, espermatozoide helduetan duen kokapenak iradokitzen du azpifamilia bakoitzak funtzio desberdina izan lezakeela. Bestalde, SPANX-A/D familia antzutasunarekin erlazionatu izan da.

Bereziki, SPANX-A/D adierazpen baxua espermatozoideen mugikortasun baxuarekin (astenozoospermia) (Hashemitabar et al., 2015; Shen et al., 2013), akrosomaren malformazioarekin (Liao et al., 2009) edota lagunduriko ugalketan zeharreko haurdunaldi tasa baxuekin erlazionatzen da (Wang et al., 2018). Beraz, SPANX-A/D azpifamilia zuzenean gameto arraren ugalkortasunarekin erlazionatuta badago ere, gaur egun, proteina hauen mekanismo molekularrak zein funtzio biologikoa guztiz ezezagunak dira. Hori dela eta, horren inguruko ikerkuntza ezinbestekoa litzateke espermatozoideen emankortasun gaitasuna zein antzutasun kasuak ulertzeko. Gainera, azken honen tratamendurako zein antisorgailu berriengarapenerako itu terapeutiko interesgarriak lirateke.

Arestian azaldu denez SPANX-A/D azpifamilia espermatozoidearen ahalmen ugalkorrarekin erlazionatuta dago baina gaur egun gero eta ikerketa gehiagok erakutsi dute proteina hauek CTA delako taldean sar daitezkeela. Izan ere, gizakiaren zenbait tumoreetan adierazten dira (Goydos et al., 2001; Wang et al., 2003; Westbrook et al., 2004). Azken urteotako lanen arabera, proteina azpifamilia hau melanoman, mieloman, glioblastoman; ugatz, prostata, eta obarioko kartzinoman eta birika zein gernu-maskuriko minbizian adierazita dago (Almanzar et al., 2009; Wang et al., 2003; Westbrook et al., 2004; Zendman et al., 1999). Esaterako, SPANX-A/D melanoma metastatikoen zitoplasma edota nukleoan adierazten da (kasuen %50) (Westbrook et al., 2004).

Horrez gain, zenbait tumoreetan SPANX-A/D adierazpena inbasioarekin eta metastasi espontaneoarekin erlazionatu bada ere (Hsiao et al., 2016; Maine et al., 2016), minbizian duen funtzio fisiopatologikoa ez da oraindik argitu. Hori dela eta, SPANX-A/D proteina azpifamiliak minbizian betetzen duen funtzioa zein induzitzen dituen mekanismo molekularrak sakonean

aztertzea interesgarria litzateke immunoterapia zein minbizia tratatzeko droga farmakologien garapenerako.

2.Hipotesia eta Helburuak

Hypothesis and Aims

3. Materialak eta Metodoak
Materials and Methods

3. MATERIALAK ETA METODOAK

Ondorengo atalean tesian zehar erabilitako material zein teknika desberdinak deskribaturik agertzen dira. Aipatzekoa da emaitzen atalean, kapitulu bakoitzean egindako esperimentuen deskribapen zehatzagoa ematen dela.

3.1. MATERIALAK

3.1. Taula. Teknika desberdinak burutzeko erabilitako gailuen zerrenda.

Gailua	Etxe komertziala
CO_2-dun inkubagailua	MCO-15AC Sanyo
Errotore baskulantedun zentrifuga	Heraeus MegaFuge 40R
FLUOstar OPTIMA fluorimetroa	BMG Labtech
Fluxu laminar bertikaleko Kanpaia	Faster Bio 48
Mikroskopio alderantzikatua	AxioscopeEclipseTS100 Nikon
Mikroskopio fase-kontrasteduna	H550S
Bideokamera	Basler
Mikroskopioari atxikitako kamera	D07739 Jenoptik
Mr Frosty edukiontzi izoztzailea	Thermo Fisher Scientific
Nitrogeno likidorako tankea	GT-40 Air Liquide
Ur-bainu termostatikoa	TBN-06/100, ICT SL
Shorting bidezko Fluxuzko zitometroa	BD FACSJazz (2B/4YG)
Mikroskopio konfokala	Zeiss Apotome 2 (Jena)
Heraeus Fresco 21 mikrozentrifuga	Thermo Fisher Scientific
NanoDrop ND-1000 espektrofotometroa	Thermo Fisher Scientific
Synergy HT espektrofotometroa	Bio-Tek
Termozikladoreak	Mycycler Life technologies
	BioRad Laser scanner
PerkinElmer CFX96 Real Time detekzio sistema	BioRad
SLM AMinco-Bowman espektrofluorimetroa	Microbeam
Chemidoc XRS sistema	Bio-Rad
FluoStar OPTIMA fluorimetroa	BMG Labtech
Heraeus Fresco 21 zentrifuga	Thermo Scientific
Hotcold S Inkubagailu lehorra	Selecta
Elektroforesi eta transferentzia euskarriak	Bio-Rad
PowerPac Basic Power Supply Energia hornidura	Bio-Rad
Labsonic Sonikatzaileaa	B.Braun International
Zelula homogenizatzailea	MICCRA D-1 30170
Magnetic Rack	NVIGEN
Labnet Mini Labrollerra	Labnet International
Apotome 2 mikroskopio konfokala	Zeiss
Olympus Fluoview FV500 mikroskopio konfokala	Olympus
Fluxuzko zitometroa	FACScalibur Becton Dickinson
Qubit Fluorimetroa	Thermo Fisher Scientific
Speedvac huts makina	Eppendorf
Q Exactive HF Mas espektrometroa	Thermo Scientific

3.2 Taula. Erabilitako erreaktiboen zerrenda.

Erreaktiboa	Erreferentzia	Etxe komertziala
G-IVF kultibo medioa	10135	Vitrolife
FBS S0415	Biochrom	GmbH
DMEM kultibo medioa	H3BE12-741F	Lonza
DMSO	D8418	Sigma
EMEM kultibo medioa	H3BE12-611F	Lonza
XtremeGENE 9 transfekzio erreaktiboa	XTG9-RO	Roche
Lipofectamine 2000 transfekzio erreaktiboa	11668027	Thermo Fisher Scientific
OPTI-MEM transfekzio medioa	31985062	Thermo Fisher Scientific
Polybrene transfekzio erreaktiboa	TR-1003	Sigma
L-glutamina 100X	G7513	Sigma
McCoy's 5A medioa	12-688F	Lonza
Pretoblue Viability erreaktiboa	A13261	Thermo Fisher Scientific
PBS 10X	BE17-517Q	Lonza
Penizilina-estreptomizina	P4333	Sigma
pEYFP-C1 plasmidoa		Clontech
Mutanteak eratzeko gBlocks DNA fragmentuak		IDT
SPANXA/D-ren shRNA lentibirusa		Origene
RPMI140 kultibo medioa	BE12-702F	Lonza
Tripsina-EDTA	T3924	Sigma
Mitomizina	Y0000378	Sigma
Crystal Violet	C3886	Sigma
Puromizina	P9620	Sigma
DEPCrekin trataturiko H_2O	750023	Thermo Fisher
Etanol absolutua	121086	Panreac Applichem
PureLink® RNA Mini kit	12183018A	Life Technologies
Quantitect Reverse Transcription Kit	205313	Qiagen
DNA polimerasa		Immolase
iScript cDNA Synthesis Kit	170-8891	Bio-Rad
Isopropanola	A3928	Panreac Applichem
Kloroformoa	CL01981000	Scharlau LabelIT
SYBRGreen AmpliTaq	1725121	BioRad
TRIzol erreaktiboa	15596026	Invitrogen
Fura-2 2AM		Molecular Probes
Azido pluroniko detergente ez-zitotoxikoa		Molecular Probes
30% akrilamida-bis akrilamida	1610158	Bio-Rad
Bicinchoninic Acid (BCA)	23225	Thermo Fisher Scientific
Bovine serum albumin (BSA)	A7906	Sigma Aldrich
PVDF mintza	10600021	GE Healthcare Life
TEMED	T9281	Sigma Aldrich
Proteasen inhibitzaile koktela	04693159001	Roche
Fosfatasen inhibitzaile koketela	04906845001	Roche
Merck Fluoromont G muntatzailea	00-4958-02	Thermo
Ponceau	P3504	Sigma Aldrich
Vectashield Mounting Medium with DAPI	H-1200	Vector Laboratories
TMT-6 plex Isobaric Labeling Reagent	90061	Thermo Fisher Scientific

Trietilamonio bikarbonato tanpoia (TEAB)	T7408	Sigma Aldrich
Urea	U5378	Sigma Aldrich
Guanidinio klorhidrikoa	G4505	Sigma Aldrich
Ditiotreitola (DTT)	DTT-RO	Sigma Aldrich
Kloroazetamida	C0267	Sigma Aldrich
Sekuentziaziorako Tripsina eraldatua		Promega
Pierce LysC Proteasa		Promega
Azetonitriloa HPLC	A998-1	Fisher Chemicals
Azido Triofluoroazetikoa	T6508-M	Sigma Aldrich
Amonio Bikarbonatoa (ABC)	285099	Sigma Aldrich
Azido Azetikoa	A6283	Sigma Aldrich
Bikarbonato tanpoia	T7408	Sigma Aldrich
NH3 tanpoia	09682	Sigma Aldrich
ReproSil-Pur, 1,9 μm, 120A beads		Dr. Maich, GmbH
Titansphere (5 μm) TiO2 beads	IN5020-75000	Life Science
Sodio Fluoridikoa (NaF)	S7920	Sigma Aldrich
Beta-glizerofosfato disodio gatz hidratoa	251291	Sigma Aldrich
Sodio ortobanadatoa	S6508	Sigma Aldrich
PureProteome Protein A Bolatxo magentikoak	LSKMAGA02	Millipore
Qubit Proteina neurtzeko Kit-a	Q33211	Thermo Fisher Scientific
Azeton	BPA181	Fisher Chemicals
L-Lysin	L5501	Sigma Aldrich
Nu- PAGE 4-12% Gelak	NP031BOX	Thermo Fisher Scientific
Coomassie Urdina	LC6065	Thermo Fisher Scientific
C18 diskoak		3M Empore™ SPE Extraction Disks
C8 diskoak		3M Empore™ SPE Extraction Disks
C18 diskoetako xiringak		Waters

3.3 Taula. Laborategian prestatutako soluzioen zerrenda.

Soluzioa	Osagaiak
Ripa eraldatua (mRIPA)	50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium pervanadate, 5 mM beta-glycerophosphate, 5 mM NaF.
Detergenterik gabeko Ripa eraldatua	50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM sodium pervanadate, 5 mM beta-glycerophosphate, 5 mM NaF.
Blokeo soluzioa	Blotto; %5 BSA
Urea tanpoia	8M urea, 10 mM TrisHCl pH 7,5.
Guanidinio hidroklorikoa tanpoia	8M Gu-HCL, 25mM ABC pH 7,5.
Garbiketa indargetzailea (blotto)	Tris 20 mM, NaCl150 mM, Triton X-100 % 0,1
Elektroforesi-indargetzailea – 10X	pH 8,3 250 mM Tris-Base; %1 (p/b) SDS; 2 M Glizina
Kimioluminiszentzia areagtotzailea (ECL)	1,25 mM luminol; 0,2 mM azido p-kumariko 0,1 M TrisHCl; pH: 8,5 *Erabiltze-egunean, nahasturara %0,3 H_2O_2 (%30) gehitu behar zaio.
Kargatzeko tanpoia 5X SDS	%10 p/b, Tris pH 6,8 %25 b/b,bromofenol urdina %0,025 p/b, glizerola %50 b/b, DTT %5 p/b Persulfato amonikoa (PSA) - %10 1 g persulfato amoniko 10 ml H_20-etan
Persulfato amonikoa (PSA) - %10	1 g persulfato amoniko 10 ml H_20-etan
Sodio dodezil sulfatoa (SDS) %10	10 g SDS 100 ml H_2O-etan
Transferentzia indargetzailea - 10X pH 8,3	250 mM Tris-Base; %0,25 (p/b) SDS; 1,92 M Glizina. *Diluitzean, % 20 (b/b) metanol gehitu behar zaio.
Tris-HCl 0,5M pH 6,8	1L: 62,07 g Tris-Base; HCl pH 6,8-an kokatzeko
Tris-HCl 1M pH 8,8	1L: 121,14 g Tris-Base; HCl pH 8,8-an kokatzeko

3.4 Taula. Tesi honetan erabilitako antigorputzen zerrenda. WB: Western Blot; IF: Immunofluoreszentzia; ON: Overnight; RT: Room Temperature.

Antigorputz Primarioak	Erreferentzia	Etxe komertziala	Ostalaria	Diluzioa	Inkubazio Denbora
Anti-Propio Melanokortina (POMC)	sc-20148	Santa Cruz Biotech.	Untxia	1:500 (IF) 1:200 (WB)	ON
Anti-FITC-CD46	555949	BD Biosciences	Sagua	5 µl/100 µl	RT 1 ordu
4G10 Antifosfo-Tirosina	05-231	Millipore	Sagua	1:1000 (WB)	RT 1 ordu
Anti-Fosfo PKC sustratuak	#2261	Cell Signaling	Untxia	1:500/ 1:750 (WB)	ON
Anti-Fosfo PKA sustratuak	#9624	Cell Signaling	Untxia	1:750 (WB)	ON
Anti-Fosfo MAPK sustratuak	#2325	Cell Signaling	Sagua	1:750 (WB)	ON
Anti-alfa Tubulina	T5168	Sigma Aldrich	Sagua	1:4000 (WB)	RT 1 ordu
Anti-KOR	ABN456	Millipore	Untxia	1:500 (IF) 1:1000 (FC)	ON
Anti-pKOR	PA5-40216	Thermo Scientific	Untxia	1:1000 (WB)	ON
Anti-MOR	ABN5509	Millipore	Untxia	1:1000 (FC)	ON
Anti-DOR	ABN1560	Millipore	Untxia	1:1000 (FC)	ON
Anti-SPANX	ab119280	Abcam	Untxia	1:500 (WB/IF)	ON
Anti-NUP98	sc-74578	Santa Cruz Biotech.	Sagua	1:50 (IF)	ON
Anti-TEKT1	sc-398507	Santa Cruz Biotech.	Sagua	1:50 (IF)	ON
Anti-Fumarato Hidratasa	sc-393992	Santa Cruz Biotech.	Sagua	1:50 (IF) 1: 200 (WB)	ON
Anti-Lamin A/C	A0249	Abclonal	Untxia	1:100 (IF) 1: 1000 (WB)	ON
Anti-GFP-Trap_MA	gtma-20	Chromotek	Alpaca	25 µl	ON/3 orduz RT
Untxi IgGa	X0903	DAKO	Untxia	1:200 (IF)	ON
Anti-GFP	3H9	Chromotek	Arratoia	1:1000	ON

Antigorputz Sekundarioak	**Erreferentzia**	**Etxe komertziala**	**Ostalaria**	**Diluzioa**	**Inkubazio Denbora**
2° Anti-untxia HRP	sc-2004	Santa Cruz Biotech.	Ahuntza	1:1000	RT 1 ordu
2° Anti-untxia HRP	ab6112	Abcam	Ahuntza	1:3000	RT 1 ordu
2° Anti-sagua HRP	sc-2314	Santa Cruz Biotech.	Astoa	1:2000	RT 1 ordu
2° Anti-rat HRP	sc-2006	Santa Cruz Biotech.	Ahuntza	1:1000	RT 1 ordu
Alexa Fluor 488 Anti-untxia	A1108	Thermo	Astoa	1:2000	RT 1 ordu
Alexa Fluor 555 Anti-sagua	A28180	Thermo	Ahuntza	1:1000	RT 1 ordu
Alexa Fluor 663 Anti-untxia	A21070	Thermo	Ahuntza	1:1000	RT 1 ordu
Ioduro Propidioa	81845	Sigma-Aldrich	----------	100 µg/ml	RT 10 min
Hoescht	H3569	Thermo	----------	0,1µg/ml	RT 2 min

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3.2. ZELULAK

Jarraian tesia burutzeko erabilitako zelula motak azaltzen dira.

3.2.1. Espermatozoideak

Espermatozoideen laginak Gurutzetako Unibertsitate Ospitaleko Lagunduriko Ugalketa Unitatean edota Quiron Bilbao ugalkortasun klinikan jasotzen ziren Euskal Herriko Unibertsitatearekin (UPV/EHU) xedatutako hitzarmenari esker. Emaile guztiak osasuntsuak eta Osasunerako Munduko Erakundearen (OME) (ingelesezko World Health Organization, WHO) (WHO, 2010) arabera normozoospermikoak ziren. Aipatzekoa da ikerketa hau UPV/EHUren Batzorde Etikoaren oniritziarekin burutu dela (CEISH-UPV/EHU (M10/2016/254)).

Hazi-laginak masturbazio bidez lortu ziren 3 edo 4 eguneko abstinentzia eta gero. Hazi gordinetik espermatozoide ez-mugikorrak eta haziaren bestelako zelulak baztertzeko, klinikan bertan *swim-up* teknika aplikatzen zitzaien lagin guztiei. Horretarako, hazia eppendorf saio-hodi etan banatzen da (250 µl eppendorfeko) eta horren gainean 10% BSArekin aberastutako G-IVF medioa gehitzen da (500-600 µl), 2 fase eratuz. 3 orduz 37°C eta %5 CO_2tan mantentzen dira. Ondoren, eppendorf saio-hodi bakoitzaren goiko geruzan geratzen diren espermatozoide mugikorrenak berreskuratzen dira. Horren ostean, espermatozoideen morfologia eta kontzentrazioa aztertzen dira fase-kontrasteko mikroskopioarekin (3.1 Irudia).

3.1 Irudia. Swim up teknikaren deskribapena. Iturria: http://www.jinepol_ivf.com. Moldatua.

3.2.1.1. Espermatozoideen tratamenduak

3.5 Taula. Espermatozoideak tratatzeko erabilitako konposatu kimikoak.

Konposatu kimikoa	Kontzentrazioa	Funtzioa	Etxe komertziala
Beta-endorfina	10^{-5}M, 10^{-7}M, 10^{-9}M	MOR eta DOR hartzaileen agonista	Sigma-Aldrich
Naloxona	10^{-5}M, 10^{-8}M	10^{-5}M: MOR, DOR eta KOR hartzaileen antagonista 10^{-8}M: MOR hartzailearen antagonista	Sigma-Aldrich
Morfina	1 µM	MOR hartzailearen agonista	Sigma-Aldrich
CTOP	1 µM	MOR hartzailearen agonista	Sigma-Aldrich
DPDPE	1 µM	DOR hartzailearen agonista espezifikoa	Sigma-Aldrich
U50488H	1 µM	KOR hartzailearen agonista espezifikoa	Sigma-Aldrich
U73122	3 µM	Fosfolipasa C inhibitzailea	Sigma-Aldrich
Mibefradil	30 µM	Kaltzio kanale aktibatzailea	Tocris Biosciences
NNC55-0395	10 µM	CatSper inhibitzailea	Sigma-Aldrich
SQ2336	200 µM	tmAC inhibitzailea	Sigma-Aldrich
Forskolina	50 µM	tmAC aktibatzailea	Sigma-Aldrich
HCO_3^-	50 mM	SACY aktibatzailea	Sigma-Aldrich
BARK1	126 µM	GRK inhibitzailea	Calbiochem
IBMX	0,5 mM	Fosfodiesterasa inhibitzailea	Sigma-Aldrich
Progesterona	1µM/10µM	Catsper kanalaren aktibatzailea. Erreakzio akrosomikoaren kontrol positiboa	Sigma-Aldrich
DMSO	10 µM	Progesteronaren disolbentea	Sigma-Aldrich

Giza espermatozoideen hartzaile opioideen azpitik induzitzen diren seinaliztapen bidezidor intrazelularrak aztertzeko eta hartzaileek ahalmen ugalkorrean duten funtzioa ikasteko, konposatu desberdinak erabili ziren (3.5.Taula). Alde batetik, MOR eta DOR hartzaileetara lotzen den beta-endorfina erabili zen. Horrez gain, naloxona izeneko antagonista ere erabili zen. Bestalde, (−)-trans-(1S,2S)-U-50488 hydrochloride hydrate (U50488H) izeneko konposatua erabili zen KOR hartzailearen agonista espezifikotzat. Kaltzioaren bidezidor intrazelularra aztertzeko U73122 fosfolipasa C inhibitzailea, Mibefradil kaltzio kanaleen aktibatzailea eta NNC55-0395 CatSper kanalearen aktibatzailea erabili ziren.

cAMP/PKA bidezidorra aztertzeko, SQ2336 mintzeko adenilato ziklasaren (tmAC) inhibitzailea, forskolina mintzeko adenilato ziklasaren (tmAC) aktibatzailea eta HCO_3^- adenilato ziklasa solugarriaren (SACY) aktibatzailea erabili ziren. MAPK-en bidezidorra ikasteko ordea, BARK1 GRK inhibitzailea eta IBMX fosfodiesterasa inhibitzailea erabili ziren. Aipatzekoa da, analisi funtzionaletarako konposatuak 1 edota 60 minutuz inkubatu zirela.

3.2.2. Zelula-lerroen kultiboa

Lan hau aurrera eramateko zelula-lerro desberdinak erabili ziren (3.6. Taula). Zelula-lerro guztiak komertzialak dira.

3.6. Taula. Biologia zelularreko teknikak burutzeko erabilitako zelula-lerroen zerrenda.

Zelula mota	Zelula lerroa	Etxe komertziala	Kultibo medioa
Melanoma Primarioa	A375	ATCC (CRL-1619)	DMEM 2mM L-Glutamina + 10% FBS+ 1% Pen/Strep
	Mel-Ho	Innoprot (ACC62)	RPMI 1640 Glutamax+ 10% FBS+ 1% Pen/Strep
Melanoma mestatatikoa Nodo linfatikoak	HS294T	ATCC (HTB140)	DMEM 2mM L-Glutamina + 10% FBS+ 1% Pen/Strep
	COLO-800	Innoprot (ACC153)	RPMI 1640 Glutamax+ 10% FBS+ 1% Pen/Strep
Melanoma Metastatikoa Larruazalpekoa	HT144	ATCC (HTB63)	Mc Coy´s+ 2mM L-Glutamina+ 10% FBS+ 1% Pen/Strep
Ondesteko Adenokartzinoma	HCT-8	ATCC (CCL244)	DMEM 2mM L-Glutamina + 10% FBS+ 1% Pen/Strep
	SW480	ATCC (CCL-228)	
Ugatzetako Adenokartzinoma	MCF-7	ATCC (HTB22)	
Giza enbrioiaren giltzurrun zelulak	HEK293T	ATCC (CRL-1573)	DMEM 2mM L-Glutamina + 10% FBS+ 1% Pen/Strep
Cervix-eko epitelioko kartzinoma	HeLa	ATCC (CCL2)	EMEM 2mM L-Glutamina + 10% FBS+ 1% Pen/Strep
Neuroblastoma	SMS-KCNR	Texas Tech University	RPMI 1640 Glutamax+ 10% FBS+ 1% Pen/Strep

Aipatzekoa da zelula guztiak behi umekiaren sueroa edo FBS (ingelesetik fetal bovine serum), penizilina-estreptomizina (Pen/Strep) eta L-Glutaminarekin osatutako kultibo espezifikoarekin, 37°C eta %5 CO_2 inkubatzailean mantendu zirela.

Zelulak hazkuntzan mantentzeko, 25 edo 75 cm^2-etako fraskoak erabiltzen ziren eta %90 inguruko konfluentziara heltzean, zelulak frasko berrira eramaten ziren. Horretarako, zelula-lerroei EDTA-dun tripsina gehitzen zitzaien eta 37°C-tako inkubadorean sartzen ziren 1-3 minutuz.

Ondoren, zelulak 5 minutuz zentrifugako tubo konikoetan sartu eta 1500rpm-tan zentrifugatzen ziren hauek hodiaren hondoan sendimentatzeko. Bukatzeko, zelulak medio berrian birsuspenditzen ziren ondoren frasko berrira pasa eta bertan hazten jartzeko.

3.3. TEKNIKAK

3.3.1. Espermatozoideen mugikortasunaren analisia

Mugikortasunaren analisia fase-kontrastedun mikroskopioarekin eta ordenagailu bidezko Sperm Class Analyzer 2005® softwarearekin neurtu zen, 3.7. taulako konfigurazioa erabiliz. Aztertu beharreko konposatua espermatozoideekin kontaktuan jarri ondoren, espermatozoideen mugikortasuna 1 eta 60 minutuz neurtu zen.

3.7.Taula: erabilitako CASA sistemaren konfigurazioa.

Parametroak	
Frame/segundoko: eskuratutako irudiak segundoko	25
Frame zenbatekoa: grabatutako irudiak	25
LVV muga (µm/s): espermatozoide geldo baten abiadura maximoa	10
MVV muga (µm/s): espermatozoide ertain baten abiadura maximoa; hortik gora, azkartzat hartuko da	35
STR minimoa (%): azkarrak ez ezik, progresibotzat hartzen diren espermatozoideen kopurua adierazteko balioa	80
Fase-kontrastea: mikroskopioak erabilitakoa irudiak grabatzean	PH+
Handipena	x20

Giza espermatozoideak 50 $x10^6$ zelula/ml-ko kontzentraziora egokitu ziren eta lagin bakoitzetik Makler zenbaketa kameran 10 µl jartzen ziren. Espermatozoideen mugikortasuna adierazteko, Osasunerako Munduko Erakundearen (WHO, 2010) irizpideen araberako nomenklatura erabili zen:

- Mugikortasun progresiboa (lerro zuzena eta azkarra) (abiadura ≥ 10 µm/s).
-Mugikortasun ez-progresiboa (abiadura < 10 µm/s) (zirkulu txikietan igerian).
-Mugimendurik ez duten espermatozoideak.

Mugikortasun hiperaktiboa espermatozoidearen higiduraren aldaketa da zeinetan espermatozoidea azkarrago mugitzen da eta flageloa indar handiagoarekin astintzen da. Mugikortasun hiperaktiboa ondorengo parametroen arabera finkatu zen: abiadura lerromakurra (VCL) 100 m/s, linealtasuna (LIN) %60 eta buruaren albo-desplazamenduaren anplitudea (ALH) 5 µm.

Aipatzekoa da espermatozoideen mugikortasun azterketatik zetozen emaitzak ondorengo erara normalizatu zirela: [(Tratamendua-Kontrola)/(Kontrola)x100]. Ondoren emaitzak SPSS (v.22) programa estatistikoarekin aztertu ziren, T-Student eta ANOVA (Bonferroni post hoc-a) test estatistikoak aplikatuta. Emaitza estatistikoki esanguratsuak zirela onartu zen *p < 0.05 eta estatistikoki oso esanguratsuak **p < 0.01 zenean.

3.3.2. Fluxuzko zitometria

Alde batetik, beta-endorfinak, U50488H agonista espezifikoak eta beste konposatuek espermatozoideen erreakzio akrosomikoan duten eragina aztertzeko, fluxuzko zitometria erabili zen. Laburki, fluxuzko zitometriak laserraren erabilpenaren bidez, zelulen zenbaketa eta sailkapena egiten du denbora laburrean ezaugarri morfologikoen edota biomarkatzaileen presentziaren arabera. Horrela, likido batean dauden zelulak kolorerik gabeko hoditxo batean zehar igarotzen dira, zeinetan laser batek argia igortzen dien. Gailuak daukan detektagailuak zelulek igorritako eta dispertsatutako argia detektatuko dute zelulen konplexutasunaren eta tamainaren araberako informazioa jasoz.

Kasu honetan, zelulen tindaketarako Fluoreszeina Isotiozianato (FITC)-anti-CD46 antigorputz konjokatua (5 µg/ 100 µl) erabili zen ordu betez ingurugiro tenperaturan. Espezifikoki, erreakzio akrosomikoa pairatu duten espermatozoideen barne-mintz akrosomalean CD46 proteina eskuragarri dago antigorputzarentzat. Horrez gain, bideragarritasun espermatikoaren test bat egin zen soilik bizirik zeuden zelulekin lan egiteko. Beraz, hilik zeuden zelulak baztertzeko Hoechst 33258 (0,1 µg/ml) markatzailea erabili zen. Izan ere, markatzaile hau hilda dauden zelulen nukleoan barneratzeko eta DNAn batzeko gai da. Ondoren, lagin guztiak fluxuzko zitometriaren bidez aztertuak izan ziren.

Aipatzekoa da erreakzio akrosomikoaren azterketatik zetozen emaitzak ondorengo erara normalizatu zirela: [(Tratamendua-Kontrola)/(Progesterona-DMSO)x100. Ondoren emaitzak SPSS (v.22) programa estatistikoarekin aztertu ziren, T-Student eta ANOVA (Bonferroni post hoc-a) test estatistikoak aplikatuta. Emaitza estatistikoki esanguratsuak zirela onartu zen *p < 0.05 zenean eta estatistikoki oso esanguratsuak **p < 0.01 zenean.

Beste alde batetik, beta-endorfinak giza espermatozoideen PKC proteinaren seinaliztapen bidezidor intrazelularrean zuen efektua aztertzeko, fluxuzko zitometria erabili zen. Kasu honetan, PKC-ren sustratu fosforilatuak zelularen barnean daudenez, espermatozoideak %4 paraformaldehidoarekin fixatu ziren 30 minutuz eta ondoren %0,5 Tritoi X-100 erabili zen 10 minutuz zelulak permeabilizatzeko. Zentrifugazioz hiru garbiketa egin ondoren, laginak %10 FBS-ko soluzioarekin blokeatu ziren 30 minutuz. Ondoren anti-PKC ren sustratu fosforilatuen aurkako antigorputza (1:500) (Cell Signalling) gehitu zen eta laginak gau osoan zehar inkubatu ziren 4°C-tan. Hurrengo egunean, antigorputz primarioa kendu eta laginak zentrifugazioz garbitu ziren PBS erabiliz. Ondoren, astoan sortutako eta untxiaren aurkakoa den Alexa Fluor 488 antigorputz sekundarioa (1:2000) gehitu zen ordu betez giro tenperaturan eta iluntasunean inkubatuz. Nukleoak tindatzeko Hoechst markatzailea erabili zen. Kontrol negatiboak soilik antigorputz sekundarioa gehituz eta untxiaren

immunoglobulinak erabiliz egin ziren. Fluxuzko zitometriatik eratorritako emaitza guztiak aztertzeko Summit v.4.3 softwarea erabili zen.

3.3.3. Kaltzio intrazelularraren neurketa

Beta-endorfinak eta U50488H agonistak, giza espermatozoideen kaltzio intrazelularraren kontzentrazioan duen eragina aztertzeko, Fura-2 zunda erabili zen. Horretarako, espermatozoideak 10 x 10^6 zelula/ml-ko kontzentraziora egokitu ziren giza albumina seruan. Ondoren, espermatozoideak azido pluroniko detergente ez-zitotoxikoaren (%0,1) presentzian zegoen Fura-2 zundaren azetoxymetil ester formarekin (Fura-2/AM 8x 10^{-6}M) inkubatu ziren 60 minutuz giro tenperaturan. Ondoren, zelulak 2-7 orduz G-IVF medioan birsuspenditu ziren. Horren ostean, espermatozoideen alikuotak (1 ml) kuartzozko kubeta batean sartu eta espektrofluorimetroaren bidez aztertu ziren 37 °C-ra. Igorritako fluoreszentzia 510nm-tan neurtu zen. Kaltzioaren kontzentrazioa neurtzeko laginak bi uhin luzeratan kitzikatu ziren (340nm eta 380nm) eta kitzikatutako bi uhin luzeren argi fluoreszentea 510nm-ko filtroa duen foto-biderkatzaileari esker neurtu zen. Autofluoreszentziaren seinalea kentzeko 5 nM $MnCl_2$ erabili ziren eta F340/F380 ratioa kaltzio intrazelularraren kontzentrazioaren adierazle bezala hartu zen.

Progesterona (1 µM) erabili zen beta-endorfinak eta U50488H agonistek progesteronak-induzitutako kaltzio intrazelularraren mailan duten eragina aztertzeko. Kaltzio intrazelularraren kontzentrazioaren kalibraziorako Tritoi X-100 (%5) detergentea erabili zen, kaltzio kontzentrazioaren erantzun maximoa sustatzen duena. Erantzun minimoa, ordea, EGTA ioi kelantearen (40 nM) gehipenarekin lortu zen.

3.3.4. Western blot (WB)

Western Blot edo immunoblot teknikaren bidez edozein proteina nahasketan intereseko proteina ezagutzen duen antigorputz espezifikoen erabilpenean datza. Horrela, intereseko proteinaren identifikazioa lortzeaz gain, kuantifikazio erlatiboa ere azter daiteke.

3.3.4.1. Proteinaren erauzketa

Espermatozoideen proteinaren erauzketarako, 60x10^6 zelula zenbatu ziren eta zentrifugazioz (1600rpm, 5 minutu) kultibo medioa kendu zitzaien. 3 aldiz PBS-arekin garbitu eta gero proteasa eta fosfatasen inhibitzaileak zituen modified RIPA lisi medioa (detergentearekin) gehitu eta izotzean 10 minutuz utzi ziren. Ondoren, 30 segunduz zelula homogenizatzailearekin zelulak apurtu ziren eta laginak 30 minutuz izotzetan mantendu ziren. Horren ostean, zelulak sonikatu ziren (%70 anplitudea, 0,5 ziklo, 10 pultso, behin eta %40 anplitudea, 0,5 ziklo, 10 pultso, birritan) eta berriz ere izotzetan jarri ziren, 45 minutuz, astintzen. Ondoren, zelulak 4°C-tan, 15.000 x g-etan, 15 minutuz zentrifugatu ziren proteina zatiki solugarria eta solugaitza banatzeko. Proteina zatiki desberdinak 80°C-tan mantendu ziren hurrengo erabilera arte.

Zelula lerroen proteinaren erauzketarako, zelulak tamaina desberdineko plaketan hazi (P6, P24 edo T25) eta %80-90-ko konfluentziara heltzean, izotzean jarri ziren. PBS hotzarekin zenbait garbiketa egin eta gero, proteasa eta fosfatasen inhibitzaileak zituen modified RIPA lisi medioa gehitu eta

izotzetan utzi ziren 10 minutuz. Zelula guztiak apurtzeko scraper bat erabili zen eta zelulak altxatu ostean mikrozentrifugako hodi batera transferitu ziren ondoren 4°C-tan, 15.000 x g-etan, 15 minutuz zentrifugatzeko. Laginen proteina solugarria edo gainjalkinak, -80°C-tan mantendu ziren hurrengo erabilerara arte.

3.3.4.2. Proteinaren kuantifikazioa

Proteinaren kuantifikaziorako BCA edo ingelesezko Bicinchoninic acid metodo fotometrikoa erabili zen. Proteina estandartzat BSA (ingelesetik Bovine serum albumin) erabili zen eta hau ur distilatuan disolbaturik erabili zen hurrengo kontzentrazioetan: 0; 0,2; 0,4; 0,6; 0,8 eta 1 µg/µl. Metodoaren detekzio mailaren barruan lan egiteko, espermatozoide zein zelula lerroetako lagin guztiak uretan diluitu ziren. Ondoren, 12,5 µl estandar eta lisatuak birritan plakeatu ziren P96 plaka batean. Lagin bakoitzaren gainean 100 µl BCA nahasketa (BCA erraktiboa eta CuSO4-ren arteko nahasketa, 50:1 proportzioan) gehitu zen eta plaka 37°C-tan, 30 minutuz inkubatu zen.

Ondoren plaka OPTIMA espektrofotometroan sartu zen eta absorbantzia 562nm-tan neurtu zen, absorbantziaren balioak laginen proteina mailarekin zuzenki erlazionatzeko. Hau estandarraren absorbantzia eta kontzentrazioa erreferentzi bezala izanda egin zen. Proteina kontzentrazioa kalkulatu ondoren, laginak eraldatutako RIPA lisi tanpoiarekin diluitu ziren konparatu beharreko lagin guztiek proteina kantitate berdina izan zezaten. Ondoren, lisatu bakoitzari 4X karga tanpoia edo karga tanpoia gehitu zitzaion kontzentrazio finala 1X izan zedin. Laginak 10 minutuz 95°C-tan termoblokean berotu ziren proteinen desnaturalizazioa lortzeko. Aipatzekoa da esperimentu batzuetarako, espermatozoideen proteina ez zela erauzi ezta kuantifikatu ere egin. Jarraian 500.000 zelula zenbatu eta lisi tanpoia gehitu zitzaien, ondoren desnaturalizatzeko eta gelean kargatzeko.

3.3.4.3. Western Blot-aren prozedura

Western Blot teknikaren prozedurarekin hasteko lehenengo eta behin elektroforesirako akrilamidazko gelak prestatu behar dira. Gelen osagaiak eta proportzioak gel banatzailerako eta gel kontzentratzailerako 3.8. eta 3.9. tauletan biltzen dira hurrenez hurren.

3.8. Taula. Western blot teknikarako erabilitako gel banatzailearen osagaiak.

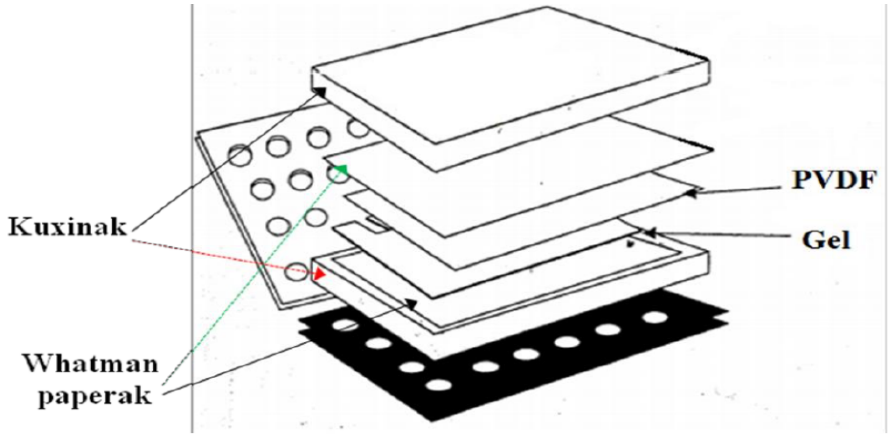
	%10eko akrilamida	**%12ko akrilamida**
H_2O	Bolumena bete arte	Bolumena bete arte
Tris-HCl pH 8,8	%25 (bol)	%25 (bol)
Akrilamida %30	%33,3 (bol)	%40 (bol)
SDS %10	%1 (bol)	%1 (bol)
PSA %10	%0,5 (bol)	%0,5 (bol)
TEMED	5 µl	5 µl

3.9. Taula. Western blot teknikarako erabilitako gel kontzentratzailearen osagaiak.

	Edozein %-ko gela
H_2O	Bolumena bete arte
Tris-HCl pH 6,8	%25 (bol)
Akrilamida %30	%13,3 (bol)

SDS %10	%1 (bol)
PSA %10	%0,5 (bol)
TEMED	5 µl

Behin konparatu nahi ziren laginen proteina kantitatea berdindu eta desnaturalizatu zenean, hauek akrilamidazko gelean kargatu ziren pisu molekular ezaguneko markatzaileekin batera. Ondoren 100V-tako potentzial diferentzia aplikatu zitzaien proteinak tamainaren arabera banatu ziren arte. Behin elektroforesia bukatuta, proteinak PVDF mintz batera transferitu ziren Mini Trans-Blot III euskarriak erabilita eta hotzetan ordu betez 100V-tako potentzial diferentzia aplikatuz. Aipatzekoa da, sandwich itxurako muntaia egin zela 3.2. irudian deskribaturik agertzen den bezala.



3.2. Irudia. Western blot teknikan tranferentziarako erabilitako sandwicharen osagaiak. Iturria: Ekaitz Agirregoitia.

Transferentzia bukatzean, PVDF mintza %5-eko BSAdun blokeo soluzioarekin ordu betez inkubatu zen giro tenperaturan, antigorputz primarioaren espezifikotasuna bermatzeko. Ondoren, mintza antigorputz egokiarekin inkubatu zen gau osoan zehar 4°C-tan. Antigorputz bakoitzaren diluzioa eta inkubazio denborak 3.4. taulan agertzen dira. Hurrengo egunean antigorputz primarioa kendu eta mintza Blotto tanpoiarekin garbitu zen 5 minutuko hiru garbiketa eginez. Ondoren HRPari (ingelesezko Horseradish peroxidase) konjokaturiko antigorputz sekundario egokiarekin ordu betez inkubatu zen giro tenperaturan eta bukatzean 5 minutuko hiru garbiketa egin ziren. Mintza errebelatzeko ECL sustratu kimioluminiszentea erabili zen, HRParen aktibitatea neurtuz. Kimioluminiszentzia detektatzeko ChemiDoc XRS gailua erabili zen. Proteinen banden intentsitatea eta tamainaren arteko ratioa kalkulatzeko Image J softwarea erabili zen. Aipatzekoa da, lagin bakoitzaren banda *housekeeping* gene batekiko normalizatu zela, kasu guztietan alfa-tubulinarekin.

3.3.5. Proteina (Co)-immunoprezipitazioa (IP) (Co-IP)

SPANX-A/D proteina familiaren interaktoma aztertzeko, proteina (co)-immunoprezipitazioaren teknika erabili zen. Espermatozoideen eta A375 melanoma zelulen proteina erauzketa 3.3.4.1. atalean azaltzen da. Aipatzekoa da A375 zelulak YFP-SPANX-A mutantearekin transfektatu zirela aurretiaz.

Espermatozoideen SPANX-A/D proteina endogenoa prezipitatzeko 500 µg proteina hartu (lau aldiz) eta 25µl anti-SPANX antigorputzarekin inkubatu zen 4 orduz, 4°C-tan biraka. Aipatzekoa da, antigorputz primarioa aurretiaz bola magnetikoetara konjokatu zela. Horretarako, antigorputz primarioa Pure Proteome Protein A bolatxo magnetikoakin ordu betez biraka inkubatu zen giro tenperaturan. Ondoren, 45 minutuz giro tenperaturan borato tanpoiarekin biratzen inkubatu zen eta hori bukatzean, dimetil pimelimidato dihidroklorhidrikoa erreaktiboarekin inkubatu zen, beste 45 minutuz (biraka, giro tenperaturan). Bukatzeko, 0,2 M trietanolaminan, pH 8,2an disolbatuta zegoen 20 mM DMP-tan inkubatu zen ordu betez giro tenperaturan. Kontrol negatibotzat untxiaren-IgG inespezifikoak erabili ziren, eta hauek ere aurretiaz bolatxo magnetikoekin konjokatu ziren arestian azaldu bezala (lau aldiz). Behin immunoprezipitazioa bukatuta, bola magnetikoak lisi tanpoiarekin 3 aldiz eta detergenterik gabeko mRIPA soluzioarekin beste 3 aldiz garbitu ondoren, proteinak eluitzeko 8M Gu-HCL (100 µl) gehitu zitzaien, 70°C-tan 15 minutuz inkubatuz.

A375 melanoma zelulen kasuan, proteina erauzi ondoren, YFP-SPANX-A proteina mutantea prezipitatzeko 500 µg proteina hartu (lau aldiz) eta 25 µl anti-GFP trap_MA antigorputzarekin (bolatxo magnetikoetara konjokatua, komertziala) inkubatu zen 3 orduz, 4°C-tan biraka. Kontrol negatibotzat YFP bektorea erabili zen (lau aldiz). Behin immunoprezipitazioa bukatuta, bola magnetikoak lisi tanpoiarekin 3 aldiz garbitu eta gero, Western Blot teknikarako bideratutako laginak eluitzeko karga tanpoian x1 birsuspenditu ziren eta arestian azaldutako protokoloarekin jarraitu zen. Masazko espektrometriarako bideratutako laginak eluitzeko 8M Gu-HCL (100 µl) gehitu zitzaien, 70°C-tan 15 minutuz inkubatuz.

Ondoren, 3.3.14.3.2 atalean azalduko den protokoloarekin jarraitu zen.

3.3.6. Zeharkako immunofluoreszentzia (IF)

Immunofluoreszentzia teknika zeluletan proteina espezifiko baten presentzia eta bere sakabanaketa aztertzeko teknika da. Intereseko proteina detektatzeko fluoreszentea den antigorputz bat erabiltzen da eta ondoren horrek igorritako fluoreszentzia mikroskopioaren bidez aztertzen da.

Espermatozoideen kasuan, 50.000 zelula zenbatu eta polilisinadun estalkien gainean itsatsi ziren, ondoren estalkiak 24 putzutzutako plaketan sartzeko. Zelula-lerroeidagokienez, zelulak estalkidun 24 putzutzutako plaketan hazten jarri ziren eta %60-70 bitarteko konfluentziara heltzean kultibo medioa kendu eta PBS-arekin garbitu ziren. Ondoren, zelula mota guztiak %4 paraformaldehidoarekin 30 minutuz giro tenperaturan fixatu ziren. Horren ostean, paraformaldehidoa kendu eta zelulak hiru aldiz garbitu ziren PBS-arekin. Espermatozoideak %0,5 tritoi X-100 detergentearekin 10 minutuz permeabilizatu ziren. Zelula-lerro desberdinen kasuan permeabilizazioa suabeagoa izan zen, %0,2 tritoi X-100 10 minutuz erabiliz. Permeabilizazioaren ostean, laginak PBS-arekin garbitu eta gero, zelulak %10ko FBSan ordu betez blokeatu ziren. Behin laginak blokeatuta, %3ko FBSan diluituriko antigorputz primarioa gehitu zen, 4°C-tan hurrengo egunera arte inkubatzen utziz.

Hurrengo egunean, antigorputz primarioa kendu eta PBSarekin hiru garbiketa egin ondoren, konposatu fluorogenikoarekin konjokatutako antigorputz sekundario egokia gehitu zen ordu betez

giro tenperaturan inkubatuz. Zelula mota bakoitzaren nukleoak tindatzeko, Hoechst 33258 markatzailea erabili zen giro tenperaturan 2 minutuz inkubatuz. Behin tindaketa bukatuta, estalkiak banaka eta buruz behera porta baten gainean muntatu ziren Fluoromont muntai medioa erabiliz. Ondoren portak ilunetan eta -20°C tan gorde ziren mikroskopioan analizatu arte. Laginak mikroskopio konfokalaren bitartez (Apotome) aztertu ziren Euskal Herriko Unibertsitateko (UPV/EHU) mikroskopia zerbitzu orokorrean (SGIker) eta irudien analisia Image J softwarea erabiliz egin zen.

3.3.7. Polimerasaren kate-erreakzio kuantitatiboa (RT-qPCR)

Espermatozoideetan POMC prekurtsorearen presentzia ikusteko eta SPANX-A/D Sh A375 zeluletan SPANX-A genearen isilpena berresteko RT-qPCR teknika erabili zen. Hau adierazpen genikoaren neurketan datza polimerasaren kate-erreakzioaren (ingelesezko Polymerase Chain Reaction) edo PCR teknikaren bidez. Bereziki, DNAren anplifikazio esponentzialean eta kuantifikazio erlatiboan datza, polimerasa entzimaren bitartez intereseko oligonukleotidoen kate osagarria sintetizatuz.

3.3.7.1 RNAren erauzketa

Zelulen RNA, PureLink® RNA Mini kit-a erabiliz erauzi zen hornitzailearen argibideak jarraituz.

3.3.7.2. RNAren kontzentrazioa, purutasuna eta integritatearen neurketa

RNAren kuantifikazioa NanoDrop eta Synergy HT izeneko espektrofotometroaren bidez egin zen. Laginaren RNAren kontzentrazioa neurtzeaz gain, absorbantzia ere neurtu zen eta uhin-luzera desberdinen arteko ratioa kalkulatu zen laginen purutasuna zehazteko. RNA purua zela onartzeko A260/280 eta A260/230 ratioen balioak 2 inguruan egon behar dira. Beste alde batetik, RNAren integritatea berresteko, laginak %1eko agarosa gelean kargatu ziren eta elektroforesia 125V-ra burutu zen. 18S eta 28S bandak integritatearen adierazle ziren.

3.3.7.3. Alderantzizko transkripzioa (RT)

Espermatozoideen DNA osagarriaren sintesia burutzeko Quantitect Reverse Transcription Kit-a erabili zen hornitzailearen argibideak jarraituz.

A375 melanoma zelulen DNA osagarriaren sintesia burutzeko iScript cDNA Synthesis Kit komertziala erabili zen. Laburki, 1 µg RNA, 1 µl iScript alderantzizko traskriptasa eta 4 µl iScript erreakzio nahasketarekin termozikladorean inkubatu zen hurrengo zikloetan zehar: 5 minutu 25°C-etan, 30 minutu 42°C-etan eta 5 minutu 85°C-etan.

3.3.7.4. Polimerasaren kate-erreakzio kuantitatiboa (qPCR)

qPCRa burutzeko POMC eta SPANX-A/C/D geneak ezagutzen dituzten primer espezifikoak diseinatu ziren. Lehenik eta behin, intereseko genea ezagutzen duen primer espezifikoa diseinatu zen Primer3 softwarea erabiliz (3.10. Taula) (Untergasser et al., 2012).

POMC-ren kasuan anplifikazioa ondorengo konposatuak zituen 25 µl PCR tanpoian egin zen: 3 µl cDNA erreakzio nahasketa, 2,5 mM $MagCl_2$, 200 µM dNTP eta 1.5U DNA polimerasa termoegonkorra. PCR-a 35 ziklotan burutu zen ondorengo parametroak finkatuz: 15 segunduz 94 °C-tan, 20 segunduz 60 °C-tan eta 20 segunduz 72 °C-tan. POMC generako diseinatutako *primer*rak exoietan lotzen ziren. Bukatzeko, RT-PCR produktuak 2,5%-ko gel elektroforesian banatu ziren. Anplikoien tamaina DNA-ren markatzaile molekularrak erabiliz kalkulatu zen eta produktuen identifikazioa anplikoien sekuentziazioaren bidez burutu zen.

SPANX-A/D-rako ordea, erreakzioak 10 µl-ko bolumen osoan burutu ziren ondorengo osagaiak izanik: 50 ng cDNA, 5 µl of SYBR Green eta 200 µM atzerako eta aurrerako primerrak.

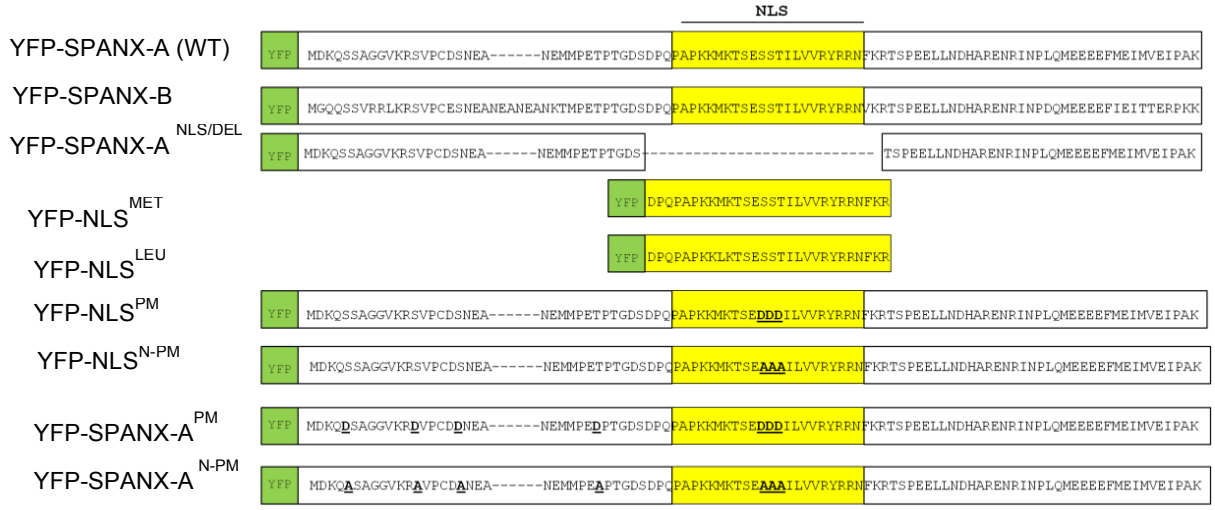
3.10. Taula. RT-qPCR teknika burutzeko erabilitako primeren sekuentzia.

Izena	**Genea**	**Sekuentzia (5'-3')**
Giza Propiomelanokortina	POMC	(f) CTCACCACGGAAAGCAACC (r) ATCGGTCCCA GCGGAAGT
Giza Beta-aktina	ACTB	(f) TCCCTGGAGAAGAGCTACGA (r) ATC TGCTGGAAGGTGGACAG
Sperm protein associated with the Nucleus A, C eta D	SPANX-A/C/D	(f) AACGAGATGATGCCGGAGAC (r) TTTGGAGGGGGTTGATTCTG

Aipatzekoa da POMC eta SPANX-A/C/D geneen espresioa hiru esperimentu independentetan neurtu ziren eta esperimentu bakoitzean hiru erreplika erabili ziren. Termozikladorean lortutako Ct-ak 2-ΔΔCt bihurtu ziren konparaketa taldeen arteko kuantifikazio erlatiboa lortzeko.

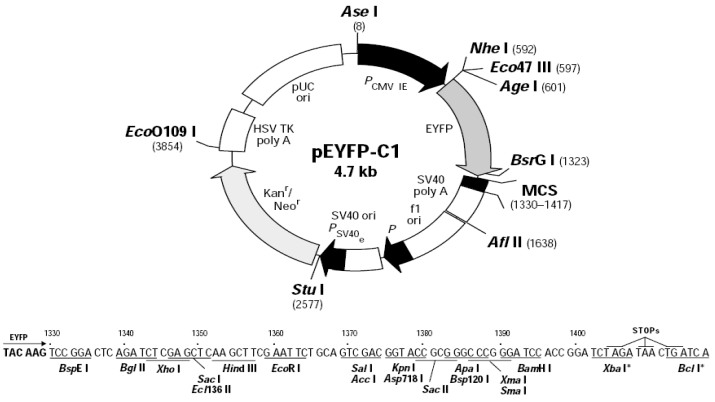
3.3.8. Klonazio entseguak

SPANX-A/D proteina familiaren funtzioa ezagutzeko mutagenesi esperimentuak burutu ziren plasmido erabiliz. Mutanteak kodetzen zituzten cDNA-k Hind III/BamHI zatiki bezala sintetizatu ziren (gBlocks Gene Fragments, IDT) eta pEYFP-C1 plasmidoan (Clontech) azpiklonatu ziren. Diseinatutako cDNA zatikiak kodetzen dituzten aminoazido sekuentziak ondorengoak dira (3.3. irudia):



3.3. Irudia. Diseinatutako cDNA zatikiak kodetzen dituzten aminoazido sekuentziak pEYFP-C1 bektorean azpliklonatuta.

Laburki azalduta, liofilizatuta zetozen cDNA fragmentu desberdinak Hind III eta Bam I entzimekin digeritu ziren 3 orduz 37°C-ra ur-bainuan, eta hornitzailearen argibideak jarraituz QIAquick PCR purifikazio Kit-a (Qiagen) erabiliz purifikatu ziren. Ondoren, cDNA-k (1 µl) aurretiaz entzima berdinez moztutako, defosforilatutako eta inaktibatutako pEYFP-C1 plasmidoarekin (1 µl) ligatu ziren gau osoan zehar (4°C) ATP (0,75 µl), Ligasa (1 µl), ligasaren tanpoia (1 µl) eta ura (4,75 µl) erabiliz. Kontrol negatiboa egiteko, ez zen cDNA-rik erabili eta plasmidoa intserturik gabe ligatu zen. Aipatzekoa da, plasmido honek kanamizina antibiotikoarekiko erresistentzia duela. Gainera fluoreszentea den EYFP proteina kodeatzen duen sekuentzia dauka.



3.4. Irudia. pEYFP-C1 bektorearen mapa. Bektoreak kanamizinarekiko erresistentzia ematen dio eta MCS izeneko gunean gure mutanteen cDNA sartzen da HindIII eta BamHI entzimentzat errestrikzio guneak dituelako.

Hurrengo egunean, ligazio bakoitza DH5α bakterio erresistenteekin transformatu zen, shock elektrotermikoaren metodoaren bidez. Horretarako, lehenengo eta behin, ligazioak bakterioekin nahastu ziren eta ordu betez izotzetan inkubatu ziren. Bakterioek ligazioak barnera zitzaten 30 segunduz 37°C-tan inkubatu ziren.

Transformazioa burutu eta gero, baldintza esteriletan, bakterioak Lysoni Broth (LB) medioarekin inkubatu ziren ordu betez, 37°C-tan (220rpm). Ondoren, bakterioak kanamizinadun agar plaketan erein ziren eta gau osoan zehar inkubatu ziren 37°C-tan.

Hurrengo goizean, agar plaketan hazitako bakterioen "mini prep" bezala ezagutzen den kultiboa egin zen anplifikatutako plasmidoak erauzteko. Aipatzekoa da, hazitako bakterio guztiek kanamizinareko erresistentea den plasmidoa barruan dutela. Izan ere, horrek ahalbidetzen die kanamizina antibiotikoarekin aberastutako medio batean haztea. Behin koloniak pikatuta, kanamizinadun LB medioarekin nahastu eta gau osoan zehar astitzen inkubatu ziren, 37°C eta 220 rpm-tan. Ondoren, hornitzailearen argibideak jarraituz QiaPrep Spin Miniprep Kit-a eta QIAquick PCR purifikazio Kit-ak (Qiagen) erabili ziren plasmidoen erauzketarako eta purifikazioarako. Horren ostean, plasmido bakoitzaren kontzentrazioa neurtu zen Nanodrop espektrofotometroa erabiliz.

Aipatzekoa da, mutante guztien DNA sekuentziatu zela (Stabvida, Caparica, Portugal) eta nahigabeko mutazioen presentzia berretsi zela.

3.3.9. Mutante desberdinen gainadierazpena

SPANX-A/D proteina familiaren funtzioa aztertzeko zenbait esperimentu burutu ziren A375, HEK293T eta HeLa zelulak plasmido desberdinekin transfektatuz. Horretarako, zelula mota desberdinak transfekzioa baino 24 ordu lehenago 24 putzutako plaketan hazi ziren estalki esterilen gainean eta kultibo medio oso egokiarekin inkubatuz (3.6. Taula).

HEK293T eta HeLa zelula lerroen transfekzioak XtremeGENE 9 izeneko transfekzio erreaktiboa erabilita egin ziren. Transfekzio medioa prestatzeko, FBSrik gabeko kultibo medioa XtremeGENE erreaktiboarekin nahastu zen (100 µl kultibo medioko, 3µl XtremeGENE). Mutante bakoitzaren 600 ng transfekzio medioarekin inkubatu zen 25 minutuz. Bitartean, zelulei kultibo medioa aldatu zitzaien (750 µl). Ondoren, kultibo medio berriaren gainean tantaka plasmido + transfekzio medioaren nahasketa gehitu zen (37 µl) eta plakak 37°C-ko inkubagailuan sartu ziren gau osoan zehar.

A375 zelula lerroaren transfekzioak Lipofectamine 2000 izeneko transfekzio erreaktiboa erabiliz egin ziren. Transfekzio medioa prestatzeko, lehenengo eta behin OPTI-MEM medioa (putzu bakoitzeko 50 µl) Lipofectamine 2000 erreaktiboarekin (putzu bakoitzeko 3 µl) nahastu zen. Beste alde batetik, plasmidoa (1 µg) OPTI-MEM medioarekin (putzu bakoitzeko 50 µl) nahastu zen. Ondoren, 5-10 minutuz, transfekzio medioa eta plasmido diluitua nahastu ziren. Bitartean zelulei kultibo medioa aldatu zitzaien (500 µl) eta gero, putzu bakoitzean nahasketa (90 µl) gehitu zen tantaka. Plakak 37°C-ko inkubagailuan sartu ziren gau osoan zehar.

Transfekzioak egiaztatzeko, estalki esterilen gainean ereindako zelulak, PBS %3,7 formaldehidoarekin fixatu ziren 30 minutuz eta PBS-a erabiliz hirutan garbitu ziren. Ondoren, estalkiak portetan muntatu ziren Vectashield Antifade muntaketa medioa erabiliz. Aipatzekoa da erreaktibo honek zelulen nukleoak tindatzeko 4',6-diamidino-2-fenilindola edo DAPI-a duela. Mutanteen lokalizazioa eta kuantifikazioa aztertzeko Zeiss Apotome 2 mikroskopio konfokala erabili zen erresoluzio handiko mikroskopia zerbitzuan (SGIker UPV/EHU).

3.3.10. Knock down bidezko isilpena

Proteina jakin batek zelulan betetzen duen funtzioa aztertzeko, proteina horren adierazpena gutxitzeko edo isiltzeko metodoak ezagunak dira. Horietariko bat shRNA zelulan transfektatzearena da. Izan ere, hori intereseko proteinaren mRNAra itsatsi eta bere transkripzioa blokeatzen du.

Kasu honetan, proteina jakin baterako eskasa den zelula-lerro egonkorra eratzeko, A375 giza melanoma zelulak GFP eta puromizinarako erresistentzia lortzeko geneak dituzten scramble edo shRNA jakinerako lentibirus partikulekin infektatu ziren. Partikula lentibirikoen zelula barnerako sarrera bultzatzeko polybrene (4ng/ml) izeneko transfekzio erreaktiboa erabili zen. Ondoren, zelula transfektatuen hautatzea egiteko, GFP maila altua zuten zelulak, zelulen sailkapenean (ingelesezko *sorting*) oinarritutako fluxuzko zitometroa erabili zen. Zelula-lerro egonkorrak eratzeko, hauek puromizinarekin (5µg/ml) mantendu ziren eta proteina jakinaren isilpena aldizka egiaztatzen joan zen Western Blot bidez.

Jarraian lan honetan erabilitako hiru shRNA-en sekuentziak zehazten dira:

SPANX-A/D shRNA 1: CCAAATGGAGGAGGAGGAATTCAT
SPANX-A/D shRNA 2: AAGAACATCTCCAGAGGAACTG
SPANX-A/D shRNA 3: CTAGTGGTTCGCTACAGGAGGAAC

3.3.11. Zelulen bideragarritasuna egiaztatzeko entsegua

Zelulen bideragarritasuna Prestoblue izeneko bideragarritasun erreaktiboarekin neurtu zen hornitzailearen argibideak jarraituz. Espezifikoki, erreaktibo honek resazurina izeneko osagaia dauka zeina zelulen barruan sartzen den eta zelula bizien ahalmen erreduzitzailearen bidez bideragarritasun indikatzaile bezala jokatzen duen. Izan ere, zelula bideragarrien ingurunearen erredukzioa bultzatzen du, fluoreszentzia altua duen kolore gorria xurgatuz edo igorriz.

SPANX-A/D proteína familiaren isilpenak A375 melanoma zeluletan duen efektua ikasteko, Scramble Sh eta SPANX-A/D Sh zelulak 96 putzutako plaketan hazi ziren (5 x 10^3 zelula) kultibo medio osoarekin (%10 FBSeta L-Glutaminarekin osagarritutako DMEM kultibo medioa) gau osoan zehar. 18 ordura T=0a finkatu zen. 24 ordura, zelulen bideragarritasuna Prestoblue bideragarritasun erreaktiboarekin neurtu zen. Laburki, kultibo medioa, Prestoblue-rako 1/10 diluiturik zeukan medio osoarengatik aldatu zen eta bideragarritasuna neurtu zen 2 orduz inkubatu ondoren. FLUOstar OPTIMA fluorimetroan produktuaren absorbantzia 570 nm-etan neurtu zen, absorbantzia maila bideragarritasun mailarekin zuzenki erlazionatzeko. Proteinaren isilpenak eragindako absorbantzia aldaketa portzentaietan kalkulatu zen. Baldintza bakoitzaren bideragarritasuna hiru esperimentu independentetan neurtu zen eta esperimentu bakoitzean sei erreplika erabili ziren. Esperimentu independenteen errepliken batazbestekoa kalkulatu zen eta bideragarritasuna portzentaietan adierazi zen kontrolarekiko erlatibizatuz (Scramble Sh).

3.3.12. Wound Healing bidezko migrazio entsegua

Wound healing teknika zelulen migrazioa aztertzeko teknika da. Hau, zelulek zonalde hutsa betetzeko gaitasunean oinarritzen da. Bereziki, SPANX-A/D proteina familiak zelulen migrazioan betetzen duen funtzioa aztertu genuen. Horretarako, SPANX-A/D Sh eta Scramble Sh A375 melanoma zelulak P24 plaka batean hazi ziren 18 orduz. Ondoren, kultibo medioa aldatu zitzaien mitomizinaz (5µg/ml) osatutako kultibo medioaz ordezkatuz eta 1h eta 30 minutuz inkubatu ziren. Ondoren, 20-200 µl-tako pipetaren punta batekin plakaren erdian zauri bertikala eratu zen. Altxatutako zelulek zeramaten kultibo medioa deuseztatu eta PBSarekin bi aldiz garbitu ziren, ondoren, kultibo medio garbia gehitzeko. Horren ostean, zelulak inkubatzen utzi ziren plakaren hondoan sortutako zauria zelulez guztiz bete arte.

Ondoren, eta T=0 finkatzeko, zelulei argazkiak atera zitzaizkien alderantzizko argiko mikroskopioarekin (Axioscope). 24 ordura zelulei argazkiak atera zitzazkien zauriaren azaleraren aldaketa neurtzeko (T=24). Zauriaren azalera denboran zehar aztertzeko, Image J softwarearen *MRI Wound Healing Tool* tresna erabili zen. Baldintza bakoitzaren migrazio gaitasuna hiru esperimentu independenteetan neurtu zen (T=0 – T=24) eta esperimentu bakoitzean hiru erreplika erabili ziren. Esperimentu independenteen errepliken batazbestekoa kalkulatu zen eta bideragarritasun maila kontrolarekiko (Scramble Sh) erlatibizatu zen.

3.3.13. Zelulen transwell migrazioa neurtzeko entsegua

Zelulen migrazio ahalmena neurtzeko transwell migrazio entsegua erabili zen. Honetan, zelulek 8 µm-tako poroak zeharkatzeko duten ahalmena neurtzen da. Espezifikoki, SPANX-A/D proteína familiak tumoreen migrazioan duten funtzioa aztertu zen. Horretarako, SPANX-A/D Sh eta Scramble Sh A375 zelulak ($1,5 \times 10^4$) 8 µm-tako poroak dituzten mintz intsertuen gainean erein ziren kultibo medio osoarekin eta zelulak 20 orduz migratzen utzi ziren. Ondoren, intsertuak %4 paraformaldehidoarekin fixatu, PBS-rekin hidratatu eta % 0,5 Crystal Violet izeneko erreaktiboarekin tindatu ziren, 15 minutuz, mikroskopioaren bidez aztertu ahal izateko.

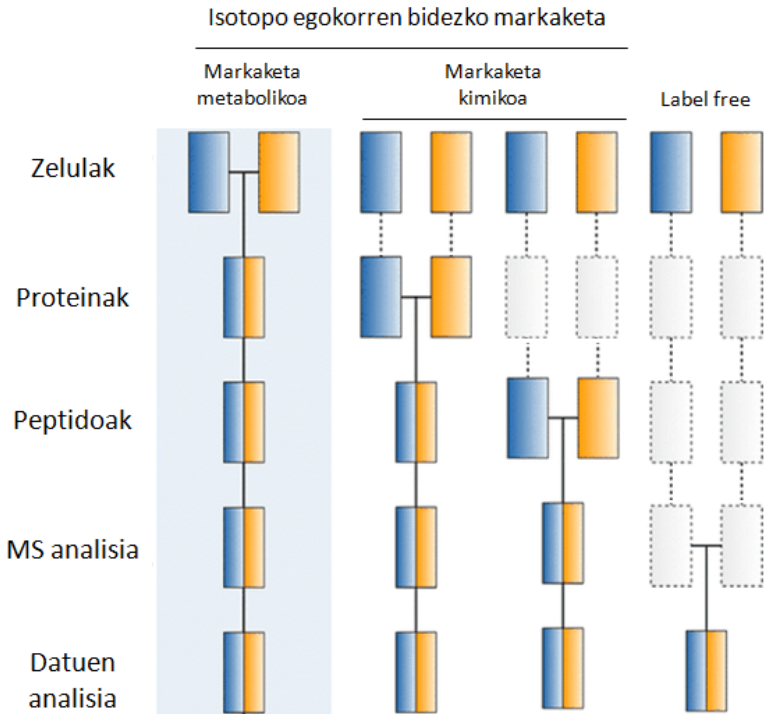
Baldintza bakoitzaren inbasio gaitasuna hiru esperimentu independenteetan neurtu zen esperimentu bakoitzean ausazko sei eremu aztertzen zirelarik. Esperimentu independenteen errepliken batazbestekoa (migratutako zelulen zenbakiarena 20x-ko eremuan) kalkulatu zen eta inbasio maila kontrolarekiko erlatibizatu zen (Scramble Sh).

3.3.14. Masa espektrometria (MS)

Masa espektrometria proteomikan erabiltzen den ioi molekularren masa/karga (m/z; ingelesetik mass-to-charge) ratioak neurtzen dituen teknika analitikoa da. Teknika honek proteinen presentzia edo absentzia, abundantzia eta identifikazioa ahalbidetzen ditu.

Aipatzekoa da proteinen kuantifikazio zehatzerako, laginak egoera esperimental berdinetan oinarrituta egon behar direla. Horretarako, esperimentuaren diseinuan eta emaitzen analisirako

estrategietan arreta handia jartzea beharrezkoa da (Hu et al., 2005). Gaur egun, markaketarik gabeko (ingelesezko *Label-free*) edota isotopo egonkorrez markatutako (ingelesezko stable isotope labelling) kuantifikazio estrategiak ezagunak dira (Schulze and Usadel, 2010).



3.5. Irudia. Masa espektometria kuantifikazio estrategia komunak. Lauki urdinek eta laranjek bi lagin desberdinei egiten diete erreferentzia. Lerro horizontalak agertzen direnean laginak konbinatzen direla esan nahi du. Lerro etenak agertzen direnean, esperimentazioan aldaketak eman daitezkeela esan nahi du eta ondorioz kuantifikazio akatsak sor daitezke (Bantscheff et al., 2007-tik moldatua).

Tesi honetan, bi motako kuantifikazio estrategiak aurrera eraman dira.

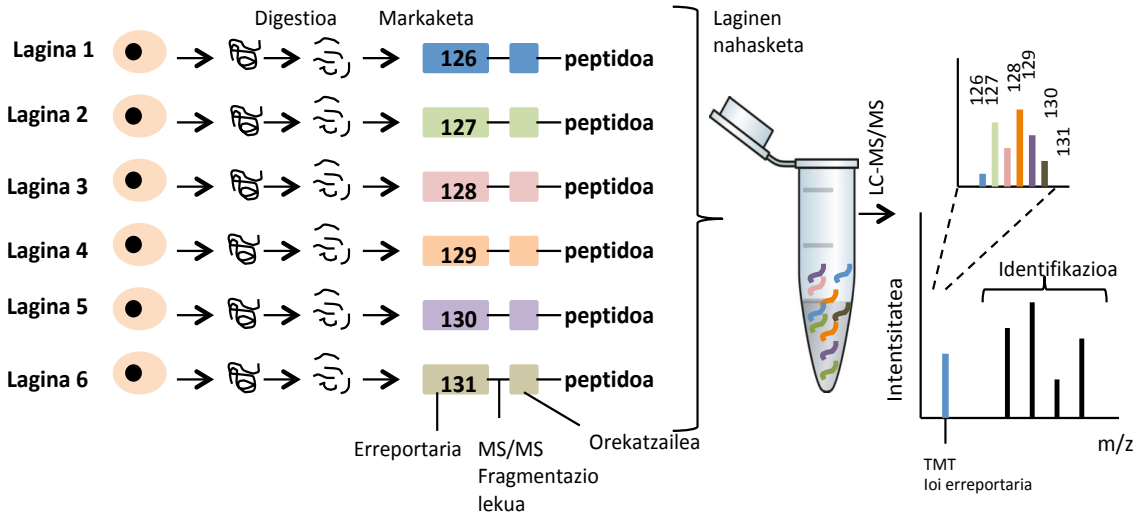
3.3.14.1. Markaketarik gabeko kuantifikazio estrategia edo *Label-free* kuantifikazioa

Teknika honetan laginak independenteki prozesatzen dira eta banaka sartzen dira masazko espektrometroan.

3.3.14.2. Isotopo egonkorrez markatutako kuantifikazio estrategia

Isotopo egonkorrez markatutako kuantifikazioaren bitartez, bi lagin desberdinetan aurkitzen den peptido bera isotopo desberdinarekin marka daiteke eta bi laginak nahastu ondoren, laginaren prozesamendua elkarrekin egin daiteke. Peptido biak kromatografian eta MS analisian berdinak izango dira eta soilik masan desberdinak izango dira. Hori dela eta masa espektrometroak bi laginak desberdindu ditzazke. Kuantifikaziorako peptido berdinaren bi bertsioen intentsitateak konparatzen dira MS edo MS/MS mailan, jarraitutako estrategiaren arabera.

Tesi honen atal batean, markaketa kimikoaren (ingelesezko *chemical labeling*) estrategia erabili zen, eta bereziki Tandem Mass Tag-ean oinarritutako teknika burutu zen. Laburki, teknika hau masa isobarikodun etiketen (ingelesezko *isobaric mass tags*) erabilpenean datza. Etiketa isobariko bakoitza moztu daitekeen aminoazido jakin batekin erreakzionatuko duen talde funtzional batez, masa erreportari batez eta masa orekatzaile talde batez loturik dago. Etiketa hauek amino talde askeekin erreakzionatzen dute eta markaketa peptido mailan gertatzen da. Aipatzekoa da etiketa bakoitzaren erreportari eta orekatzaileen masa desberdina bada ere, bien arteko gehiketa berdina dela. Beraz, etiketa guztien masa osoa berdina da. Ondorioz, era desberdinean markatutako peptidoak kromatografia likidoan ko-eluitzen dira eta ezin dira MS mailan desberdindu. Hori dela eta, peptidoen fragmentazioa beharrezkoa da, izan ere, prozesu horretan zehar masa erreportari desberdinak askatuko dira eta MS/MS mailan desberdinduko dira. Erreportarien intentsitateek, lagin bakoitzean zegoen peptido kopuruari buruzko informazioa emango digute. Hori dela eta, lagin bakoitzean zegoen peptido berdinaren kuantifikazioa erreportarien intentsitateak konparatuta egingo da MS/MS mailan (3.6. irudia).



3.6. Irudia. Marketa isobarikoan oinarritutako kuantifikazioa. Etiketa isobarikoen bidez markatutako peptidoak masa osoan berdinak dira, horregatik kromatografia likidoan era berean eluitzen dira eta ezin dira MS mailan desberdindu. Fragmentazioa eman eta gero, erreportariaren eta orekatzailearen arteko lotura mozten da. Hori dela eta erreportarien masak masa espektrometroaren bidez desberdindu daitezke eta intentsitateen konparazioaren bidez MS/MS mailan peptidoen kuantifikazioa ahalbidetzen du.

3.3.14.3 Masa espektrometria prozedurak

Jarraian estrategia bakoitzean jarraitutako prozedurak azalduko dira:

3.3.14.3.1. Gelean egindako liseriketa edo *In-gel digestion*

Alde batetik, giza espermatozoideetan eta A375 melanoma zeluletan endogenoki aurkitzen diren SPANX-A/D proteina familiaren isoformak eta fosforilazio lekuak aztertu ziren. Horretarako, bi zelula moten proteina zatiki solugarria eta solugaitzak Nu-PAGE 4-12% gelean kargatu eta gero gelean zeharreko liseriketa edo ingelesezko *in-gel digestion* deritzon metodologia jarraitu zen.

Akrilamidazko gela Coomassie urdinarekin tindatu zen proteinen bandak antzemateko eta baldintza esteriletan zenbait zatitan moztu ondoren, hauek banaka liseritu ziren. Laburki, akrilamidazko gel zatiak hodietan jaso eta garbiketa tanpoia (25 amonio bikarbonatoa, %50 etanola) eta etanola erabiliz, kolorantea kendu zitzaien giro tenperaturan. Behin gel zatiak gogor zeudela eta kolore zuria zutela, 10 mM DTT erabiliz proteinak erreduzitu ziren 45 minutuz, 56 °C-tan. 5 minutuz etanolarekin inkubatu eta gero, proteinak 55 mM kloroazetamida (25mM amonio bikarbonatoan) erabiliz alkilatu ziren, 30 minutuz, giro tenperaturan eta iluntasunean. Ondoren, gel zatiak 20 minutuz garbiketa tanpoiarekin garbitu eta 10 minutuz etanola gehitu zitzaien. Prozesu hau gelak erabat deshidratuta egon arte errepikatu zen. Bukatzeko, gel zatiak 12.5 ng/µl tripsinarekin gau osoan zehar inkubatu ziren, 37 °C-tan. Liseriketa eta gero, peptidoak gel zatietatik atera ziren 10 minutuko %30 azetonitrilo (ACN) eta %3 azido triorofluoriko (TFA) eta 10 minutuko %100 ACN inkubaketekin (bi tanpoi bakoitzarentzat). Ondoren, zatikiak konbinatu egin ziren eta Speedvac hutsean sikatu ziren.

3.3.14.3.2. Soluzioan egindako liseriketa edo *In-solution digestion*

Arestian azaldutako immunoprezipitazio prozeduratik zetozen laginak 8M Gu-HCl-an birsuspenditu eta 75 °C-tan 15 minutuz inkubatu ziren proteinak antigorputzarengandik eluitzeko. Ondoren, bolatxo magnetikoak kendu eta gainjalkinak beste hodi batera transferitu ziren. Ondoren, 2 mM DTT erabiliz proteina 30 minutuz giro tenperaturan erreduzitu ziren. Hori bukatzean, proteinak 11 mM kloroazetamida erabiliz alkilatu ziren 30 minutuz giro tenperaturan eta iluntasunean. Liseriketarako, laginak 8M Gu-HCL-an izatetik, 2M-ko kontzentrazioa izateko 25mM ABC-rekin diluitu ziren. Ondoren, LysC (1mg/ml stock-a) entzima gehitu zen 1:100 proportzioan eta 4 orduz inkubatu zen giro tenperaturan. Denbora hori pasa eta gero, tripsina (1mg/ml stock-a) entzima gehitu zen 1:100 proportzioan eta gau osoan zehar inkubatu zen giro tenperaturan. Hurrengo egunean, tripsina entzima inaktibatzeko %10 TFA gehitu zen eta pH-a azidikoa zela frogatu zen.

3.3.14.3.3. C18 Stage Tip bidezko purifikazioa

Masa espektrometroan kargatu beharreko lagin guztiak lehenik eta behin purifikaturik eta gatzik gabe egon behar ziren. Horretarako Stage Tip deritzon purifikazioa aurrera eraman zen. Lehenengo eta behin, C18 diskoez egindako stage tip-ak metanolarekin garbitu ziren. Ondoren %60 ACN eta %0.5 azido azetikoa zuen nahasketa gehitu zitzaien eta horren ostean, bi aldiz garbitu ziren %0.5 azido azetikoarekin. Ondoren, lagina C18 diskoaren gainean kargatu zen eta xiringa baten laguntzaz lagina diskoan barrena sartu zen. Hori egitean, peptidoak %60 ACN eta %0.5 azido azetikoa zuen nahasketarekin eluitu ziren. Jasotako eluzioak Speedvac deritzon hutsean sikatu ziren 2 µl geratu arte

eta lagin bakoitzaren gainean %2 ACN eta %0.3 TFA-z osaturiko 7.5 µl buffer gehitu zen, masa espektrometroan kargatzeko prest egon zitezen.

3.3.14.3.4. Tandem Mass Tag 6-plex

Espermatozoideen Kappa-opioide hartzailearen azpitik dauden seinaliztapen bidezidor intrazelularrak aztertzeko fosfoproteomika kuantitatiboa burutu zen. Horretarako, markaketa isotopikoan oinarritutako Tandem Mass Tag 6-plex erreaktiboa erabili zen.

Laburki, U50488H (1 µl) agonista espezifikoak 1 eta 60 minutuan giza espermatozoideen fosfoproteomikan duen eragina aztertu zen. Horretarako, agonistarekin tratatutako eta tratatu gabeko (kontrola) giza espermatozoideen lagin bakoitzeko hiru erreplika biologiko erabili ziren, esperimentua denbora bakoitzerako sei laginekin hasiz. Ondoren, lagin bakoitzaren proteina solugarriaren eta solugaitzaren zatikiak erauzi ziren 3.3.4.1. atalean azaltzen den bezala.

3.3.14.3.4.1. Espermatozoideen proteomaren azterketa

Proteina solugarriari dagokionez, *in-solution digestion* protokoloa jarraitu zen (3.3.14.3.2 atalean), proteinak DTT-arekin (2mM) erreduzituz eta kloroazetamidarekin (11mM) alkilatuz. Proteina soluziotik detergenteak kentzeko laginak azetonarekin prezipitatu ziren eta ondoren urea 8M tanpoian disolbatu ziren. Proteina solugaitzari dagokionez, laginak 8M GuaHCL tanpoiarekin nahastu eta sonikatu ziren. Ondoren proteinak erreduzitu eta alkilatu ziren. Puntu honetan, jarraian deskribatzen den protokoloa berdina izan zen proteina zatiki solugarri zein solugaitzarentzat (1 eta 60 minututik eratorriak). Ondoren, lagin guztiak 1 mg proteinara egokitu ziren. Ondoren LysC eta tripsina bidezko liseriketarekin jarraitu zen eta hurrengo egunean, SekPak C18 zutabe batekin laginak purifikatu eta garbitu ziren.

Lagin bakoitzeko 100 µg peptido hartu ziren espermatozoideen proteoma osoa aztertzeko eta hori TMT 6-plex markaketa kimikorako erreaktiboa erabiliz egin zen. Laburki, proteinak Speedvac izeneko hutsean lehortu eta TEAB tanpoian (100 µl) disolbatu ziren. QUBIT espektrofotometroa erabilita peptidoen kontzentrazioa neurtu zen eta lagin guztiak 25 µg-ra egokitu ziren. TMT kit bakoitzak 100 µg peptido markatzeko ahalmena dauka eta gure peptidoen kontzentrazioa laurdena zenez, kit erdia erabili genuen. Horretarako, markaketa hodi bakoitzari 41 µl azetonitrilo gehitu zitzaion eta 5 minutuz giro tenperatura orekatzen mantendu eta gero, lagin bakoitzari 20 µl zegokion markaketa erreaktiboa gehitu zitzaion. Jarraian lagin bakoitzean gehitutako markaketa etiketa isotopikoak aurkezten dira:

3.11. Taula. Lagin bakoitzean gehitutako markaketa isotopoa.

Lagina	Markaketa etiketa isotopikoa
Kontrol 1	126
Kontrol 2	127
Kontrol 3	128
U50488H 1	129
U50488H 2	130
U50488H 3	131

Ordu betez eta giro tenperaturan, laginak eta markaketa etiketa isotopikoak nahastu ondoren, erreakzioa gelditu zen 8 µl %11 L-lisina gehituz (15 minutuz). Ondoren, lagin guztiak elkartu ziren eta pH altuko frakzionamendua egin zen nahasturaren izaera oso konplexua zelako.

Pausoz pausoko pH altuko alderantzizko fasean oinarritutako frakzionamendua

Proteinen identifikazioa eta kuantifikazioa handitzeko, lagin guztien nahastura 17 zatikitan banatu zen. Horretarako lagin bakoitzeko 100 µg hartu eta 10 mM amonio hidroxido erabiliz, pH 10-ra egokitu ziren. Ondoren laginak ReproSil-Pur, 1.9 µm 120A bolatxoez osatutako punta baten gainera transferitu ziren. Horren ostean 1.75, 3.5, 5.25, 7, 8.75, 10.5, 12.5, 14, 15.5, 17.5, 21, 24.5, 28, 31.5, 35, 50 eta %70 azetonitrilo edukia zuten tanpoiarekin eluzio sekuentzialak egin ziren eta hauek C18 Stage Tip desberdinetan purifikatu eta garbitu ziren.

3.3.14.3.4.2. Espermatozoideen fosfoproteomaren azterketa

Fosfoproteomaren azterketarako, TiO_2 bidezko fosfopeptidoen aberasketa protokoloa jarraitu zen. Horretarako, liseriketaren ostean proteina solugarri eta solugaitzetik eratorritako (1 eta 60 minutuetatik eratorriak) lagin bakoitzeko 900 µg peptido erabili ziren. Lehenengo eta behin, TiO_2 bolatxoak tanpoi orekatzailearekin (%50 ACN, %0.1 TFA, 30 g/l DHB) orekatu ziren eta 30 minutuz giro tenperaturan biratzen utzi ziren lagin bakoitzeko peptidoekin. Zentrifugazio azkarren bidez, bolatxoak garbiketa tanpoiarekin garbitu ziren (%50 ACN, %0.1 TFA) hiru aldiz. Fosfopeptidoen eluziorako, bolatxoak C8 diskoz egindako Stage Tip baten gainera transferitu ziren. Ondoren, eluzio tanpoia (%40 CAN, %15 NH3) gehitu eta xiringa baten laguntzarekin laginak diskoan barrena pasa ziren. Eluzio tanpoiak zeukan amonioa neutralizatzeko 10 µl %2 ACN, % 0.3 TFA tanpoia gehitu zen. Bukatzeko laginak Speedvac hutsean lehortu ziren eta TMT bidezko markaketa kimikorako egokitu ziren. Horretarako laginei 100 µl TEAB tanpoia gehitu eta ondoren arestian aipatutako isotopoen bidezko markaketa protokolo berdina jarraitu zen (3.3.14.3.4.1. atala, 3.11. Taula). Kasu honetan ez zen laginaren frakzionamendurik egin eta laginen nahasketei C18 Stage Tip bidezko purifikazioa egin zitzaien ondoren masa espektrometroan sartzeko.

3.3.14.4. Masa espektrometroa eta datuen analisi estatistikoa

Aurretiaz azidifikatutako peptidoen nahasturak C18-z osatutako alderantzizko fasedun kromatografia likido bidez banatu eta tandem bidezko masa espektrometria erabiliz (LC-MS/MS) aztertu ziren. Masa espektrometria (MS) analisi guztiak EASY-nanoLC 1000 Sistemara konektatutako Q-Exactive HF masa espektrometroan burutu ziren, *nanoelektrospray* ioi iturria erabiliz (Proxeon Biosystems, Odense, DK).

MS espektro osoak (m/z irismena, 200-2000; erresoluzioa 60,000 m/z 400-ean), Orbitrap-a erabiliz lortu ziren eta karga anitzeko 12 ioi intentsoenen fragmentazioa burutu zen. MS/MS-rako hautatutako ioiak 45 segunduz esklusio dinamiko lista batean utzi ziren. Masa zehaztasuna hobetzeko, masa kalibrazioa burutu zen. Horrez gain, 2.3 kV-ko spray boltaia erabili zen, ez zen gas fluxu osagarririk erabili eta kapilarraren tenperatura 275 °C-takoa izan zen. Fitxategi gordin (ingelesezko *raw file*) guztiak 2015.08 Uniprot (42122 sekuentzia sarrerekin) eta TrEMBL (49496 sekuentzia sarrerekin) datubaseekin konparatu ziren Andromeda bilatzailea duen MaxQuant plataforma (1.5.2.8 eta 1.5.3.30 bertsioak) erabiliz.

Prekurtsoreen eta fragmentuen tolerantziak 4.5 eta 20ppm-takoak izan ziren, hurrenez hurren. Pikuen lista bat eratu zen MaxQuant programaren Quant elementua erabiliz eta ondorengo parametroak finkatuz. Entzima espezifikotasuna tripsina entzimarako ezarri zen, prolinaren N-muturrean eta azido aspartikoaren eta prolinaren erdian mozketa ahalbidetuz eta gehienez 2 mozketa akats (ingelesezko *missed cleavage*) baimenduz. Horrez gain, aldaketa finkotzat karbamidometilazioa (C) kontuan hartu zen, aldaketa aldagarritzat Oxidazioa (M), deamidazioa (NQ) eta Phospho_STY (STY) aintzat hartu ziren bitartean. Peptido eta proteina FDR-a (ingelesezko false discovery rate) 0,01-ean ezarri zen, gune FDR-a 0,01-ean, peptido luzera minimoa 7-an, eta peptido unikoen eta peptidoen kantitatea 1-ean finkatu zen bitartean.

Proteina kuantifikaziorako, soilik aldatu gabeko eta azetilatutako (proteinak N-terminalean), oxidatutako (Met) eta deamidatutako (NQ) peptidoak kontuan hartu ziren. MaxQuant programaren bidez esleitutako proteina taldeei dagokienez, identifikatutako proteina zerrendari kontaminanteak, errebertsoak eta soilik gune bidez identifikatutakoak baztertu zitzaizkien. Horrez gain, soilik aintzat hartu ziren identifikatutako peptidoak ≥ 2 eta peptide unikoak ≥ 1 zituzten proteinak.

Beste alde batetik, fosfopeptidoei zegozkien emaitzak FDR < 1% zenean eta soilik 0,75 baino handiagoko lokalizazio probabilitatea zuten fosfopeptidoak fidagarritzat definitu ziren (Class I sites).
Masa espektrometriatik eratorriak ziren emaitzen estatistika egiteko eta laginen arteko ratioak (ingelesezko Fold change) kalkulatzeko Perseus programa erabili zen. Horrez gain, gene ontologian oinarritzen diren analisiak egiteko Panther tresna informatikoa erabili zen. Bestalde, proteinen arteko elkarrekintzak aztertzeko, GENEMANIA sare interaktiboa erabili zen. Elkarrekintza hauek irudikatzeko Cytoscape plataforma bioinformatikoa erabili zen.

Aipatzekoa da, emaitzen atalean kapitulu bakoitzean egindako masa espektrometria esperimentuen analisi bioinformatikoaren deskribapen zehatzagoa ematen dela.

3.3.15. Datuen analisi estatistikoa

Tesi honetatik eratorritako emaitzen analisi estatistikoa SPSS Statistics (v.22), GraphPAD Prism (v.6.2) eta Perseus (v.1.6.0.7) softwareekin egin zen. Emaitza estatistikoki esanguratsuak kontsideratu ziren*$p < 0.05$ eta estatistikoki oso esanguratsuak **$p < 0.01$. Horrez gain, emaitzen analisirako eta tratamendurako ondoren deskribatzen diren tresna informatikoak erabili ziren.

3.12. Taula. Erabilitako tekniketatik eratorritako emaitzen azterketarako erabilitako trensa informatikoen zerrenda.

Tresna informatikoa	**Bertsioa**	**Erabilpena**
Computer-assisted sperm analysis (CASA)	2005	Mugikortasun espermatikoa
IBM SPSS Statistics	v.22	Analisi Estatistikoa
Image J Softwarea	v.1.49	Irudien tratamendua
Summit Softwarea	v. 4.3	Fluxuzko zitometria analisia
Perseus Softwarea	v. 1.6.0.7	Emaitzen tratamendua
GraphPAD Prism Softwarea	v.6.2	Emaitzen tratamendua
Panther tresna bioinformatikoa	v.13.1	Gene Ontology analisiak
MaxQuant softwarea	v.1.5.2.8 v1.5.3.30	Proteomikaren analisia
Genemania tresna bioinformatikoa		Interaktomaren analisia
Cytoscape plataforma	v.3.5.1	Emaitzen tratamendua

4.Emaitzak
Results

1.Kapitulua

Chapter 1

4. RESULTS

4.1. CHAPTER 1

The opioid peptide beta-endorphin stimulates acrosome reaction in human spermatozoa

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SUMMARY

The acrosome reaction occurs *in vivo* following sperm capacitation and is essential for the acquisition of sperm fertilization ability. However, little is known about the molecular identity of the physiological acrosome reaction regulators. In addition to progesterone, which is produced by cumulus oophorus cells and known to regulate acrosome reaction by activating the specific calcium channel CatSper, endogenous opioid peptides such as beta-endorphin and met-enkephalin are present at high concentrations in the follicular fluid suggesting that the opioid system may be involved in the mechanisms regulating the acrosome reaction in humans. By using Reverse Transcription-PCR, western blot and immunofluorescence approaches, we described the presence and localization of the beta-endorphin precursor, pro-opiomelanocortin in the middle section and in flagellum of human spermatozoa, and inside the seminiferous tubules of human testis. Flow cytometry and intracellular calcium analyses showed that beta-endorphin causes an inversely dose-dependent increase in the percentage of acrosome-reacted sperm cells by a calcium-independent protein kinase C pathway. These findings are important for future studies of sperm physiology and provide new insight into the function of the opioid system as a target of fertility management.

INTRODUCTION

After ejaculation, human sperm cells are immature and infertile and must undergo many modifications to become fertilization competent (Suarez, 2008a, 2008b). Several morphological and biochemical changes occur during the transit through the female tract. These sperm modifications include different processes such as capacitation (sperm membrane reorganization), hyperactive motility (changes to the motility pattern needed to penetrate oocyte vestments) and acrosome reaction. The acrosome reaction of spermatozoa is a complex calcium-dependent process and is essential for the spermatozoa to fertilize an egg. Fusion at multiple sites between

the outer acrosomal membrane and the cell membrane causes the release of the acrosomal contents and the loss of the membranes surrounding the acrosome (Florman et al., 2008). Progesterone produced by cumulus oophorus cells is known to be the main physiological regulator of acrosome reaction (Baldi et al., 2009) as the binding and the respond of progesterone are compromised in spermatozoa derived from infertile men (Gadkar et al., 2002; Smith et al., 2013).

Progesterone-induced acrosome reaction causes a multicomponent intracellular Ca^{+2} increases (Darszon et al., 2011). In mammalian spermatozoa, the progesterone-induced intracellular Ca^{+2} increase is controlled by a sperm-specific Ca^{+2} channel called CatSper (cation channel of spermatozoa) (Lishko et al., 2011; Quill et al., 2001; Tamburrino et al., 2014). However, several chemical molecules including vitamin D, chemokines, small peptides, the gas NO, neurotransmitters, analogues of cyclic nucleotides, and odorants can also affect the acrosomal exocytosis *in vitro* (Brenker et al., 2012; Eisenbach and Giojalas, 2006; Florman et al., 2008; Suarez, 2008a, 2008b). To date, the underlying signalling mechanisms of acrosome reaction are ill-defined. Endogenous opioid peptides (EOPs) are a type of small peptides known to participate in the regulation of reproductive physiology at multiple sites and, particularly, the opioid system seems to be involved in the regulation of sperm physiology (Subirán et al., 2011).

Previously, we described the presence of three types of opioid receptors (mu, delta, kappa) and other components of the opioid system in human sperm cells and we described its role in sperm motility. (Agirregoitia et al., 2006; Fernández et al., 2002; Subirán et al., 2012, 2008). Nevertheless, the role of the opioid system in acrosome reaction is poorly understood and to date, there have been no relevant *in vivo* studies. Beta-endorphin immunoreactivity has been detected in spermatozoa but the main role of this peptide in human spermatozoa is completely unknown.

Together with progesterone, beta-endorphin is secreted in the oviduct (Petraglia et al., 1986, 1985), raising the possibility that EOPs may be involved in human acrosome reaction regulation. Here, we describe for the first time that the EOP beta-endorphin precursor, proopiomelanocortin (POMC), is present in human testis and sperm cells and that beta-endorphin regulates human acrosome reaction by specific calcium-independent protein kinase C (PKC) pathway.

MATERIALS AND METHODS

Samples and isolation of spermatozoa

Ethical approval for this study was obtained from the Ethics Committee of the University of the Basque Country (CEISH/61/2011). Freshly ejaculated semen was collected from 80 donors (18–35 years old) with normal sperm parameters according to World Health Organization standards (WHO, 2010). Samples were obtained by masturbation after 3–4 days of sexual abstinence and processed immediately upon liquefaction (at 37 °C for 30 min). Spermatozoa were capacitated by a swim-up procedure (Cejudo-Roman et al., 2013) and resuspended in G-IVF (Vitrolife, Goteborg, Sweden) supplemented with 1%

bovine serum albumin for 3 h at 37 °C under 5% CO_2.

Reverse transcription-PCR (RT-PCR) analysis

Total RNA was extracted from a sperm pool containing spermatozoa from eight different donors using TriReagent (Sigma, San Luis, MO, USA) and cDNA was synthesized using the Quantitect Reverse Transcription kit (Qiagen, Venlo, The Netherlands). Specific oligonucleotide primer pairs used for PCR were synthesized and purified by Sigma Genosys (Cambridge, UK) and their sequences were as follows: human Pomc, forward 5′-CTCACCACGGAAAGCAACC-3′ and reverse 5′-ATCGGTCCCA GCGGAAGT-3′ (151-bp product); and human Actb (b-actin), forward 5′-TCCCTGGAGAAGAGCTACGA-3′ and reverse 5′-ATC TGCTGGAAGGTGGACAG-3′ (362-bp product; exon spanning), used as an internal control. A pool of cDNAs from 20 different human tissues (human total RNA master panel, BD Biosciences, Clontech, Palo Alto, CA, USA) was used as a positive control of amplification. Amplification was carried out in 25 μL of PCR buffer containing 3 μL of cDNA reaction mixture, 2.5 mM MgCl2, 0.2 μM primers, 200 μM dNTPs and 1.5 U of heat-activated thermostable DNA polymerase (Immolase; Bioline, London, UK). PCR was performed for 35 cycles with cycling parameters being: 15 s at 94 °C, 20 s at 60 °C and 20 s at 72 °C. The primers for hPomc were located on the same exon of each respective gene (i.e. they did not span introns). Thus, we verified the possible carry-over of genomic DNA during the extraction process by performing PCR in the absence of reverse transcriptase. Expression of CD4 and acrosin was also analysed to exclude the presence of leucocyte contamination and to verify the presence of sperm complementary DNA respectively (data no shown). The RT-PCR products were separated by 2.5% gel electrophoresis. The amplicon sizes were verified by comparison with a DNA size-ladder and the identity of the products was established by sequencing of amplicons.

Western blotting

Sperm proteins were prepared as described elsewhere (Subirán et al., 2012), modifying the lysis buffer (phosphate-buffered saline, PBS and 1% [v/v] Triton-X100, with protease inhibitor cocktail). Membrane pellets were suspended in lysis buffer, and then protein extracts were diluted in Laemmly sample buffer containing b-mercaptoethanol (5% vol/vol) and boiled for 5 min. Proteins (50 μg sperm protein; 30 μg kidney protein) were loaded onto 12% resolving gels and separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene fluoride membranes using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, the membrane was blocked with Blotto (20 mM TrisHCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100) containing 5% nonfat dry milk (blocking buffer) for 1 h and then incubated with a dilution of polyclonal rabbit anti-POMC antibody (1 : 200) After washing (3 x 5 min) in Blotto buffer, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (1 : 3000) (Goat anti-rabbit IgG HRP, ab6112; Abcam, Cambridge, UK). Blots were revealed for peroxidase activity by enhanced chemiluminiscence.

Indirect immunofluorescence

Isolated spermatozoa obtained after swim-up and human frozen testis slides provided by Zyagen Company (California, USA) were used to identify the localization of POMC in human sperm cells and testis, and to analyse the effect of beta-endorphin on PKC-signalling pathways.

Cells were fixed in 4% paraformaldehyde for 10 min, permeated in 0.5% Triton X-100 for 10 min and blocked for 30 min with 10% (v/v) foetal bovine serum in PBS. For immunofluorescence staining, samples were incubated overnight at 4 °C with different primary antibodies. We used rabbit polyclonal anti-proopiomelanocortin (1 : 500) (Santa Cruz Technologies, CA, USA) and rabbit anti-phospho-PKC (Cell Signalling, Massachusetts, USA) antiserum at a dilution of 1 : 500. Secondary antibody incubations involved Alexa Fluor 488 donkey anti-rabbit IgG (1 : 2000) (Molecular Probes, Oregon USA). Nuclei were stained with Hoechst 33258 at 10 µg/mL (spermatozoa) and propidium iodide at 100 µg/mL (testis), and slides were assembled with Fluoromount G (Molecular Probes). The specificity of the primary antibody was verified by using negative unspecific rabbit immunoglobulin fraction (normal) (Dako, Glostrup, Denmark) in the same concentration as the primary antibody, and pre-absorbing primary antibody immunoreactivity with beta-endorphin (10^{-5} M) for 2 h at room temperature before incubation. At the same time, controls for the specificity of the secondary antisera were performed by omitting the primary antiserum before addition of the secondary antisera.

Finally, the samples were examined using confocal microscopy (Olympus Fluoview FV500, Tokyo, Japan). Corrected total cell fluorescence (CTCF) per area was measured by ImageJ software (National Institutes of Health, Maryland, USA) using the following equation: CTCF = [Integrated density (Area of selected cell 9 Mean fluorescence of background readings)]/Area of selected cell. We measured the green fluorescence of at least 200 cells.

Incubation media and treatments

Isolated spermatozoa were treated at 37 °C under 5% CO_2 with different doses of beta-endorphin (10^{-5}, 10^{-7} and 10^{-9}M). Sperm samples were divided into aliquots of 0.1 mL in G-IVF (Vitrolife) and one of the different concentrations of beta-endorphin was added to each aliquot. An equal volume of solvent was used as control. In addition, to ascertain the specificity of the action of the peptide, beta-endorphin (10^{-9}M)-treated sperm cells and control samples were also co-incubated with naloxone, an antagonist of opioid receptors, at high (10^{-5}M) and low (10^{-8}M) concentrations. High naloxone doses (10^{-5}M) block the three opioid receptors and low doses (10^{-8}M) are able to block selectively the mu-opioid receptor. Sperm cells were treated with naloxone for 10 min before beta-endorphin addition. In all experiments, sperm cells were incubated with beta-endorphin for 60 min. Finally, to evaluate the effect of beta-endorphin on progesterone induced acrosome reaction, samples were also co-incubated with 10^{-9}M beta endorphin and 10^{-6}M progesterone. After 1 h of incubation with beta-endorphin, samples were treated with progesterone for 15 min.

Treated spermatozoa were used for subsequent experiments

Flow cytometry

In all experiments, acrosome reaction was measured by flow cytometry. We used Fluorescein IsoTioCyanate (FITC) anti-human CD46 (for 60 min at room temperature; BioLegend, California, USA) and Hoechst 33258 (2 min at room temperature; Sigma-Aldrich, Missouri, St. Louis, USA) as acrosome reaction molecular marker and viability dyes respectively. Samples were checked visually by confocal microscopy to verify the signal of the dyes. Green positive cells represented acrosome-reacted spermatozoa. Fluorescence data from at least 100.000 events were analysed in a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA). To ensure fluorescence data were from live spermatozoa, the percentage of Hoechst 33258-positive events was determined by subtraction of background fluorescence in each histogram. We also analysed the effect of beta-endorphin on PKC signalling pathways using flow cytometry. Capacitated spermatozoa obtained by Percoll gradient followed by a swim-up procedure were incubated for 3 h in GVI-F medium. A minimum of $3x10^6$ cell/mL was collected and treated as we described before. Collected spermatozoa were fixed and permeated in suspension in 0.5% Triton X-100 for 10 min. Samples were washed twice in PBS by centrifugation at 800 g for 5 min and incubated in blocking medium (PBS/10% (v/v) foetal bovine serum) for 30 min. For immunofluorescence staining, samples were incubated overnight at 4 °C with rabbit anti-phospho-PKC (Cell Signalling) antiserum at a dilution of 1:500. On the next day, the samples were centrifuged in PBS at 800 g for 5 min and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1 : 2000) (Molecular Probes) in the dark, at room temperature for 1 h. Nucleus was stained with 0.1 μg/mL Hoechst 33258 for 2 min. Finally, samples were washed twice by centrifugation in PBS at 800 g for 5 min, suspended in PBS and kept in the dark until analysis. Negative controls were performed by omitting the primary antibody before secondary antibody addition and by using negative unspecific rabbit immunoglobulin fraction (normal) (Dako) in the same concentration as the primary antibody. Fluorescence data from at least 100.000 events were analysed. To measure the green fluorescence only from spermatozoa, the percentage of Hoechst 33258-positive was determined by subtraction of background fluorescence in each histogram. Histograms were analysed using the Summit v4.3 software (Beckman Coulter, California, USA).

Measurements of sperm intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$

For measurement of $[Ca^{2+}]_i$, spermatozoa were adjusted to a concentration of $10x10^6$ cell/mL in corresponding medium. They were then incubated with the acetoxymethyl ester form of Fura-2 (Fura-2/AM, $8x10^{-6}$ M; Molecular Probes) for 60 min at room temperature in the presence of the non-cytotoxic detergent pluronic acid (0.1%; Molecular Probes). After loading, the cells were washed and resuspended in G-IVF solution and used within the next 2–7 h. Sperm aliquots (1 mL) were placed in the quartz cuvette of a spectrofluorometer (SLM Aminco-Bowman, Series 2; Microbeam, Barcelona, Spain) and magnetically stirred at 37 °C. The emitted fluorescence was measured at 510 nm.

Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 as previously described (Cejudo-Roman et al., 2013). To measure $[Ca^{2+}]_i$ samples were alternatively illuminated with two excitation wavelengths (340 nm and 380 nm) and the fluorescence ratio (F340:F380) was recorded continuously. The emitted fluorescent light from the two excitation wavelengths was measured by a photomultiplier through a 510-nm filter. After subtracting the autofluorescence signal, obtained by adding 5 mM $MnCl_2$ at the end of the experiment, the F340/F380 ratio was used as an indicator of $[Ca^{2+}]_i$. The effect of beta-endorphin was studied on sperm aliquots incubated with this peptide at different doses (10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M). Progesterone 10^{-6} M was added to the same sperm aliquot to analyse the effect of beta-endorphin on the progesterone-induced intracellular Ca^{2+} levels. Calibration of $[Ca^{2+}]_i$ was achieved according to the equation of Grynkiewicz et al. (Grynkiewicz et al., 1985) adding Triton X-100 (5%), to obtain the maximal response, followed by addition of ethylene glycol tetraacetic acid (EGTA) (40 mM) to obtain the minimal response.

Statistics

Acrosome-reacted data were normalized as [(Treatment-Control)/(Control)] x 100 and evaluated using the Kruskal–Wallis non-parametric test followed by Mann–Whitney U-tests. These procedures were undertaken using GraphPad PRISM (version 5.0) (GraphPad Company, California, USA) program. Differences were considered significant at *p < 0.05 and highly significant at **p < 0.01. Data are expressed as mean SEM.

RESULTS

Expression and localization of POMC in human sperm cells

POMC transcript was not detected in human spermatozoa using RT-PCR. The expected 151-bp fragment for POMC was undetectable in human spermatozoa. We only observed the fragment corresponding to the pool of DNA from 20 different human tissues used as a positive control (pc). The housekeeping gene ACTB was detected in all tissues and the absence of amplicons in the retrotranscriptase negative controls confirmed the absence of contaminating genomic DNA in each sample (Fig. 1a). The absence of CD4 in the spermatozoa preparation indicates no leucocyte contamination, whereas the presence of ACR verifies the presence of sperm complementary DNA (data not shown). On the other hand, using western blot a band of 55 kDa was observed in human sperm protein fraction as well as in human kidney protein fraction, which was used as a control (Fig. 1b). The molecular weight corresponds to the theoretical molecular weight of POMC in humans. We did not detect any signal in the absence of primary antibody (data not shown). Analysis by immunofluorescence confirmed that POMC was present in human sperm cells (Fig. 1c) and in human testis (Fig. 1d).

We found a strong immunoreactivity of POMC in the middle section and in the tail of the sperm cells Fig. 1c). In human frozen testis, POMC immunoreactivity was also detected inside the seminiferous tubules, where spermatogenic cells are present (Fig. 1d). In both cases, no fluorescent signal was detected using pre-absorbing primary antibody and non-specific rabbit antibody

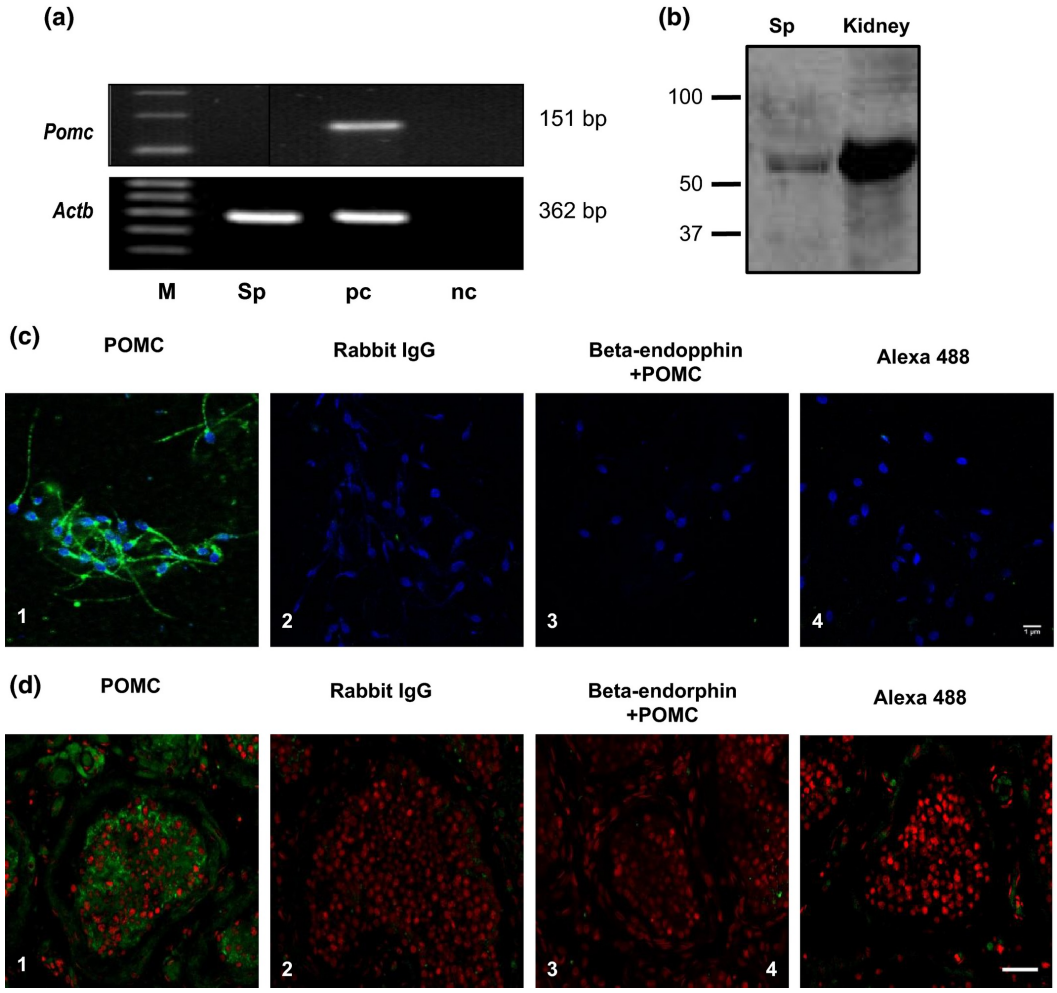

Figure 1. Expression of POMC in human spermatozoa. (a) RT-PCR analysis of proopiomelanocortin (POMC) precursor in human spermatozoa (sp1 and sp2); nc: primers without cDNA were used as negative control and pc: pool of DNA from 20 different human tissues used as a positive control. (b) Western blotting analysis of POMC in human spermatozoa (Sp) and kidney (kd) using a rabbit anti-POMC polyclonal antiserum. The molecular mass markers (kDa) are indicated on the left. Molecular weights of pre-stained markers proteins are indicated. Representative blot obtained from four normozoospermic donors is shown (c) Immunofluorescence analysis of POMC in human sperm cells (panel 1). Negative controls incubating with unspecific rabbit immunoglobulin fraction (panel 2) and pre-adsorbing the anti-POMC antibody with beta-endorphin (panel 3). Incubation with secondary antibody alone (panels 4). DNA of controls was stained with Hoechst 33258. Representative photomicrographs are shown; n = 5. Scale bar for all panels, 1 lm. (d) Immunofluorescence analysis of POMC in human testis (panel 1). Negative controls incubating with unspecific rabbit immunoglobulin fraction (panel 2) and preadsorbing the anti-POMC antibody with betaendorphin (panel 3). Incubation with secondary antibody alone (panels 4). DNA of controls was stained with Propidium Iodide. Representative photomicrographs are shown; n = 3. Scale bar for all panels, 50 µm.

immunoglobulin confirming the specificity of primary antibody. When the primary antibodies were omitted before secondary addition, the fluorescent staining pattern was also abolished.

Effect of beta-endorphin on acrosome reaction

Beta-endorphin induced an inversely dose-dependent increase in acrosome-reacted spermatozoa in capacitated samples (Fig. 2a). The incubation with 10^{-9} M beta-endorphin caused the highest increase in the percentage of acrosome reacted cells ($p < 0.01$). Incubation with higher doses (10^{-7} M) led to a smaller increase in the percentage of acrosome-reacted cells ($p < 0.05$) and beta-endorphin 10^{-5} M caused no significant effect. To further analyse the specificity of the beta-endorphin effect, we co-incubated this pentapeptide with the opioid receptor antagonist naloxone. After pre-incubation with naloxone the effect of beta-endorphin on the percentage of acrosome-reacted cells was blunted by the high dose of naloxone (10^{-5} M, $p < 0.05$), but not by the low doses (10^{-8} M, Fig. 2b). The coincubation of beta-endorphin with 10^{-8} M naloxone caused a partial non-significant reversion of the acrosome reaction. High or low doses of naloxone, added alone, had no effect on the acrosome reaction. To evaluate the effect of beta-endorphin on progesterone-induced acrosome reaction, we co-incubated spermatozoa with beta-endorphin and progesterone. As expected, progesterone increased the percentage of acrosome-reacted sperm cells ($p < 0.05$, Fig. 2c). Additional incubation of this samples with beta-endorphin (10^{-9} M) caused a greater stimulation of the acrosome reaction ($p < 0.01$). The percentage of acrosome reacted sperm cells was higher after the treatment of both substances compared to progesterone exposure, being the difference statistically significant ($p < 0.05$).

Effects of beta-endorphin on intracellular free Ca2+ concentration $[Ca^{2+}]_i$

Beta-endorphin (10^{-9} M) did not modify $[Ca^{2+}]_i$ in Fura2-loaded human sperm cells (Fig. 3a). Higher doses of beta-endorphin assayed (10^{-8}, 10^{-7} or 10^{-6} M) neither caused any effects, even after prolonged periods of incubation (30 min, not shown). Subsequent addition of 10^{-6} M progesterone to the same sperm aliquot caused a typical biphasic $[Ca^{+2}]i$ progesterone response, consisting of a rapid transient peak followed by a decay to $[Ca^{+2}]_i$ levels slightly above basal and a lower sustained plateau phase. Beta-endorphin was not able to modify the progesterone-induced intracellular Ca^{+2} response (Fig. 3a). The area of the progesterone-induced $[Ca^{+2}]_i$ signal was not modified in sperm aliquots pre-incubated with 10^{-9} , 10^{-8} , 10^{-7} or 10^{-6} M beta-endorphin (Fig. 3b).

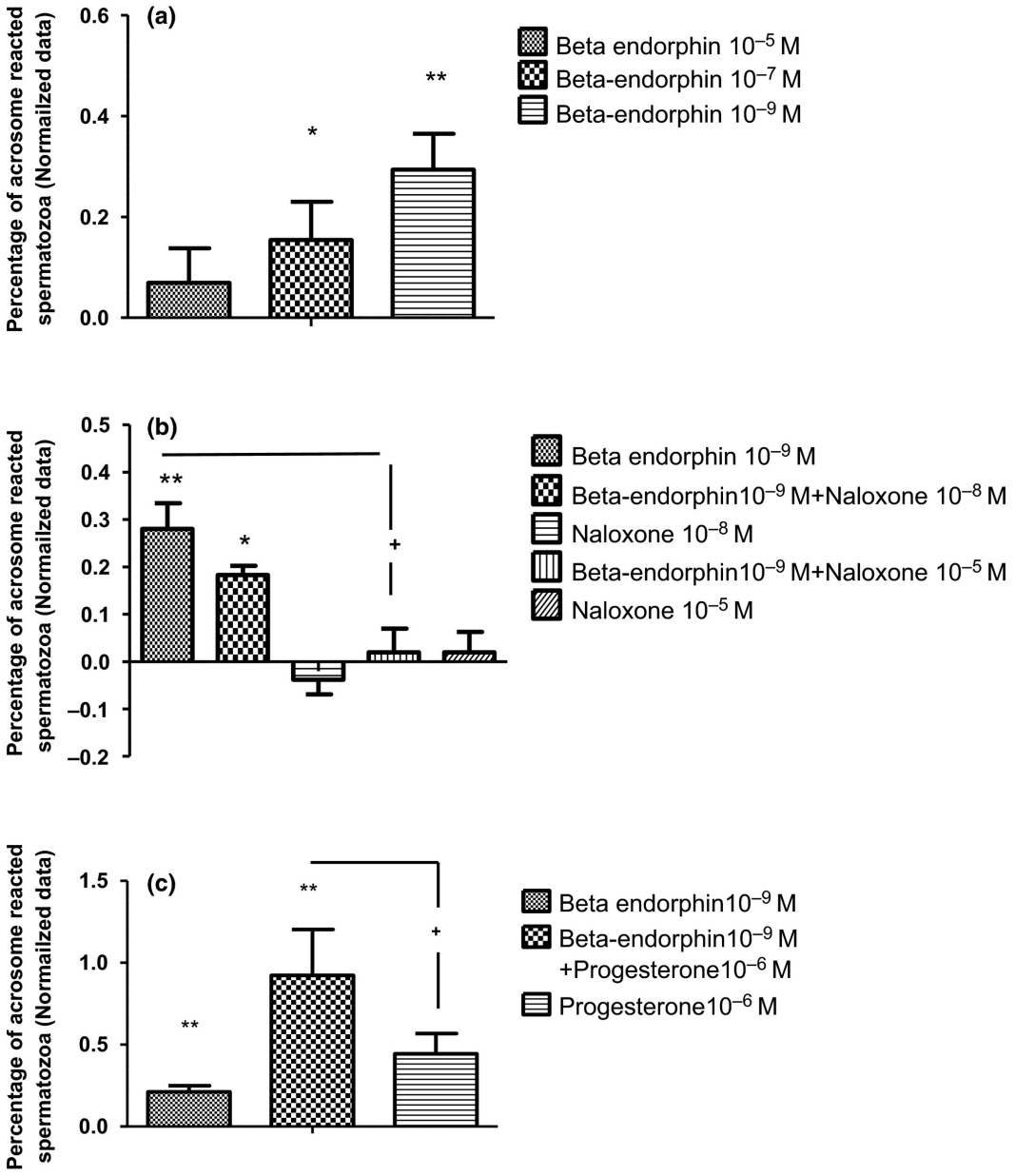

Figure 2. Effect of beta-endorphin on human acrosome reaction. (a) Dose-dependent effect of beta-endorphin on the percentage of acrosome-reacted sperm cells for 1 h. (b) Percentage of CD46-positive sperm cells after co-incubation with beta-endorphin (10^{-9} M) and high (10^{-5} M) and low doses (10^{-8} M) of naloxone for 1 h. (c) Percentage of CD46-positive sperm cells after co-incubation with beta-endorphin (10^{-9} M) and progesterone (10^{-6} M) for 1 h. *$p < 0.05$, significant difference vs. control responses; **$p < 0.01$, significant difference vs. control responses; and +$p < 0.05$ significant difference vs. beta-endorphin responses. (n = 12). Normalized data as [(Treatment − Control)/(Control)] × 100.

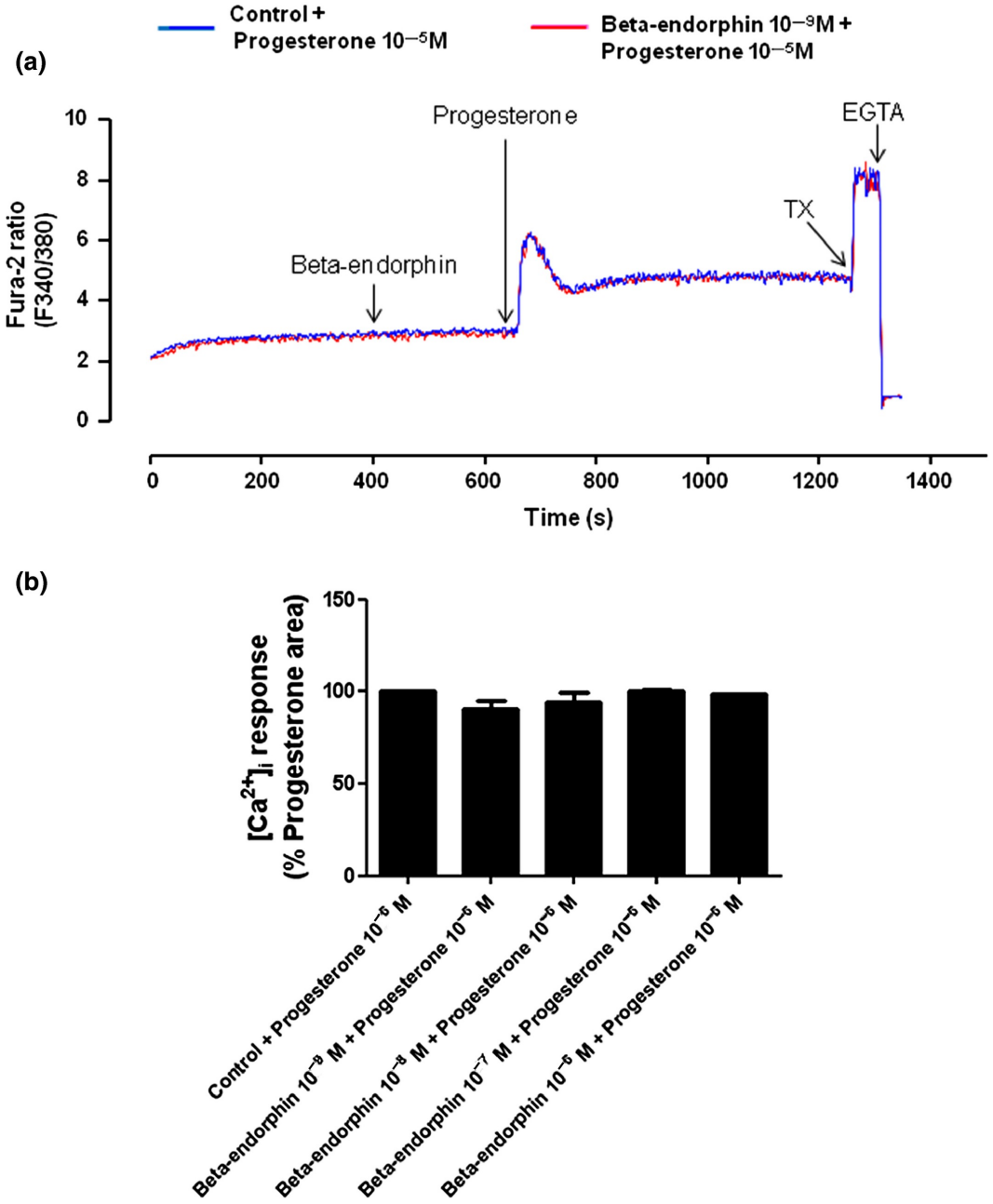

Figure 3. Effects of beta-endorphin on intracellular free Ca2+ ($[Ca^{2+}]_i$). (a) Intracellular free Ca2+ measurement in human sperm cells loaded with Fura-2 in response to beta-endorphin (10^{-9} M) (red line) and control (black line). Subsequent addition of 10^{-6} M progesterone to the same sperm aliquot caused a typical biphasic $[Ca^{2+}]_i$ progesterone response that had not been modified by beta-endorphin. The X axis shows time in seconds and the Y axis shows $[Ca^{2+}]_i$ data expressed by the F340/F380 ratio. Traces are representative of typical results obtained in five different experiments for each blocker. (b) Dose-dependent effect of beta-endorphin on progesterone-induced intracellular Ca^{2+} response. Data expressed the area of the progesterone-induced $[Ca^{2+}]_i$ signal measured by the ratio of F340/F380 signals. Calibration of $[Ca^{2+}]_i$ was achieved adding Triton X-100 (TX), to obtain the maximal response, followed by addition of EGTA to obtain the minimal response n = 5.

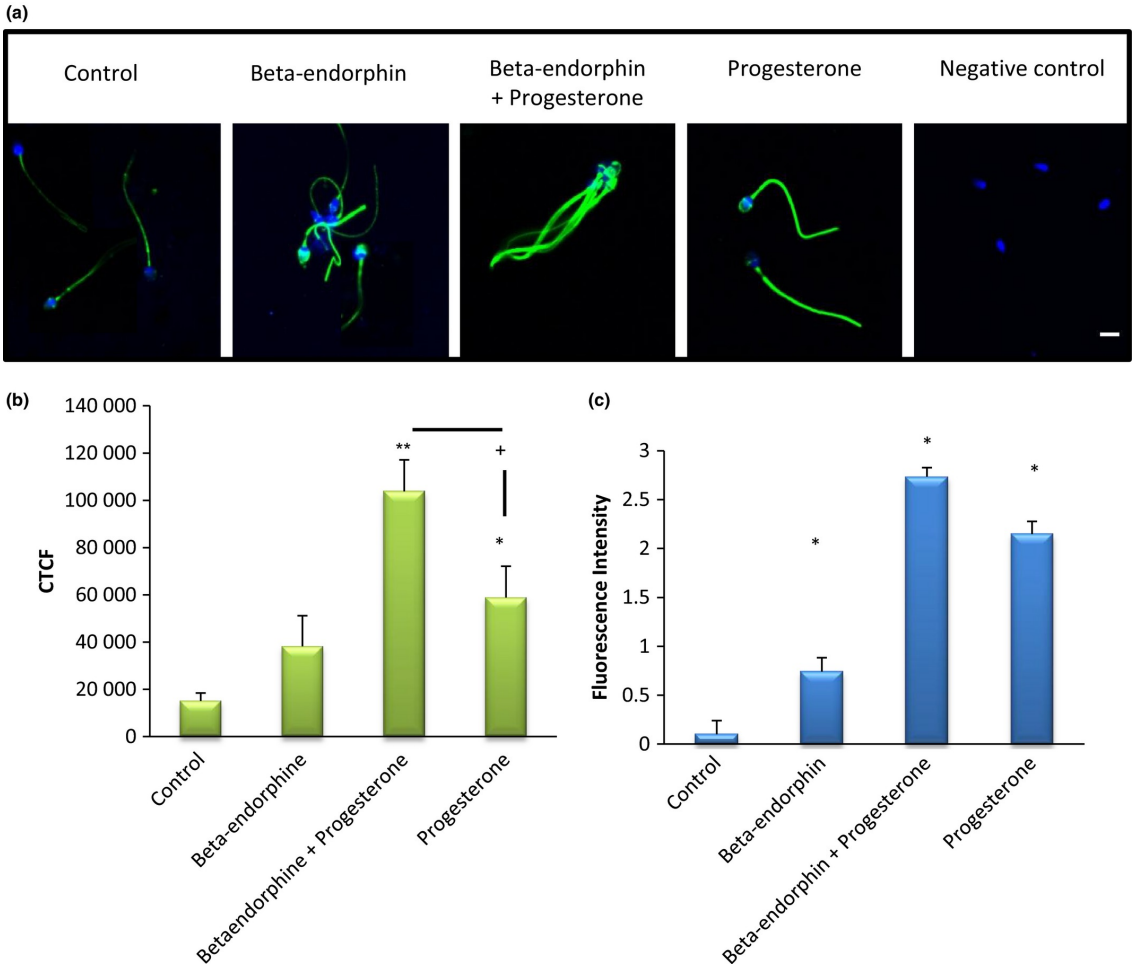

Figure 4. Effect of beta-endorphin on Ca^{+2}/protein kinase C (PKC)-signalling pathway. (a) Immunofluorescence analysis of the PKC-induced substrate phosphorylation in samples treated with beta-endorphin, beta-endorphin and progesterone, and progesterone. DNA of controls was stained with Hoechst 33258. Representative photomicrographs are shown; $n = 5$. Scale bar, 2 µm. **(**b) Percentage of phospho-PKC substrates positive spermatozoa and (c) fluorescence intensity measured by flow cytometry in samples treated with beta-endorphin, beta-endorphin and progesterone, and progesterone. Fluorescence data from at least 100.000 events were analysed. *$p < 0.05$, significant difference vs control responses; **$p < 0.01$, significant difference vs control responses; and + $p < 0.01$, significant difference vs progesterone responses.

Effect of beta-endorphin on sperm PKC-signalling pathways

Figure 4a shows PKC-substrate phosphorylation using an anti-phospho-PKC. In control samples, immunofluorescence was detected along the tail and we observed an increase in the phospho-PKC immunoreactivity after progesterone treatment, as we expected. CTCF analysis showed also a significant increase in PKC-induced phosphorylated substrates after progesterone treatment ($p < 0.05$) (Fig. 4b). Beta-endorphin also increased significantly the staining of the phospho-PKC substrates in the tail and induced appearance of positive immunoreactivity over the acrosome region (Fig. 4a,b). The co-incubation of beta-endorphin with progesterone caused a stronger increase in the immunoreactivity of phosphorylated substrates induced by PKC (Fig. 4a). Measured by CTCF, beta-endorphin caused a 1.7-fold increase in the phosphorylation of PKC substrates respect to progesterone (Fig. 4b).

Experiments were repeated at least five times and non-specific binding was not observed in the negative controls that were not exposed to the primary antibody. Flow cytometry analysis was carried out to verify the positive effect of beta-endorphin on phosphorylation of PKC substrates. As we expected, the intensity of fluorescence of PKC-induced phosphorylated substrates (Fig. 4c) increased after progesterone treatment ($p < 0.05$). Compared with controls, beta-endorphin caused also a positive effect on the phosphorylation of PKC substrates. The fluorescence of phospho-PKC substrates increased in beta-endorphin-treated semen samples ($p < 0.05$). A synergic effect was observed after the co-incubation of beta-endorphin and progesterone on the phosphorylation of PKC substrates status. The fluorescence intensity of PKC-induced phosphorylated substrates was significantly higher (1.2-fold) compared to progesterone ($p < 0.05$) and controls ($p < 0.01$) (Fig. 4c).

DISCUSSION

Endogenous opioid peptides participate in the regulation of reproductive physiology at multiple sites and appear to be increasingly important in the regulation of sperm physiology. In this study, we showed that beta-endorphin exerted a regulatory effect on sperm function.

Expression and localization of POMC in human spermatozoa and testis

beta-endorphin has been described over the acrosome reaction of human spermatozoa (Fraioli et al., 1984). However, the presence of its protein precursor – POMC – was completely unknown. RT-PCR revealed the absence of POMC mRNA in human spermatozoa, consistent with the fact that mature mammalian spermatozoa are not transcriptionally active because of their highly condensed chromatin and the scarcity of cytoplasm capable of supporting translation (Miller and Ostermeier, 2006; Ostermeier et al., 2004). However, recent findings have shown that a limited pool of RNA could be selectively maintained in mature sperm cells to be subsequently translated into protein upon fertilization.

The absence of POMC mRNA in human sperm cells suggests that the transcript of the precursor may not be important during the first steps of embryogenesis as reported for other sperm transcripts

(Agirregoitia et al., 2010; Ravina et al., 2007).

Despite this, immunoblotting and immunofluorescence analysis revealed the presence of POMC protein in human sperm cells – specifically, there was immunoreactivity in the tail of spermatozoa. In agreement with previous studies (Garrett et al., 1989; Kilpatrick et al., 1987), we showed the presence of POMC inside of human seminiferous tubules, where spermatogenic cells are present. Cathepsin L, the major proteolytic enzyme for the production of POMC-derived peptides (Funkelstein et al., 2008), is also present in mice male germ cells and haploid cells (Wright et al., 2003). This suggests that spermatozoa may be able to synthesize POMC-derived peptides *de novo*, such as beta-endorphin, through processing their precursor POMC.

Beta-endorphin stimulates acrosome reaction by PKC pathway

The acrosome reaction is an exocytosis process triggered by very complex signalling pathways involving the activation of protein kinases, intracellular protein activation and the activation of ionic channels (Ickowicz et al., 2012). Progesterone, ZP3, prostaglandins, sterol sulphates and glycosaminoglycans are some inductors of the acrosome reaction and are found in the cumulus oophorus cells and in the follicular fluids (Vigil et al., 2011). These inductors promote the sperm penetration and a rise in calcium concentration of the cytosol that is required for the acrosomic reaction (Ickowicz et al., 2012; Vigil et al., 2011).

Our results suggest that the opioid peptide beta-endorphin can be a physiological inductor of the acrosome reaction. We found an inverse dose-dependent activation of acrosome reaction induced by beta-endorphin. In fact, the physiological doses of beta-endorphin (10^{-9} M) caused the most potent effect on acrosome reaction. High doses (10^{-5} M) of the specific antagonist, naloxone, blunted the activation of acrosome reaction, suggesting that the effect of beta-endorphin is specifically mediated by activation of the opioid receptors. However, low doses of naloxone (10^{-8} M) -at which this compound acts selectively on the mu-opioid receptor- only partially blocked the effect of beta-endorphin on acrosome reacted spermatozoa, raising the possibility that more than one receptor might be involved in this process. The activation of more than one type of opioid receptor also can explain the inverse dose dependent inhibition, as the mu- and delta-opioid receptors can activate opposite responses, as we observed in human sperm motility (Agirregoitia et al., 2006).

Together with progesterone, beta-endorphin is present at high concentrations in the follicular fluid and in the vicinity of the egg (Petraglia et al., 1986, 1985). To elucidate whether beta-endorphin modulates progesterone action, we co-incubated sperm cells with both beta-endorphin and progesterone. Beta-endorphin modified the progesterone response. The percentage of acrosome-reacted sperm cells in samples co-incubated with beta-endorphin and progesterone was 1.5-fold higher that in samples with only progesterone. Owing to the fact that the progesterone response is totally dependent on $Ca^{+2/}$PKC pathways (Chen et al., 2000; O'Toole et al., 1996; Rathi et al., 2003), we investigated whether beta-endorphin may stimulate

acrosome reaction by activation of the Ca^{+2}/PKC pathway.

A common and fundamental feature of physiological and pharmacological acrosome reaction inducers is that they provoke intracellular multicomponent Ca^{+2} increases (Darszon et al., 2011). Thus, we investigated whether beta-endorphin stimulates an increase in intracellular Ca^{+2}. Progesterone caused a typical biphasic wave of intracellular Ca^{+2} stimulation in spermatozoa, composed of a transient increase followed by a sustained elevation as previously reported (Baldi et al., 2009; Gadkar et al., 2002). We failed to detect any change in Ca^{+2} after addition of beta-endorphin. Beta-endorphin did not cause any effect on spermatozoa $[Ca^{+2}]_i$ in Fura-2-loaded sperm suspensions and none of the doses assayed was able to modify the progesterone-induced calcium response. In spite of that, beta-endorphin caused an activation of the PKC-induced substrates phosphorylation. By immunofluorescence and flow cytometry approaches, we observed an increase in the phosphorylation of PKC-induced substrates after beta-endorphin exposure. In addition, we also reported a further activation of the PKC-signalling pathway in semen samples co-incubated simultaneously with beta-endorphin and progesterone. Compared to progesterone alone, the co-incubation of beta-endorphin and progesterone caused a 1.7-fold and 1.2-fold increase in the phosphorylation of PKC substrates, measured by CTCF and flow cytometry respectively. This result was also consistent with the increase observed in the percentage of acrosome-reacted sperm cells. Thus, beta-endorphin may stimulate the acrosome reaction via PKC-signalling pathway activation, as have been reported for other inductors (O'Toole et al., 1996; Vigil et al., 2011).

Moreover, our data suggest that beta-endorphin can activate the PKC-signalling pathways through a Ca^{+2}-independent pathway. Mouse and rat eggs can express the atypical Ca^{2+} independent PKC isoforms ζ and λ (Baluch et al., 2004; Pauken and Capco, 2000) but further analyses will be necessary to analyse the presence of Ca^{+2}-independent PKC isoforms in human sperm cells. In conclusion, the present data allow us to identify a new physiological acrosome reaction inductor and described its signalling pathways in human spermatozoa. Beta-endorphin may be involved in the regulation of acrosome reaction by a Ca^{2+}-independent PKC pathway in humans. These findings are important for future studies of sperm physiology and provide new insight into the function of the opioid system as a target for fertility management.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

I.U-A., H.E. and I.M-H carried out and analysed the experiments, F.M.P. and L.C. carried out the experiments and provided

conceptual support, R.M and A.E evaluated the samples. A.V and J.I. provided conceptual support. N.S. designed the study, analysed the experiments and wrote the manuscript.

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2.Kapitulua

Chapter 2

4.2. CHAPTER 2

Phosphoproteomic and functional approaches reveal changes in sperm-specific proteins downstream KOR in human spermatozoa

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Summary

G-protein coupled receptors (GPCRs) belong to the seven transmembrane receptors superfamily that in response to external stimuli, transduce signals via G proteins to initiate different intracellular signaling pathways which culminate in specific cellular responses. Although in somatic cells the GPCR participate in a broad variety of physiological processes, the expression of diverse GPCRs at the plasma membrane of human spermatozoa suggests their involvement in the regulation of sperm fertility. Mature spermatozoa could present unique features in their molecular mechanisms downstream GPCRs due to the fact that they possess sperm-specific proteins and are transcriptionally and translationally silent. In order to decipher the signaling pathways engaged by this receptor superfamily in human spermatozoa, we selected the kappa-opioid receptor (KOR) as a study model and applied, for first time, phosphoproteomic approach based on TMT labeling and LC-MS/MS analyses combined with functional studies using a specific agonist of KOR, U50488H. Data are available via ProteomeXchange with identifier PXD011290.

INTRODUCTION

Ejaculated mammalian sperm cells are immature and infertile and must undergo many physiological and biochemical modifications to become fertilization competent. These processes as the acquisition of sperm motility, capacitation, hyperactivation and acrosome reaction occur sequentially inside the female

reproductive tract and are considered key functions in the control of the reproduction as well as essential for spermatozoa to become fertile (Visconti et al., 2002). It is well documented that ionotropic modulation through rapid responses are the main regulators of sperm physiology (Hille, 1992). However, the presence of a high number of GPCRs described over the last decade in human spermatozoa, suggests that metabotropic mechanisms could also be important in the acquisition of the sperm fertilizing capacity (Spehr et al., 2006).

The GPCRs are seven transmembrane receptors which represent approximately the 1% of the human genome and are considered to be one of the best therapeutic targets (Nambi and Aiyar, 2003). In somatic cells, the canonical (G-protein dependent pathways) and non-canonical (G-protein independent pathways) are the major signaling pathways initiated downstream GPCRs and have an important role in the regulation of basic cellular activities as well as in the coordination of cell actions. Over the last 20 years, several GPCRs have been described in human spermatozoa providing clear evidences of their involvement in the regulation of sperm fertility (Jiménez-Trejo et al., 2012; Köhn et al., 1998; Pinto et al., 2012; Rossato et al., 2005; Schaefer et al., 1998). Furthermore, different components from the G-protein dependent transduction pathways such as the cAMP-dependent and the Ca^{+2}/PKC signaling cascades have been described to influence aspects of sperm function such as sperm motility, capacitation and acrosome reaction (Hess et al., 2005). In addition, in respect to the non-canonical signaling pathway, β-arrestin that promotes desensitization and internalization of the GPCRs via a G-protein independent signaling pathway, has been demonstrated to act as a signal transducer, capable of modulating the sperm motility and acrosome reaction (Almog et al., 2008). Moreover, since spermatozoa are transcriptionally and translationally silent cells, it has been suggested that they may possess unique, sperm-specific signaling pathways (Almog et al., 2008; Hess et al., 2005). In 2006, although we described for the first time the presence of functional Mu-, delta- and kappa- opioid receptors (MOR, DOR and KOR, respectively) in human spermatozoa (Agirregoitia et al., 2006), the signaling events downstream of these receptors remain uncharacterized.
Taking all this into account, the aim of this study was to elucidate the existence of sperm-specific molecular mechanisms of GPCRs signaling in human spermatozoa. For this purpose we combined for the first time phosphoproteomic approaches together with functional analyses in human spermatozoa, by using the kappa-opioid receptor as a study model.

EXPERIMENTAL PROCEDURES

Spermatozoa isolation and treatments

Ethical approval for this study was obtained from the Ethics Committee of the University of the Basque Country (CEISH-UPV/EHU (M10/2016/254)). Freshly ejaculated semen was collected from patients undergoing routine semen analysis at the Cruces University Hospital (Bilbao, Spain). The donors had normal sperm parameters according to World Health Organization standards (World Health Organization, 2010). Semen samples were obtained by masturbation after 3–4 days of sexual abstinence and

immediately processed upon liquefaction (at 37 °C for 30 min). Spermatozoa were capacitated by the swim-up procedure and resuspended in G-IVF (Vitrolife, Goteborg, Sweden) supplemented with 1% bovine serum albumin for 3 h at 37°C under 5% CO_2.Isolated spermatozoa were treated at 37 °C and 5% CO2 in G-IVF culture media. The spermatozoa were treated with 1 µM U50488H (the specific agonist of the receptor) (Sigma) for 1 and 60 minutes independently for proteomic and functional analyses.

For functional analyses the samples were co-incubated for 60 minutes with U50488H (Sigma-Aldrich) and the different activators and inhibitors of different signaling pathways. To study the calcium signaling pathway we used U73122 (3 µM) (Sigma-Aldrich), a phospholipase C inhibitor; Mibefradil (30 µM) (Tocris Biosciences), a calcium channel activator and NNC55-0395 (10 µM)(Sigma-Aldrich), a CatSper specific calcium channel inhibitor. To study the cAMP/PKA signaling pathway we used the SQ2336 (200 µM) (Sigma-Aldrich), the transmembrane adenylate cyclase (tmAC) inhibitor; Forskolin (50 µM) (Sigma-Aldrich), the tmAC activator and the HCO_3^- (50 mM)(Sigma-Aldrich), the SACY activator. In order to study the MAPK signaling pathway we used the BARK1 (126 µM)(Calbiochem), the GRK inhibitor and IBMX (0.5 mM)(Sigma-Aldrich),the phosphodiesterases inhibitor.

Experimental Design and Statistical Rationale

Human spermatozoa that had normal parameters were treated with 1 µM U50488H (the specific agonist of the receptor) for 1 and 60 minutes independently for proteomic and functional analyses. For proteomic analyses, we adopted a Tandem Mass-Tag (TMT) 6-plex isotopic labeling strategy followed by phosphopeptide enrichment by consecutive incubations with titanium dioxide beads (TiO_2) and generated samples for LC-MS/MS. The experiment was performed in three biological replicates. Peptide and protein searches were performed using the Andromeda search engine (integrated in MaxQuant, version 1.5.3.30) at a FDR threshold of 1%. Perseus software (v.1.6.0.7) was employed for the calculation of the statistical significance (two-sample student´s T-test) and fold changes between U50488H-treated and Control samples. We considered as U50488H-dependent phosphosites the ones that were consistently regulated presenting a 1.5 fold change (U50488H/Ctr > 1.5 or U50488H/Ctr < 0.67 and p value 0.05) in the three replicas for each timepoint and protein fraction.

Protein extraction and digestion

The followed proteomic strategy is summarized in Figure 1A. Three biological replicates of each untreated (Ctrl) and treated spermatozoa (U50488H) were used for 1 and 60 minutes. For soluble and insoluble protein extraction, treated sperm cells were lysed using ice-cold RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium pervanadate, 5 mM beta-glycerophosphate, 5 mM NaF, complete protease inhibitor cocktail. Roche). After protein homogenization and sonication (20% amplitude, 10 pulses, three times), proteins were separated in soluble and insoluble fractions after 15 min

centrifugation at 13000 g, 4ºC. To solubilize the insoluble fraction, we resuspended proteins into 8M Guanidinium chloride and sonicated them (20% amplitude, 10 pulses). Both soluble and insoluble protein fractions were reduced and alkylated using 2 mM dithiothreitol and 11 mM chloroacetamide respectively. Then the soluble proteins were precipitated using acetone to further resuspend them in urea buffer (8M urea, 10 mM TrisHCl pH 7,5) and estimate the protein abundance by bicinchoninic acid (BCA) method (Pierce, Thermo Fisher). After adjusting all the samples to 1:1:1:1:1:1 in each timepoint and protein fraction (soluble or insoluble), the samples were subjected to in-solution digestion using LysC (4 hours) and Trypsin (overnight). Proteolytic digestion products were desalted on a Sep-Pak C18 cartridge (Waters, Milford) for further sample processing.

TMT labeling

Digested peptides were labeled by Tandem Mass-Tag (TMT) 6-plex isotopic label reagent set (Thermo Scientific, Rockford) to study the total proteome. After the peptide drying, each condition was resuspended in 100 mM Triethylammonium Bicarbonate Buffer (TEAB) (Sigma-Aldrich). Peptides were incubated with each label for 1 hour at room temperature (RT) and reactions were quenched using 8 µl of 11% Lysine, following the manufacturer´s recommendations. After further 15 minute incubation, the peptide solutions were acidified 1:10 v/v with 10% trifluoroacetic acid (TFA, Sigma-Aldrich). Then we mixed the independent samples in 1:1:1:1:1:1 proportion and fractionated the samples into 17 different fractions following the stepwise high- pH reversed phase fractionation protocol. Each fraction was loaded onto a C18 Stage Tip (made in house using Empore disc –C18 Agilent Life Science) for further proteomic analyses.

Stepwise high-pH reversed phase fractionation

A part of the digested peptides (1/10) were TMT labeled and then fractionated into 17 different fractions following the Stepwise high-pH reversed phase fractionation protocol. The samples were adjusted to pH 10 with 10 mM ammonium hydroxide and transferred to a home-made tip containing ReproSil-Pur, 1,9 µm, 120A beads. Sequential elutions were made using 1.75, 3.5, 5.25, 7, 8.75, 10.5, 12.5, 14, 15.5, 17.5, 21, 24.5, 28, 31.5, 35, 50 and 70% ACN. Each fraction was loaded onto C18 StageTips. Peptides were eluted with 60% ACN to further continue with mass spectrometry analysis.

Phosphopeptide enrichment and TMT labeling

The 9/10s of the peptides were enriched in phosphopeptides by consecutive incubations with titanium dioxide beads (TiO_2). After all the samples were brought to 60% acetonitrile (ACN) and 1% TFA, the beads were added and incubated for 15 minutes rotating. Beads were then transferred to home-made C8 Stage Tips (Empore disc –C8 Agilent Life Science) and washed with 60% ACN/1% TFA. Phosphopeptides were eluted with 5% ammonia and 25% ACN/10% ammonia and loaded onto C18 StageTips. Peptides were eluted with 60% ACN and subjected to TMT labeling as previously described. Finally,

the samples were analyzed by mass-spectrometry.

Mass Spectrometry Analysis

Acidified peptide mixtures were separated by online C18- reverse-phase nanoscale liquid chromatography and analyzed by tandem mass spectrometry (LC-MS/MS). MS analysis was performed on an Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany) connected to an EASY-nanoLC 1000 System (Thermo) using a nanoelectrospray ion source (Proxeon Biosystems, Odense, DK). Survey full-scan MS spectra (m/z range, 200–2000; resolution 60,000 at m/z 400) were acquired in the Orbitrap followed by the fragmentation of the twelve most intense multiply charged ions. Ions selected for MS/MS were placed on a dynamic exclusion list for 45 s. To improve mass accuracy, internal real time lock mass calibration was enabled. Additional mass spectrometric parameters included a spray voltage of 2.3 kV, no sheath and auxiliary gas flow, and the temperature of the heated capillary was 275 °C. All raw files were searched against combined human database 2015.08 UniProt (with 42122 sequence entries) and TrEMBL (with 49496 sequence entries) using MaxQuant platform version1.5.3.30 with an Andromeda search engine.

Precursor and fragment tolerances were 4.5 and 20 ppm, respectively. A peak list was generated using the Quant element of MaxQuant using the following parameters. A maximum of 2 missed cleavages was allowed and enzyme specificity was set to trypsin, allowing for cleavage N-terminal to proline and between aspartic acid and proline. In addition, carbamidomethyl (C) was chosen as fixed modification and variable modifications included oxidation (M), deamidation (NQ) and Phospho_STY (STY). The peptide and protein FDR 0.01; site FDR 0.01; max. peptide PEP, 1; min. peptide length 7; min unique peptides and peptides, 1. For protein quantitation, only unmodified peptides and peptides modified by acetyl (protein N-terminus), oxidation (Met) and deamidation (NQ) were used. According to the protein group assignment done by MaxQuant, the identified proteins were determined after removing the contaminants, reverses and those proteins only identified by site. Moreover, we took into account those proteins with ≥2 identified peptides and ≥1 unique peptides. On the other hand, the phosphopeptide data was filtered by FDR < 1% and only the phosphosites displaying a localization probability above 0.75 were considered as confident phosphorylated sites (Class I sites).

Proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier **PXD011290.**

Bioinformatic Analysis

The Perseus software (v.1.6.0.7) was employed for the calculation of the statistical significance and Fold changes between U50488H-treated and Control samples. In order to identify the U50488H-dependent phosphosites only the data of the quantified phosphosites and proteins were considered and used further. Firstly, the data was normalized to correct the labeling variability and then it was normalized depending on phosphosite reporter ion intensities/protein reporter ion intensities. We considered as U50488H-dependent phosphosites the ones that were consistently regulated

presenting a 1.5 fold change (U50488H/Ctr > 1.5 or U50488H/Ctr < 0.67 and p value 0.05) in the three replicas for each timepoint and protein fraction.

The PANTHER (v13.1) functional annotation tool (http://geneontology.org/) was used to detect the overrepresented gene ontology (GO) term "biological process" within the total identified proteins and Class I identified phosphoproteins in human spermatozoa. The Homo Sapiens dataset was used as reference In order to construct the signaling pathways regulated by the kappa-opioid receptor, GENEMANIA (http://genemania.org/) the interactive functional association network tool was used.

Computer-assisted motility analysis

Human spermatozoa were capacitated and adjusted to a concentration of 50 x 10^6 cells/ml. Motility analysis was conducted by computer-assisted sperm analysis (CASA) (Sperm Class Analyzer, S.C.A., Microptic, Barcelona, Spain) following the WHO guidelines. For that purpose we examined the percent of motile sperm being by the manufacturer as appropriate for human species: progressive motility (rapidly progressive with velocity ≥35 µms s^{-1} at 37ºC (grade "a") + slow sluggish progressive with velocity ≥10 µms s^{-1} but <35 µms s^{-1} (grade "b"), non-progressive motility with velocity <10 µms s^{-1} and immotile motility. Moreover the following kinematics parameters were also measured: curvilinear velocity (VCL, µm/s); straight-line velocity (VSL, µm/s), average-path velocity (VAP, µm/s); amplitude of lateral head displacement (ALH, µm); linearity of progression (LIN=VSL/VCL x 100); straightness (STR=VSL/VAP x 100); motility and hyperactivated motility. The percentage of hyperactive cells was defined following parameters above (Almog et al., 2008): VCL 100 m/s, LIN 60% and ALH 5 µm. To investigate the effects of drugs, sperm samples were divided in several aliquots and treated with a single concentration of U50488H (1 µM) (Sigma). Sperm motility was measured 5 min before U50488H addition (initial value) and after a contact time of 1 and 60 minutes. Additional experiments were performed in similar conditions to evaluate the effects of Mibefradil (30 µM), U73122 (3 µM) and NNC55-0395 (10 µM) and their co-incubations with the U50488H agonist.

Flow cytometry

Acrosome reaction was evaluated by using the anti-CD46 antibody by Flow cytometry. The FACScalibur flow cytometer (Becton, Dickinson, San Jose, CA, USA) was used in order to study the role of the kappa-opioid receptor in human sperm acrosome reaction. Spermatozoa were treated with U50488H for 1 and 60 minutes, and the different activators and inhibitors of different signaling pathways. The progesterone (10 µM) was used as an internal control for the acrosome reaction. For the staining of the cells the Fluorescein IsoTioCyanate (FITC) antihuman CD46 antibody (BioLegend, California, USA) (5 µl) was used for 60 min at room temperature as acrosome reaction molecular marker. As indicator of cell viability we used 0.1 µg/mL Hoechst 33258 for 2 min. Finally, samples were washed twice by centrifugation in PBS at 800 g for 5 min, suspended in PBS and kept in the dark until analysis. Fluorescence data from at least 10.000 alive sperm cells were analysed and the

green fluorescence belonging to spermatozoa was measured. Histograms were analysed using the Summit v4.3 software (Beckman Coulter, California, USA).

Western Blotting

For Western Blotting analyses, whole cell extracts (500.000 cells) were diluted in 1x Laemmly sample buffer containing Dithiothreitol (DTT) (%10v/v) and boiled for 5 min. Samples were loaded onto 12% resolving gels and separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride membranes (PVDF) (Amersham Hybond, Sigma) using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Then, membranes were blocked with Blotto (20mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1%Triton X-100) containing 5% Bovine Serum Albumin (BSA) for 1 hour and then incubated with a dilution of: the monoclonal mouse 4G10 anti-phosphotyrosine antibody (05-231, Millipore) (1:1000) as a molecular marker of the human sperm capacitation (Leclerc el al, 1997), the polyclonal rabbit anti-phosphorylated protein kinase C substrates (#2261, Cell Signalling) (1:750), the polyclonal rabbit anti-phosphorylated protein kinase A substrates (#9624,Cell Signalling) (1:750), the monoclonal mouse anti-phosphorylated MAP kinase substrates (#2325, Cell Signalling) (1:750) and the monoclonal mouse anti-alpha tubulin (T5168, Sigma, 1:4000). After washings (3x 5minutes) in Blotto buffer, the membranes were incubated for 1 hour at RT with peroxidase-conjugated goat anti-rabbit (Blotto + %5 BSA 1:1000) (Goat anti-rabbit IgG HRP, sc-2004; Santa Cruz Biotechnology) and donkey anti-mouse IgG antibodies (Blotto+ %5 BSA 1:2000) (Donkey anti-mouse IgG HRP, sc-2314; Santa Cruz Biotechnology). After washing (3x 5 minutes) blots were revealed for peroxidase activity by enhanced chemoluminiscence (ChemiDoc XRS detector, Bio-Rad). Results were analyzed by semi-quantitative Western blots densitometry analysis using Image J (Image Processing and analysis in Java) software.

Measurements of sperm intracellular Ca^{+2} concentration

Changes in $[Ca^{+2}]_i$ were monitored using the Fura-2 as previously described by Cejudo-Roman et al (Cejudo-Roman et al., 2013). Briefly, spermatozoa were adjusted to a concentration of 10 $x10^6$ cell/ml in human serum albumin (HSA) medium. They were then incubated with the acetoxymethyl ester form of Fura-2 (Fura-2/AM 8 x 10^{-6}M; Molecular Probes) for 60 minutes at room temperature in the presence of the non-cytotoxic detergent pluronic acid (0,1%; Molecular Probes). After loading, the cells were washed and resuspended in G-IVF solution and used within the next 2-7 hours. Sperm aliquots (1 ml) were placed in the quartz cuvette of a spectrofluorometer (SLM AMinco-Bowman, Series 2; Microbeam, Barcelona, Spain) and magnetically stirred at 37ºC. The emitted fluorescence was measured at 510 nm. To measure $[Ca^{+2}]_i$, samples were alternativelly illuminated with two excitation wavelenghts (340 nm and 380 nm) and the fluorescent light from the two excitation wavelengts was measured by a photomultiplier through a 510 nm filter. After substracting the autofluorescence signal, obtaining by adding 5 nM MnCl2 at

the end of the experiment, the F340/F380 ratio was used an indicator of $[Ca^{+2}]_i$. The effect of U50488H was studied on sperm aliquots incubated with this peptide at different doses (1 µM). Mibefradil (30 µM) was added to the same sperm aliquots to analyze the effect of U50488H of the mibefradil-induced intracellular calcium level. Progesterone (1 µM) was added to the same sperm aliquots as a positive control of the AR and the consequent $[Ca^{+2}]_I$ increase. Calibration of $[Ca^{+2}]_i$ was achieved adding Triton X-100 (5%) to obtain the maximal response, followed by the addition of ethylene glycol tetra-acetic acid (EGTA) (40 nM) to obtain the minimal response.

Statistical analysis

Sperm motility data were normalized as [(Treatment –Control)/(Control)] x100, and acrosome-reacted data were normalized as [(Treatment- Control)/(Progesterone-DMSO)] x100 and evaluated using Students T-test and one-way ANOVA with post-hoc Bonferroni test. These procedures were undertaken using the IBM SPSS Statistics program (version 22). Differences were considered significant at $*p < 0.05$ and highly significant at $**p < 0.01$. Data are expressed as mean ± SEM using the GraphPad PRISM (version 6.0) (GraphPad Company, California, USA) program.

RESULTS

Human sperm protein identification

To gain insights into the human sperm proteome, we followed mass-spectrometry (MS) based proteomic and phosphoproteomic approaches in parallel. Isolated spermatozoa samples, untreated or stimulated with U50488H, were divided into soluble and insoluble protein fractions and subjected to the workflow described in Figure 1A. Once we excluded reverse hits and common contaminants and accounting for the reporter ion intensities and number of peptides (≥2 identified peptides and ≥ 1 unique peptides), a total of 5109 proteins were identified of which 5070 were confidently quantified (Supplemental Table S1, See CD).

To obtain an overview of the biological functions of the identified proteins, Gene Ontology (GO) analyses were performed using the Panther classification system (Figure 1B). The most enriched functions were related to the protein and tRNA transport, metabolic processes, nuclear organization or processes related to sperm function. Specifically, we found a wide variety of proteins associated to protein folding and targeting to different organelles like the mitochondrion, endoplasmic reticulum or the plasma membrane. Moreover, we identified different enzymes involved in diverse metabolic processes like the glycolysis, the citrate catabolism or nucleobase biosynthetic processes which have an important function in the regulation of sperm fertility. Interestingly, in spite of mature spermatozoa are unable to translate genes into proteins, we also observed proteins connected to the nuclear envelope disassembly or those related to gene expression and protein synthesis (Supplemental Table S1, See CD). Furthermore, we detected key proteins for the flagellar movement and sperm-egg recognition, as well as a broad catalogue of testis- and sperm-proteins involved in different molecular functions that are essential for the accomplishment of cell-specific physiological processes. On the

other hand, we also identified particular proteins belonging to the GPCR signaling pathway. Although we found only few GPCRs in our proteomics dataset, we identified a wide variety of participants of the canonical and non-canonical signaling pathways downstream this family of receptors (Supplemental Table S1, See CD).

Identification of novel phosphosites in human spermatozoa

In order to study the human sperm phosphoproteome, the phosphopeptides belonging to the soluble and insoluble protein fractions were enriched with TiO_2 beads followed by TMT labeling (Figure 1A) and analyzed by MS, yielding a total of 4367 identified phosphorylation sites. Of those, 3527 had a localization probability above 0.75, considered as confident phosphorylated sites (Class I sites), and mapped to 1332 proteins (Figure 1C). The 3527 Class I sites comprised mainly serine phosphorylations (pSer, 77%), followed by phosphorylation on threonine (pThr, 17%) and tyrosine (pTyr, 6%) residues (Figure 1C). All the proteins containing Class I sites were categorized according to their "biological process" using the Panther classification system (Figure 1D). The most enriched biological processes were related to the cilium or flagellum-dependent cell motility, regulation of protein localization to Cajal Body, axonemal dynein complex assembly, sperm-egg recognition, binding to zona pellucida, sperm capacitation, nuclear organization and chaperone mediated protein assembly.

Although some of the phosphosites we identified have already been described and are reported in the PhosphositePlus database as well as in different studies (32) (Supplemental Table S2, See CD), we present evidences of a large number of phosphosites (2157) that have not been previously characterized (Figure 1E). This information represents a valuable source of novel information to understand the role of phosphorylation events in human spermatozoa.

U50448H-induced changes in the phosphoproteome of human spermatozoa

To have a better comprehension of the molecular mechanisms underlying GPCR signaling in human spermatozoa we chose the KOR as a study model. (Supplemental Figure S1). After the addition of its specific ligand, U50488H, we followed phosphoproteomic approach (Figure 1A) to decipher the phosphorylation changes downstream KOR. To unravel U50488H-dependent phosphorylation events, phosphosites with at least 1.5-fold increase or 0.67-fold decrease, and with a significant change of $p<0.05$, were considered as regulated. Considering these criteria, in the insoluble protein fraction 1 minute U50488H treatment resulted in changes in the phosphorylation levels of five previously undescribed phosphosites belonging to 4 different proteins (Figure 2A and 2B, Supplemental Table S3, See CD). Treatment with the agonist resulted in decrease in the phosphorylation levels of the threonine 147 and the serine 152 of the solute carrier organic anion transporter family member 13 (Thr147/Ser152 SLCO1B3), and in the serine 236 of the A kinase anchoring protein 3 (Ser236 AKAP3), while it led to an increase in the phosphorylation on serine 169 of the protein chiby (Ser169 CBY3) and on the serine 127 of the transmembrane 190

protein (Ser127 TMEM190) (Figure 2A and 2B, Supplemental Table S3, See CD). The phosphorylation levels of the human sperm soluble protein fraction, however, remained unchanged following 1 minute treatment with U50488H.

At 60 minute treatment we detected a single change in the soluble protein fraction, a decreased phosphorylation of the uncharacterized protein C6orf10 (Ser31 C6orf10). In the insoluble fraction, we observed changes in the phosphorylation levels of twenty nine phosphosites belonging to 20 different proteins. It is worth highlighting that 13 of them were sperm-specific proteins and that 19 phosphosites have not been previously reported in neither in human spermatozoa nor in other cells (Figure 2A and 2B, Supplemental Table S3, See CD).

Intriguingly, U50488H induced changes in the phosphorylation levels of different sperm-specific proteins mainly involved in the regulation of the sperm fertility. Among other, we saw both increase and decrease in the phosphorylation of different proteins related to sperm motility, capacitation and acrosome reaction like AKAP3 and 4, Outer dense fiber protein 1 and 2 (ODF1 and ODF2), Fibrous sheath interacting protein 2 (FSIP2), the family of the coiled-coil domain containing proteins (CCDC18, CCDC136 and CCDC81) and the Calcium binding tyrosine phosphorylation-regulated protein (CABYR). We also detected changes in different residues of the phosphoglycerate kinase 1 and 2 (PGK1 and PGK2) metabolic enzymes and the 26S proteasome non-ATPase regulatory subunit 3 (PSMD3), involved in protein degradation (Supplemental Figure S2, Supplemental Table S3, See CD). The biggest changes among the regulated sites were seen for the Ser464 of CABYR, the Ser236 of AKAP3 and the Ser256 of CCDC81 presenting fold changes > 2.

To assess the relationships among the U50488H-regulated phosphoproteins and KOR, we generated a network using the biological network integration platform GeneMANIA. The proposed model predominantly links proteins, which could be regulated by the U50488H with those activated downstream the Kappa-opioid receptor. As seen in Figure 2C, KOR, AKAPs, CCDC family, CABYR, ODFs, PGKs, FSIP2, PSMA3 and PSMD3 are connected either directly or indirectly. Based on the data provided by the bionformatic tool, KOR has been shown to be coexpressed together with AKAP3 and ODF1. At the same time, both proteins tend to coexpress and interact with ODF2, AKAP4 and other cytoskeletal proteins like FSIP2, SEPT12 or the ropporin 1B (ROPNB1). On the other hand, AKAP3 is expressed together with CABYR, which among other, coexpresses with RIIAD1 (Regulatory subunit of type II PKA R-subunit) or the PRKAR1A (protein kinase cAMP-dependent type I regulatory subunit alpha), a direct participant in the kappa-opioid signaling pathway. According to this information, KOR could have the potential to modulate the phosphorylation of sperm-specific proteins that participate in the construction of a functional interaction network based on the information provided by the database. Regarding the localization of the U50488H-regulated phosphoproteins within the spermatozoa, the Figure 2D gathers a schematic representation of the distribution of these proteins along the flagella, acrosome, midpiece or axoneme.

To further investigate the signaling downstream KOR, we studied the phosphorylation of different targets from the canonical and non-canonical signaling

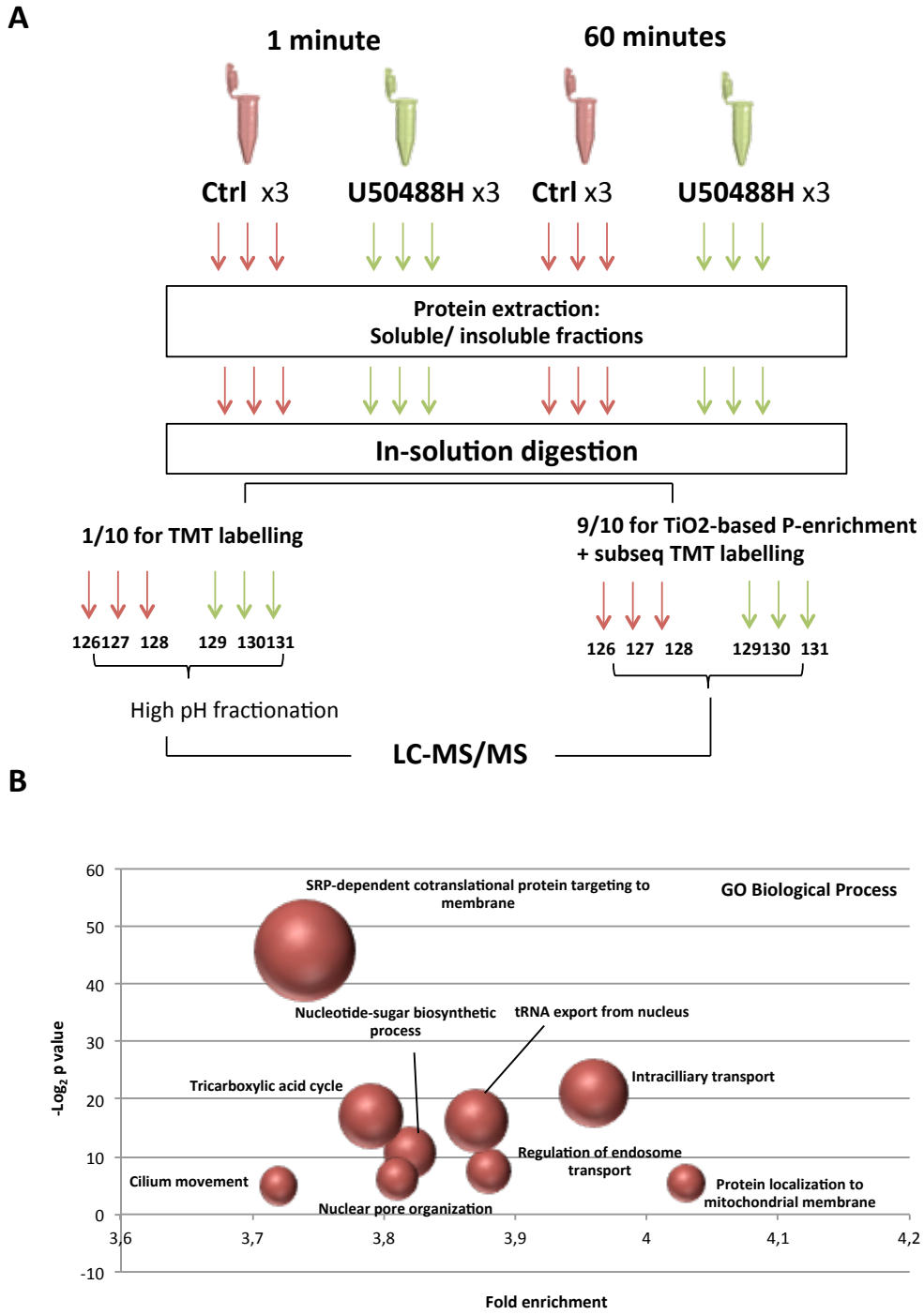
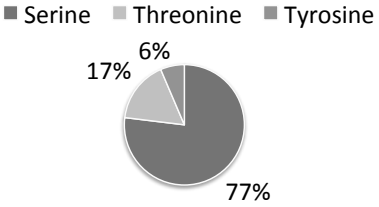


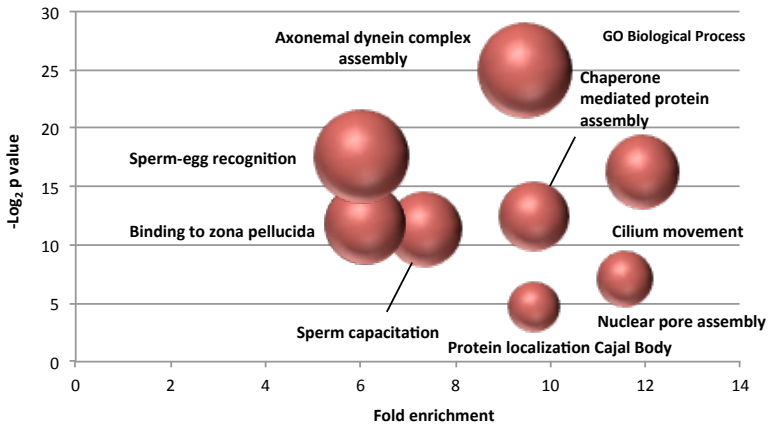
Figure 1. **Proteomic and phosphoproteomic analysis of human spermatozoa. (A)** Schematic representation of the experimental workflow followed in the study. Three biological replicates of each untreated (Ctrl) and treated spermatozoa (U50488H) were used for 1 and 60 minutes. For both timepoint, after the protein extraction of each sample, the soluble and insoluble protein fractions were separated and independently subjected to a in-solution digestion. To analyze the whole proteome in human spermatozoa, 1/10 of each sample were used for tandem mass tag (TMT-6plex) labeling and after performing a high pH fractionation, the peptide mixture was analyzed by LC-MS/MS. On the other hand, the other 9/10 of each sample was utilized for TiO_2-based phospho-enrichment followed by the subsequent TMT labeling and LC-MS/MS analysis. This procedure was followed independently with both soluble and insoluble protein fractions treated with U50488H for 1 and 60 minutes. **(B)** Gene ontology analysis (PANTHER bioinformatic tool) indicating the biological processes of the total proteins identified in the study. The fold enrichment and the statistical significance p value of the most indicative terms are indicated. The size of the dots correlate with the number of proteins grouped in the same term.

C

	TOTAL
Identified proteins	5109
Class I Site Phosphosites	3527
Phosphoproteins	1332



D



E

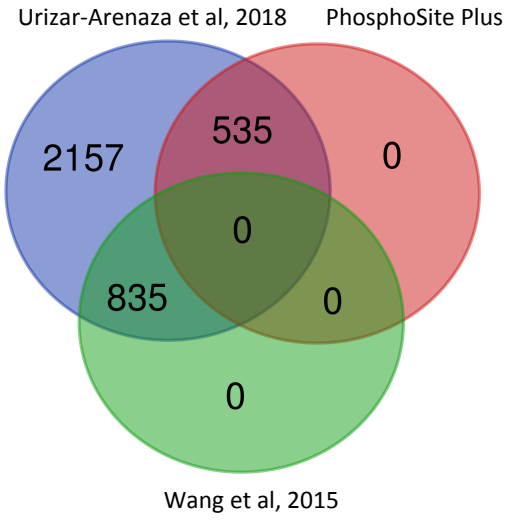


Figure 1. **Proteomic and phosphoproteomic analysis of human spermatozoa. (C)** Total Class I phosphosite (p-sites) and corresponding proteins quantified in the three biological replicates. Distribution of all quantified phosphorylated serine, threonine and tyrosine residues **(D)** Gene ontology analysis (PANTHER bioinformatic tool) indicating the biological processes of the proteins belonging to the Class I site phosphosites. The fold enrichment and the statistical significance p value of the most indicative terms are indicated. **(E)** Venn diagram comparing the number of the identified phosphosites in this study with the published studies.

pathways by immunoblotting. Considering that the AKAPs act as scaffold proteins forming multi-protein complexes that connect several signaling pathways (Michel and Scott, 2002), we analyzed the overall levels of the phosphorylated substrates of protein kinase C (PKC) (Figure 3A), protein kinase A (PKA) and MAP kinases (MAPK) (Supplemental Figure S3A and S3B) following the ligand addition. In the case of the phosphorylated substrates of PKC, U50488H resulted in an increase after both 1 and 60 minute stimulation ($p<0.01$), suggesting that U50488H could participate in the modulation of the Ca^{+2}/ PKC signaling pathway. In contrary, we did not observed any substantial change of the overall phosphorylated substrates of PKA and MAPK at either of the time points (Supplemental Figure S3A and S3B).

KOR regulates sperm motility and acrosome reaction in human spermatozoa

To elucidate the effect of the treatment with the KOR specific ligand on the physiology of human spermatozoa, we analyzed the role of this receptor in sperm motility, capacitation and acrosome reaction. For that purpose we treated the human spermatozoa with 1 µM of U50488H (KOR agonist) for 1 and 60 minutes. Cell viability studies using Hoechst 33258 did not show toxicity after U50488H addition of that dose at both time points (results not shown).

To unravel the function of the kappa opioid receptor in human sperm motility, we analyzed the progressive, non-progressive, immotile and hyperactive motilities following the recommendations by the WHO (World Health Organization, 2010). As it is shown in figure 3B, the percentage of the progressive ($p<0.05$ 1 minute), non-progressive ($p<0.01$ 60 minutes) and hyperactive sperm ($p<0.01$) cells decreased at both time points, whereas the percentage of immotile cells was increased ($p<0.01$). In accordance to this result, U50488H inhibited the human sperm motility at 1 and 60 minutes.

In regard to capacitation, the phosphorylation of tyrosine residues (pTyr) is one of the most important events that occur during the sperm capacitation (Naz and Rajesh, 2004), so in order to study the role of KOR in this physiological process we analyzed the effect of U50488H on the total pTyr levels. Specifically, the kappa-opioid receptor agonist did not have an effect on the levels of tyrosine phosphorylated proteins at both timepoints suggesting that U50488H does not induce phosphotyrosine-dependent changes during the process of human sperm capacitation (Figure 3C).

To study the role of KOR in the human sperm acrosome reaction, after treating the samples with U50488H we incubated them with the anti-CD46 antibody linked to fluorescein isothiocyanate (FITC). This antibody targets the inner acrosomal membrane for detecting a complete acrosome reaction, and allows analyzing the emitted fluorescence by flow cytometry. As it is observed in Figure 3D, the agonist showed no significant effect after the U50488H addition for 1 minute. However, U50488H inhibited the acrosome reaction following 60 minute treatment ($p<0.01$). The progesterone was used as the positive control of the acrosome reaction.

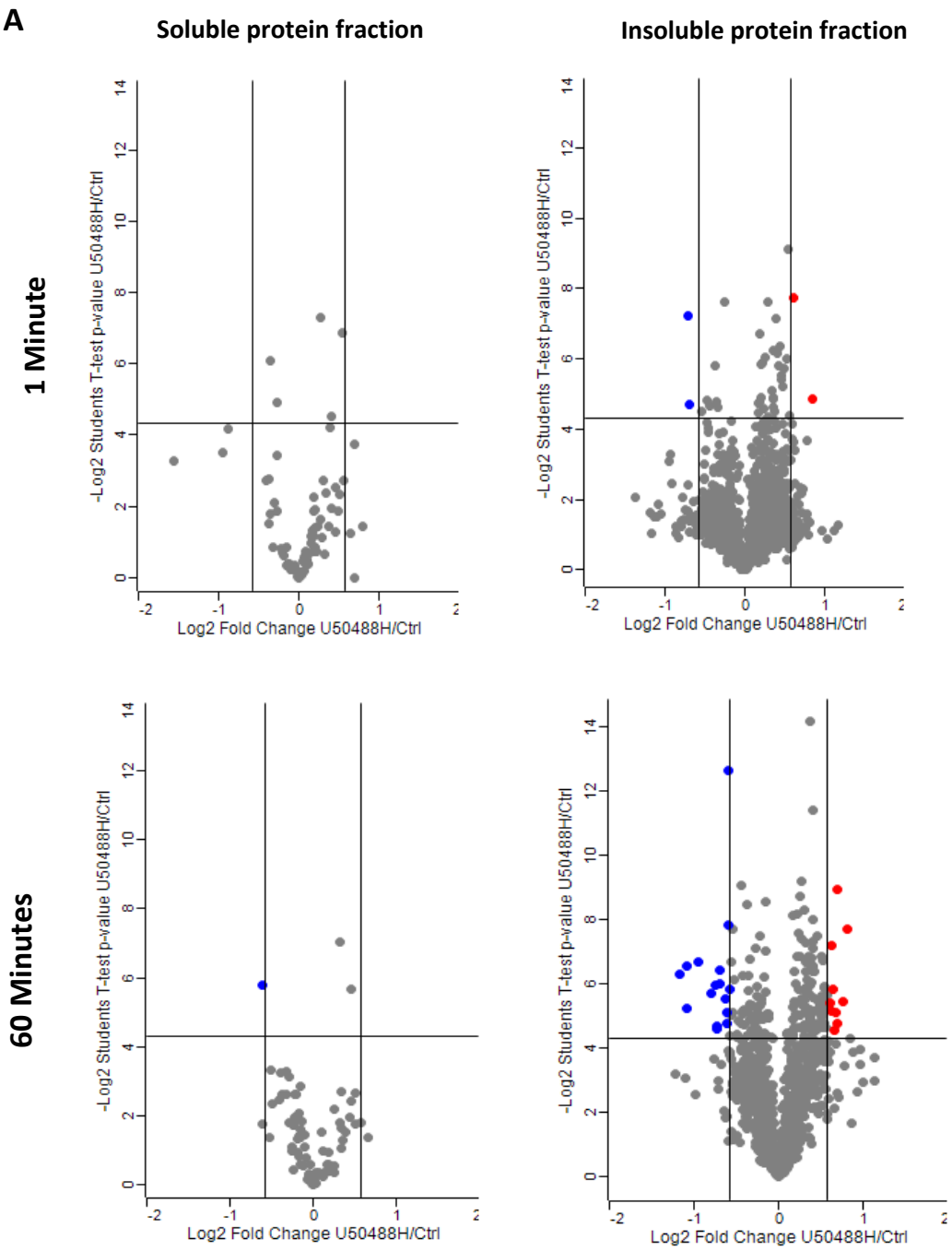


Figure 2. **Effect of U50488H in the human sperm phosphoproteome. (A)** Overall Log_2 U50488H/Control TMT fold change as a function of $–Log_2$ statistical significance of U50488H/Control (p value< 0.05) of the soluble and insoluble protein fractions after 1 and 60 minute U50488H treatment. In blue are indicated the down-regulated phosphosites by U50488H. In red are represented the U50488H-up-regulated phosphosites.

B

1 Minute	
Soluble protein fraction	
Up-regulated phosphosites	---
Down-regulated phosphosites	---
Insoluble protein fraction	
Up-regulated phosphosites	CBY3 Ser169 TMEM190 Ser127
Down-regulated phosphosites	AKAP3 Ser612 SLCO1B3 Ser152 SLCO1B3 Thr147

60 Minutes		
Soluble protein fraction		
Up-regulated phosphosites	---	
Down-regulated phosphosites	C6orf10 Ser313	
Insoluble protein fraction		
Up-regulated phosphosites	CXorf66 Ser260 SEPT12 Ser308 SPATA32 Ser93 ODF1 Thr187 FSIP2 Ser1290 FSIP2 Ser758 FSIP2 Ser1294	AKAP4 Tyr751 FAM186B S338 ODF1 S32 TMEM190 Ser140 PSMD3 Ser413 PSMA Ser243 TRIM42 Ser526
Down-regulated phosphosites	CABYR Ser464 CCDC18 Ser540 FSIP2 Ser4607 AKAP3 Ser236 AKAP4 Ser268 AKAP4 Tyr147 FSIP2 Ser882 CCDC136 Ser1074	FSIP2 Ser1224 PGK2 Ser186 PGK1 Ser153 CCDC81 Ser256 H3F3B Ser58 ODF2 Ser74 AKAP4 Thr265

Figure 2. **Effect of U50488H in the human sperm phosphoproteome. (B)** Tables containing the U50488H-regulated phosphosites following 1 and 60 minute treatment in the soluble and insoluble protein fractions. U50488H/Ctrl>1.5 in red and U50488H/Ctrl<0.67 in blue.

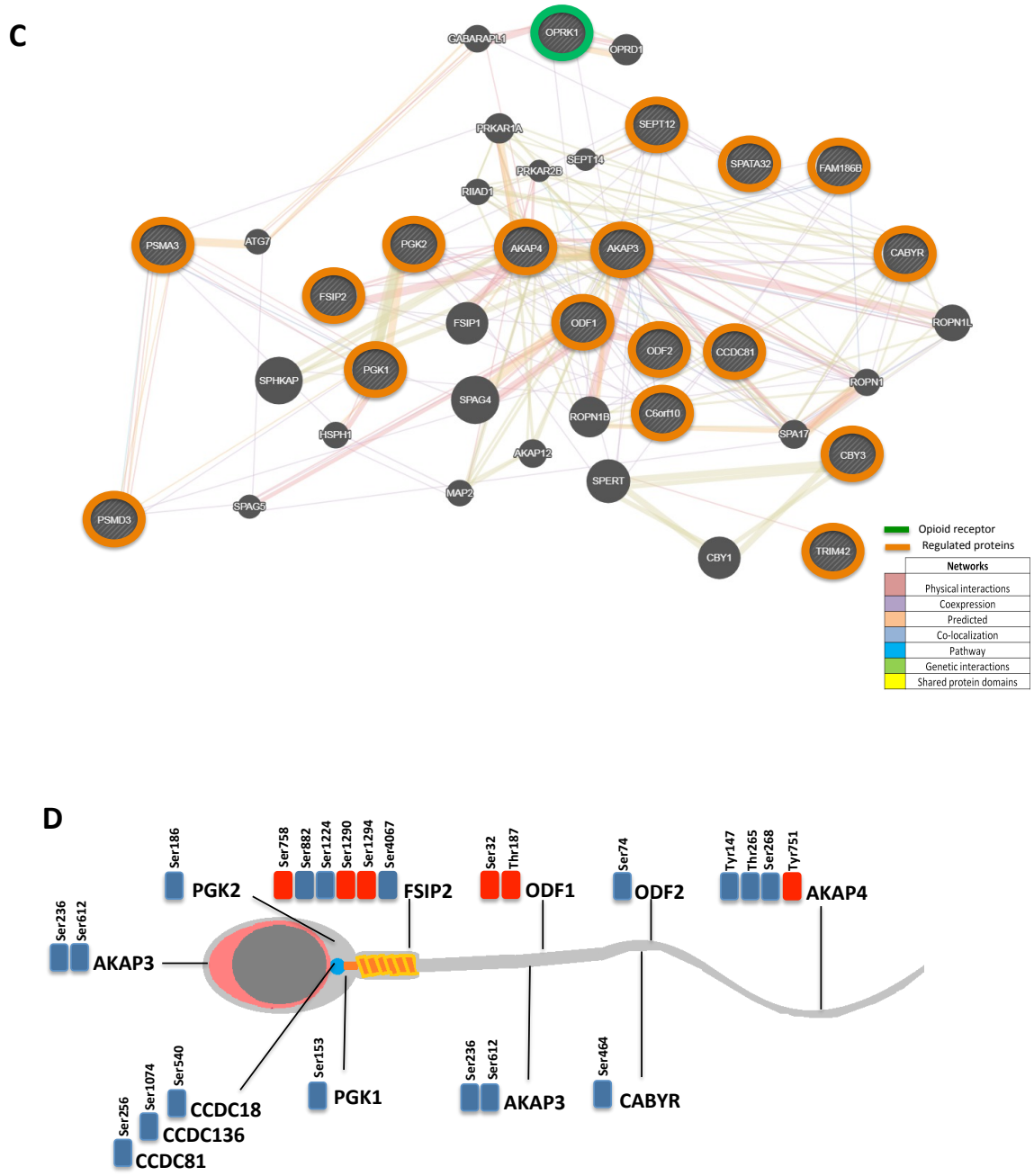


Figure 2. **Effect of U50488H in the human sperm phosphoproteome. (C)** Representation of a working model of the protein interactions belonging to the U50488H-regulated phosphosites. KOR is represented in green color while the proteins belonging to the U50488H-regulated phosphosites are colored in orange. The showing model has been constructed based on GENEMANIA (http://genemania.org/) the interactive functional association network tool. **(D)** Illustration gathering the localization of the proteins belonging to the U50488H-regulated phosphosites within the human spermatozoa. The downregulated phoshosites are represented in blue while the upregulated ones are colorized in red.

KOR regulates hyperactive sperm motility and acrosome reaction by the modulation of calcium channels in human spermatozoa

Once we observed that U50488H provoked an increase in the phosphorylated substrates of PKC and had an inhibitory effect in the hyperactive motility and acrosome reaction, we decided to analyze deeper the calcium signaling pathway as these two physiological processes are mainly regulated by the Ca^{+2} (Roldan and Shi, 2007; Yanagimachi, 1994). For that, we co-incubated both U50488H with different activators and inhibitors of diverse key proteins in the calcium cascade, for 1 and 60 minutes. We used the Mibefradil as a calcium channel activator, NNC55-0395 as the CatSper sperm specific calcium channel selective inhibitor and U73122 as the phospholipase C inhibitor.

Regarding the motility assays, at 1 minute treatment U50488H resulted in inhibition of the hyperactive motility being this effect persistent in all the analyzed samples (N=6) ($p<0.05$). As expected, the stimulation with 30 µM of Mibefradil for 1 minute, increased the percentage of hyperactive motile sperm cells ($p<0.01$). The co-incubation of Mibefradil with U50488H induced a statistically significant reversion of the effect as the percentage of the hyperactive spermatozoa decreased ($p<0.05$) (Figure 4A). Furthermore, 10 µM of NNC55-0395 itself, and together with U50488H had an inhibitory effect in the hyperactive motility after 1 minute stimulation ($p<0.05$ and $p<0.01$ respectively). The U73122 treatment and its co-incubation with the KOR agonist, provided similar results to the U50488H, as the percentage of cells with a hyperactive motility was reduced ($p<0.05$) (Figure 4A). According to these results, KOR may have the capacity to inhibit the hyperactive motility at 1 minute by the modulation of different calcium channels in human spermatozoa. However at longer times the inhibition of the hyperactive motility would not exclusively go through the same molecular mechanisms (Supplemental Figure S4).

In regard to acrosome reaction studies, the stimulation of spermatozoa with Mibefradil, yielded to the activation of the acrosome exocytosis ($p<0.01$). After the co-incubation of U50488H and Mibefradil, the kappa-opioid agonist was able to partially blunt the acrosome reaction induced by the activator ($p<0.05$) (Figure 4B). On the other hand, although NNC55-0395 had an inhibitory effect on the acrosome reaction, which was accentuated after the co-incubation with U50488H, these changes were not statistically significant. We observed similar results with the Phospholipase C inhibitor, U73122, as the number of the acrosome reacted spermatozoa were decreased compared to the control samples ($p<0.05$) (Figure 4B). This effect was enhanced after the co-incubation of U73122 and U50488H, as we observed a greater decrease in the percentage of the acrosome reacted sperm cells ($p<0.05$). The progesterone was used as the positive control of the acrosome reaction (Figure 4B). Considering these results, the U50488H may inhibit the acrosome reaction at 60 minutes, by blocking calcium channels and PLC and would not involve other canonical and non-canonical mechanisms (Supplemental Figure S3A and S3B).

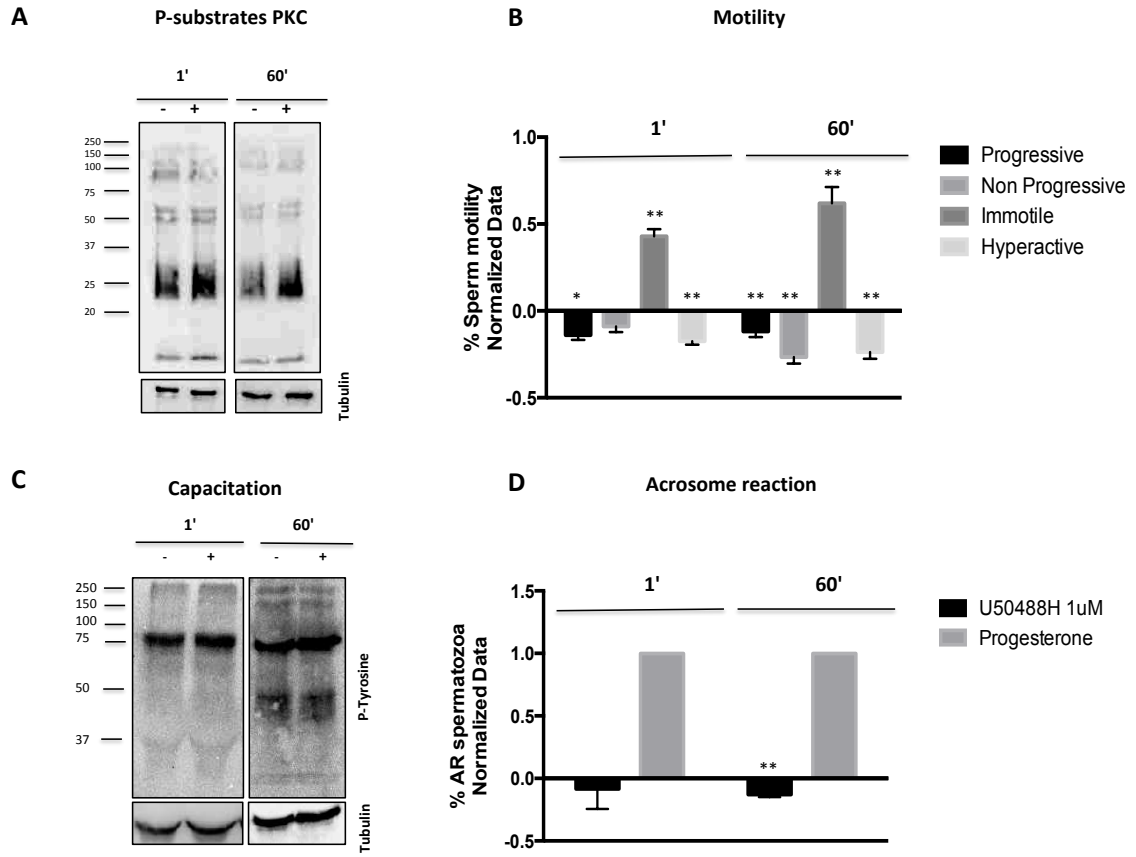


Figure 3. **Role of kappa-opioid receptor in human sperm physiology.** Effect of 1 and 60 minute U50488H (1 µM) (Kappa-opioid receptor agonist) treatments in human sperm motility, capacitation and acrosome reaction. **(A)** Immunoblotting assays showing the expression of the phosphorylated substrates of protein kinase C following 1 and 60 minute U50488H treatment. N=3. (**p<0.01 vs Control). **(B)** Motility assays using the CASA (Computer Assisted Sperm Analyzer) system for the study of 1 and 60 minutes U50488H treatment in the progressive, non-progressive, immotile and hyperactive motilities. X axis shows the motility types measured in the study and the Y axis respresents the normalized data of the % of motile spermatozoa. The normalization was performed using the untreated samples (Control). N=8. (*p<0.05 and **p<0.01 vs Control). **(C)** Immunoblotting assays for the study of U50488H in human sperm capacitation using the anti-pTyr antibody. **(D)** Flow citometry experiments for the study of U50488H in the human sperm acrosome reaction. X axis shows the different treatments used in the study and the Y axis represents the normalized data of the % acrosome reacted spermatozoa. The normalization was performed using the untreated samples and the acrosome reacted samples. The progesterone was used as the positive control of the acrosome reaction. N=7. (**p<0.01 vs Control) AR: acrosome reaction. P-subtrates PKC: Phosphorylated substrates of PKC.

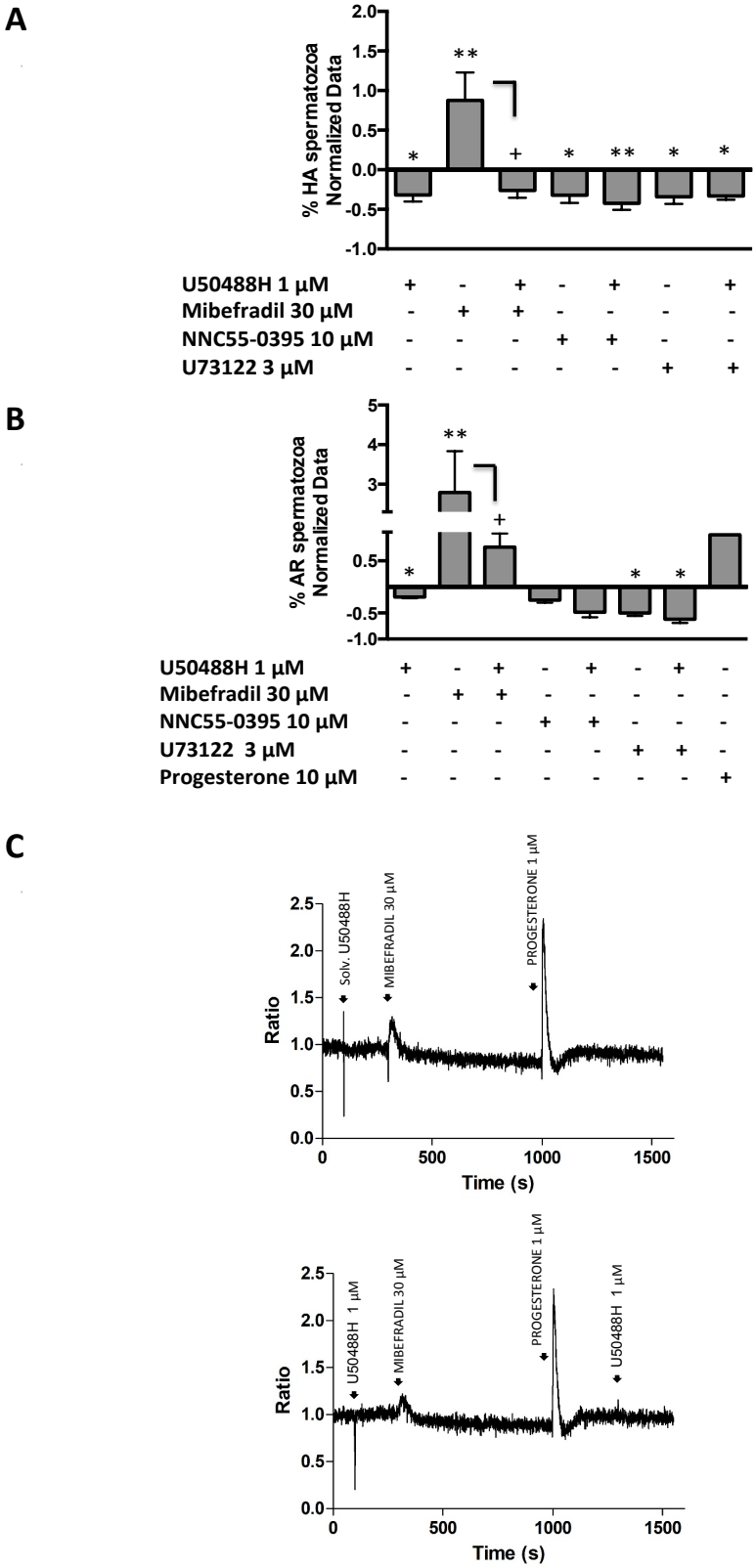


Figure 4. **Study of the hyperactive motility and acrosome reaction calcium downstream KOR in human spermatozoa. (A)** Study of the hyperactive motility by 1 minute co-incubation of U50488H (1 µM) and: NNC-55-0395 (10 µM), the Catsper inhibitor; Mibefradil (30 µM), a calcium channel activator, and U73122 (3 µM), the PLC inhibitor. X axis shows the different treatments used for this study and the Y axis represents the normalized data of the % of motile spermatozoa. The normalization was performed

using the untreated samples. N=6. (*p<0.05 and **p<0.01 vs Control; +p<0.05 vs Mibefradil). **(B)** Study of the acrosome reaction by the 60 minute co-incubation of U50488H (1 µM) and: NNC-55-0395 (10 µM), Mibefradil (30 µM) and U733122 (3 µM). X axis shows the different treatments used for this study and the Y axis represents the normalized data of the % of acrosome reacted spermatozoa. The normalization was performed using the untreated samples and the acrosome reacted samples. The progesterone was used as the positive control of the acrosome reaction. N=7. (*p<0.05 and **p<0.01 vs Control; +p<0.05 vs Mibefradil). **(C)** Intracellular free Ca^{+2} measurements in human sperm cells loaded with Fura-2 in response to the solvent of U50488H (H_2O) (control samples) and U50488H (1 µM). Subsequent addition of 30 µM Mibefradil as a calcium channel activator and 1 µM progesterone to the same sperm aliquots. The progesterone caused a typical biphasic $[Ca^{+2}]_i$ progesterone response. Calibration of $[Ca^{+2}]_i$ was achieved adding Triton X-100 (TX), to obtain the maximal response, followed by addition of EGTA to obtain the minimal response. N=5. AR= Acrosome reacted. HA= Hyperactive

According to our results, as the KOR agonist might regulate the sperm physiology by the blocking of calcium channels, we analyzed if U50488H provoked changes in the intracellular free calcium concentration. As we expected, we did not report any intracellular Ca^{+2} changes in Fura-2-loaded human sperm cells after the 1 µM U50488H treatment (Figure 4C). When we added the Mibefradil at high doses, we observed an increase in the $[Ca^{+2}]_i$ levels in the spermatozoa treated with the solvent of U50488H (H_2O) (control samples). However, compared to the control samples the U50488H was able to produce a partial reversion in the levels of $[Ca^{+2}]_i$ induced by the Mibefradil (Figure 4C). As a positive control, we added 1 µM of progesterone to the same sperm samples and this caused the typical biphasic $[Ca^{+2}]_i$ response, consisting in a rapid transient peak followed by the decay of the intracellular calcium levels slightly above basal and a lower sustained plateau phase. Moreover, U50488H was not able to modify the progesterone-induced intracellular calcium response (Figure 4C). Based on the results we obtained, the U50488H influenced the level of intracellular calcium in human spermatozoa compared to Mibefradil, demonstrating the capacity of the agonist in the modulation of calcium channels.

DISCUSSION

Identification of novel phosphosites in human spermatozoa

Proteomic studies based on chemical labeling strategies gave rise to the identification of 5109 sperm proteins of which 5070 were quantified. Although Amaral et al (Amaral et al., 2014) have presented the most complete catalogue of human sperm proteins with 6198 reported protein IDs, in the present study we have identified over the 68% of the total sperm proteome since the human spermatozoa is estimated to be composed by 7500 different proteins (Amaral et al., 2014). In regard to proteins linked to GPCR, we found proteins associated to different signaling pathways. Concretely, we identified proteins belonging to the canonical and non-canonical signaling pathways as other researchers have previously described. GPCR activation in somatic cells mainly involves the induction of the Guanine nucleotide-binding proteins for the subsequent activation of the canonical signaling pathways (Neves et al., 2002). In the present study we identified via proteomics the presence of the $G\alpha_s$, $G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_i$, $G\alpha_o$ and G_β isoforms in human spermatozoa. Although the characterization of $G\alpha_s$ and $G\alpha_q$ was previously reported (Merlet et al, 1999;

Spehr et al., 2004), we have described for the first time the existence of other isoforms in human spermatozoa suggesting that these proteins may also be involved in the regulation of different signaling pathways downstream of GPCRs in spermatozoa.

Regarding the cAMP-dependent signaling pathway, we found the sperm-specific soluble isoform of the adenylate cyclase (SACY or the adenylate cyclase type 10) whereas we did not detect the transmembrane adenylate cyclase (tmAC) in human spermatozoa by our approach. This subject has been a matter of controversy during the last twenty years as although there is one study describing a tmAC in human spermatozoa (Spehr et al., 2004), different researchers have not been able to unambiguously identify it (Aitken et al., 1984; Rojas et al., 1992; Strünker et al., 2011). This issue reinforces the hypothesis of different authors who suggest the involvement of the soluble form of the protein in the regulation of sperm fertility (Jaiswal and Conti, 2003).
In reference to the calcium-signaling pathway, interestingly we identified different isoforms of the phosphoinositide phospholipase C (PLC), such as delta and zeta (PLCD4 and PLCZ1, respectively), and both the calcium-dependent (alpha and delta) and independent (iota and zeta) protein kinase C (PKC), as previously has been described (Rotem et al., 1992, 1990; Yoon et al., 2008). According to different reports, PKC iota and zeta could be involved in the regulation of sperm fertility via a specific signaling cascade that does not involve calcium (Aitken et al., 1984; Jaiswal and Conti, 2003; Rotem et al., 1992, 1990). At the same time, we also reported by proteomic approaches the presence of different participants of the non-canonical or G-protein independent signaling pathway in human spermatozoa, like the β-arrestin, the GPCR kinases (GRKs) or the ERK1/2 (Almog et al., 2008; de Lamirande and Gagnon, 2002; Kang et al., 2005; Neuhaus, 2006).

On the other hand, our phosphoproteomic screen allowed us to uncover over 3500 phosphosites coming from 1332 proteins. The identified phosphoproteins in the present work are mainly involved in cilium and flagellum-dependent movement, nuclear pore organization or sperm-egg recognition, among other. According to this, Wang et al in 2015 (Wang et al., 2015), previously identified 3303 phosphosites belonging to 965 proteins. Comparing our data with the largest description of the sperm phosphoproteome reported so far (Wang et al., 2015), we found 2692 novel phosphosites being 835 common in both studies. From these 2692 phosphosites, 535 were previously reported in humans in the PhosphoSitePlus database. Taking all these into account, it is worth highlighting that the present study contains the total of 2157 previously not reported phosphosites in humans and specifically in human spermatozoa.

U50448H-induced changes in the phosphoproteome of human spermatozoa

In order to understand the initial steps of the signaling cascades stimulated by KOR, sperm samples were treated with a KOR selective-agonist U50488H (Taub et al., 1991) for 1 minute. Our quantitative phosphoproteomic analyses showed decrease in the phosphorylation levels of four proteins, CBY3, TMEM190, SLCO1B3

and AKAP3, after treatment. Currently, no attributable function has been determined for CBY3, TMEM190 and SLCO3. However, AKAP3 is a scaffold protein, synthesized in round sperm cells and involved in the formation of the basic structure of the fibrous sheath (Brown et al., 2003). It is localized in the main segments of the sperm flagella and the acrosome region of sperm heads, and different studies have suggested its involvement in the regulation of sperm motility, capacitation and acrosome reaction acting as a scaffold protein that assembles multiprotein signal complexes (Brown et al., 2003; Vizel et al., 2015). Although AKAP3 is known to be tyrosine phosphorylated during human sperm capacitation, the agonist showed to decrease the phosphorylation levels of AKAP3 in the Ser612, a phosphosite previously described by Ficarro et al (Ficarro et al., 2003). In 2004, Rieger-Chu et al (Rieger et al., 2004) reported the co-expression of both KOR and AKAP3 in different cell systems, which suggest that AKAP3 could be involved in different molecular processes induced by KOR in human spermatozoa.

Due to the fact that spermatozoa are transcriptionally and translationally inactive (Amaral et al., 2014; Castillo et al., 2014; Miller, 1992), we also aimed to elucidate the signaling pathways underlying KOR activation at longer time point, 60 minutes. Interestingly, U50488H induced changes in the phosphorylation levels of 29 phosphosites, belonging to 20 different proteins of which 13 of them are sperm-specific. Among the identified phosphosites we considered 19 as novel, as there are no previous studies describing their presence.

The KOR specific-agonist changed the phosphorylation levels of novel phosphosites belonging to different proteins such as CABYR, AKAP3 and AKAP4 that have different localization within the cell (Figure 2D). AKAPs family is one of the main components of sperm fibrous sheath, and it is involved in sperm fertility by sequestering PKA as it has a putative motif for binding cAMP-dependent protein kinase A (RIIa) (Michel and Scott, 2002; Vizel et al., 2015). In fact, this protein family may serve as a platform for the integration of cAMP and other signaling pathways by the binding with other protein kinases, protein phosphatases, ion channels and small GTP binding proteins (Skroblin et al., 2010). This is consistent with the fact that AKAP4 is an ERK1/2 substrate and serves as a regulator between the cAMP/PKA and the PKC/ERK1/2 signaling pathways in human spermatozoa to regulate capacitation and acrosome reaction (Ben-Navi et al., 2016). Concretely, these researchers described that ERK2 is the responsible of the *in vitro* phosphorylation of the Thr265 of AKAP4, which in our study presents a decrease in the phosphorylation levels after the ligand addition for 60 minutes. This suggests that AKAP4 could be related to the PKC/ERK1/2 signaling pathway downstream KOR. In fact, we observed that U50488H induced an increase in the phosphorylated substrates of PKC at both time points.

AKAP3 is another component of the AKAPs family, which displayed changes in the phosphorylation level at a specific phosphosite at 60 minute U50488H treatment. As already mentioned, there are evidences supporting the coexpression of both KOR and AKAP3 in humans (Rieger et al., 2004), suggesting that in human spermatozoa this GPCR could have a regulatory function in the phosphorylation of the anchoring protein. On the other

hand, there are evidences proving the interaction and coexpression of AKAP3 and CABYR in human spermatozoa (Li et al., 2011; Mallon et al., 2013). CABYR is localized throughout the entire length of the midpiece and acts as an important component belonging to the calcium-signaling pathway during the hyperactivation and capacitation. Interestingly, CABYR possesses putative motifs for self-assembly and for binding RIIa and AKAPs (Li et al., 2011), and its dephosphorylation abolishes its capacity to bind to calcium (Naaby-Hansen et al., 2002). As mentioned before, different studies suggest the role of AKAPs serving as a scaffold for integrating cAMP/PKA, Rho and calcium signaling (Skroblin et al., 2010) reinforcing that AKAP3 and CABYR could be the linkers between different transduction cascades.

Associated to this, the KOR agonist did not have effect in the phosphorylated substrates of PKA at both timepoints. In somatic cells, the PKA is activated by the cAMP second messenger (Tasken, 2004) which is usually synthetized by the tmAC (Simonds, 1999). In human spermatozoa, however, the cAMP is mainly synthetized by SACY activated by HCO_3^- (Kaupp and Strünker, 2017; Wang et al., 2007). Functional analyses using tmAC and sAC inhibitors also revealed that the KOR-induced acrosome reaction inhibition is not dependent of both enzymes. This is consistent with the fact that many GPCR ligands fail to increase the cAMP levels suggesting that GPCRs do not stimulate the PKA signaling via the typical transduction pathway in human spermatozoa (Brenker et al., 2012). Moreover, the presence of the tmAC in human spermatozoa is still under doubt since forskolin activation does not involve an increase of cAMP levels (Aitken et al., 1984; Jaiswal and Conti, 2003; Strünker et al., 2011). Although several studies have described the presence of tmAC in mouse spermatozoa (Chien et al., 2010; Livera et al., 2005), we did not succeed in the identification of the tmAC in our proteomic analyses and its presence in human spermatozoa is not reported yet (Brenker et al., 2012; Rojas et al., 1992; Strünker et al., 2011).

Regarding the non-canonical signaling pathways, U50488H did not show any effect on the phosphorylated substrates of MAPK at both timepoints. Although the opioids have been described to promote activation of the MAPK signaling pathway in different cell systems (New and Wong, 2002; Zhang et al., 1999) the KOR selective-agonist did not activate this cascade at the selected time of stimulation. It is well studied that the non-canonical signaling pathway involves the GPCR phosphorylation mediated by GRKs to promote the receptor desensitization and internalization with the support of β-arrestin protein family (Luttrell and Lefkowitz, 2002; Magalhaes et al., 2012). This proteins act as scaffolds activating the MAPKs (Luttrell and Lefkowitz, 2002; Magalhaes et al., 2012) and recruiting the cAMP phosphodiesterases into a complex with the activated receptor, where they are placed to degrade cAMP (Perry, 2002). Functional studies using U50488H together with GRKs and phosphodiesterases inhibitors fail to induce significant changes in the acrosome reaction suggesting that KOR does not activate the non-canonical transduction-signaling pathway in human spermatozoa.

Although our phosphoproteomics data did not result in a direct signaling pathway downstream KOR, we discovered that the most of the proteins having U50488H-regulated phosphosites were related to each other and could be connected indirectly with KOR signaling. ODF1 and 2 are sperm cytoskeletal structures that surround the axoneme in the midpiece and principal piece of sperm tail (Azizi and Ghafoun-Fard, 2017; Henkel et al., 1992) and their co-expression with KOR has been previously described in cancer (Bild et al., 2006). The CCDC family (CCDC81, CCDC136 and CCDC18) comprises the main components of the sperm centrosomes (Firat-Karalar et al., 2014) and the FSIP2 is a component of the fibrous sheath (Brown et al., 2003). All these proteins compose the fibrous sheath in human spermatozoa and may be involved in the regulation of the sperm fertility, as the sperm motility.

Finally, the opioid system is known to be involved in regulating carbohydrate metabolism and immune system functions in the periphery (Böttcher et al., 2017; Eyvazzadeh et al., 2009). This is consistent with the phosphorylation changes we observe in the metabolic enzymes like PGK1 and PGK2, which are implicated in an important step of the glycolysis (Dolcetta et al., 1986), and hence in sperm fertility (Danshina et al., 2010).
Although there are numerous papers describing the involvement of these proteins in the sperm function, we have not found studies describing the role of the different phosphorylations in none of the proteins. Therefore, further studies are needed to have a better comprehension of the implication of these phosphorylation events in sperm physiology.

KOR regulates human sperm fertility by inhibiting the hyperactive motility and acrosome reaction through phosphorylation changes in sperm-specific proteins

Classically, the agonist stimulation of the opioid receptors can inhibit the cyclic adenosine monophosphate (cAMP) production (Al-Hasani and Bruchas, 2011), modulate the calcium and potassium channels (Al-Hasani and Bruchas, 2011) and recruit alternate signal transduction cascades like the MAPKs (Lefkowitz and Shenoy, 2005). Although, opioid receptors are known to be critical in the modulation of pain behavior and antinociception in the central nervous system (Böttcher et al., 2017), their presence in human spermatozoa (Agirregoitia et al., 2006) has leaded us to think that may have an important function in the regulation of sperm fertility. In this paper we describe for the first time the role of KOR in the hyperactive motility and acrosome reaction in human spermatozoa, via changes in the phosphorylation of sperm-specific proteins.

Taking into account the phosphoproteomic data and the functional studies, we suggested that at short exposure times the agonist is able to regulate the human sperm hyperactive motility mainly through the calcium ion channel modulation and fast responses. At the same time, the ligand could induce phosphorylation changes in few proteins like AKAP3, which could be involved in the first steps of the inhibition of sperm motility. It is known that, AKAPs are anchor cAMP-dependent protein kinases (PKAs) presented in different subcellular

regions where they phosphorylate nearby proteins in response to cAMP signaling and often form complexes with other components of different signaling pathways (Michel and Scott, 2002; Skroblin et al., 2010). In human sperm cells AKAPs family may be a link to calcium signaling pathway as they have the capacity to bind to proteins like CABYR and connect to PKC pathway to regulate sperm fertility (Naaby-Hansen et al., 2002; Ben-Navi et al., 2016). This is consistent with our results at longer exposures times, in which KOR activation induces changes in the phosphorylation of PKC substrates, in sperm-specific proteins such as AKAP3, AKAP4 and CABYR and other sperm-specific proteins involved in sperm motility and acrosome reaction like ODFs, FSIP2 and CCDCs necessary to provoke the physiological effect by U50488H (Figure 2D).

In conclusion, the present data allows us to have a better comprehension of the phosphorylation changes in sperm-specific proteins underlying Kappa-opioid receptor by which the human spermatozoa modulate the hyperactive motility and acrosome reaction. These finding may suggest that human spermatozoa possess unique features in the molecular mechanisms downstream GPCRs which could be key regulators of sperm fertility. Improved knowledge of these specific processes may contribute in the development of useful biochemical tools for the diagnosis and treatment of male infertility. Moreover, it could also represent an innovative opportunity for reproductive management, for either enhancing the probability of fertilization or reducing it through the development of novel target contraceptives.

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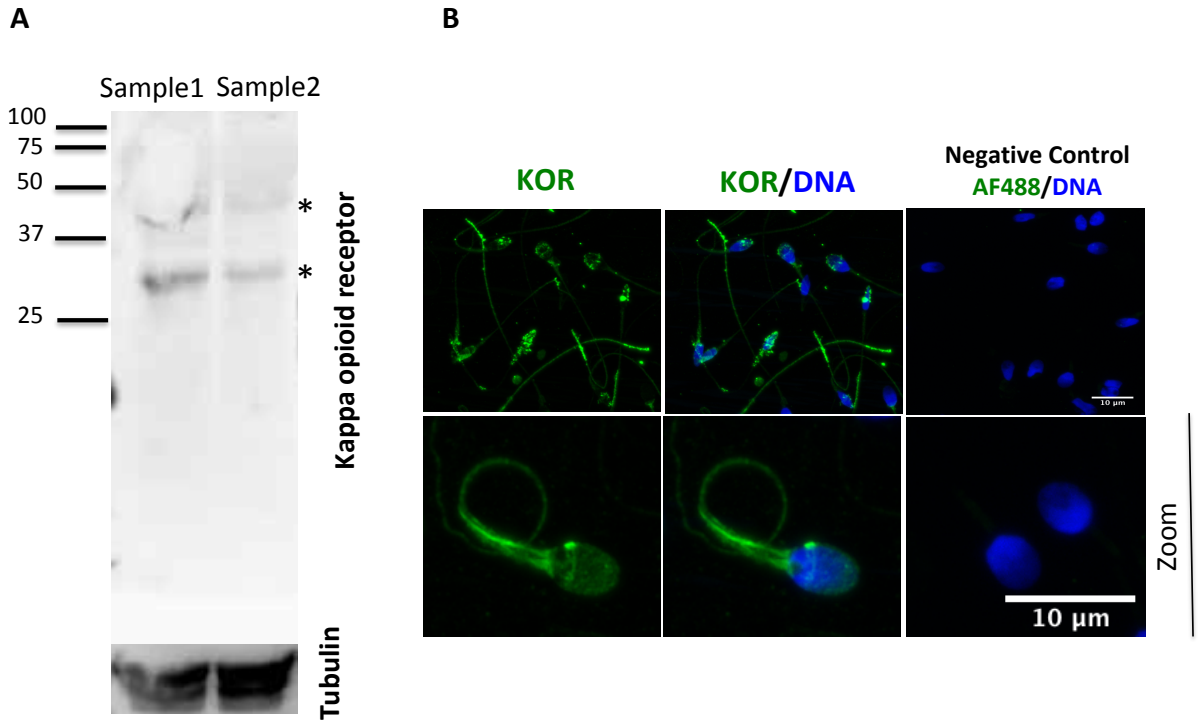
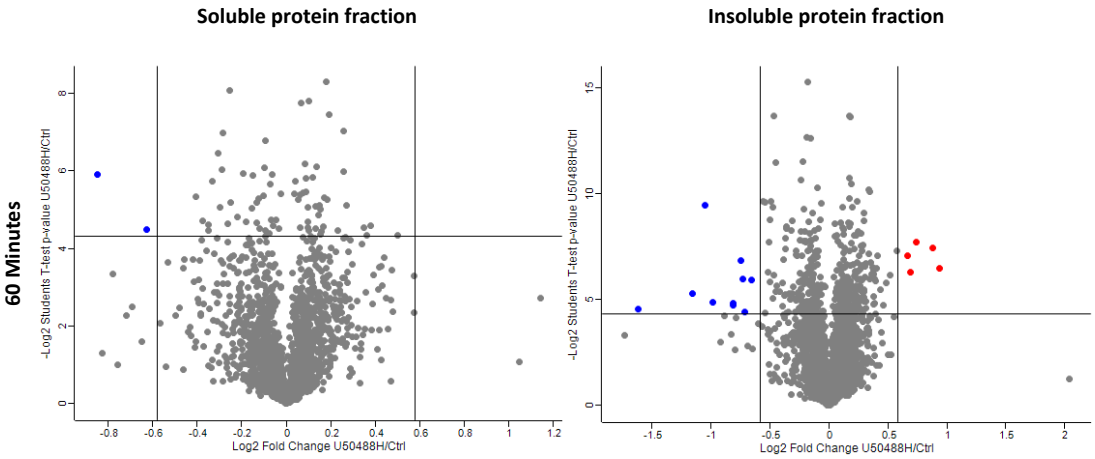
Supplemental Figures



Supplemental Figure S1. KOR expression in human spermatozoa **(A)** by Western blotting showing immunoreactivity at around 45 and 35 kDa. * indicate the different bands of KOR. **(B)** by immunofluorescence, in the sperm head, middle/postacrosomal region and tail. The nuclei are stained with Hoescht and are represented in blue (N=3). Scale bar 10 µm.

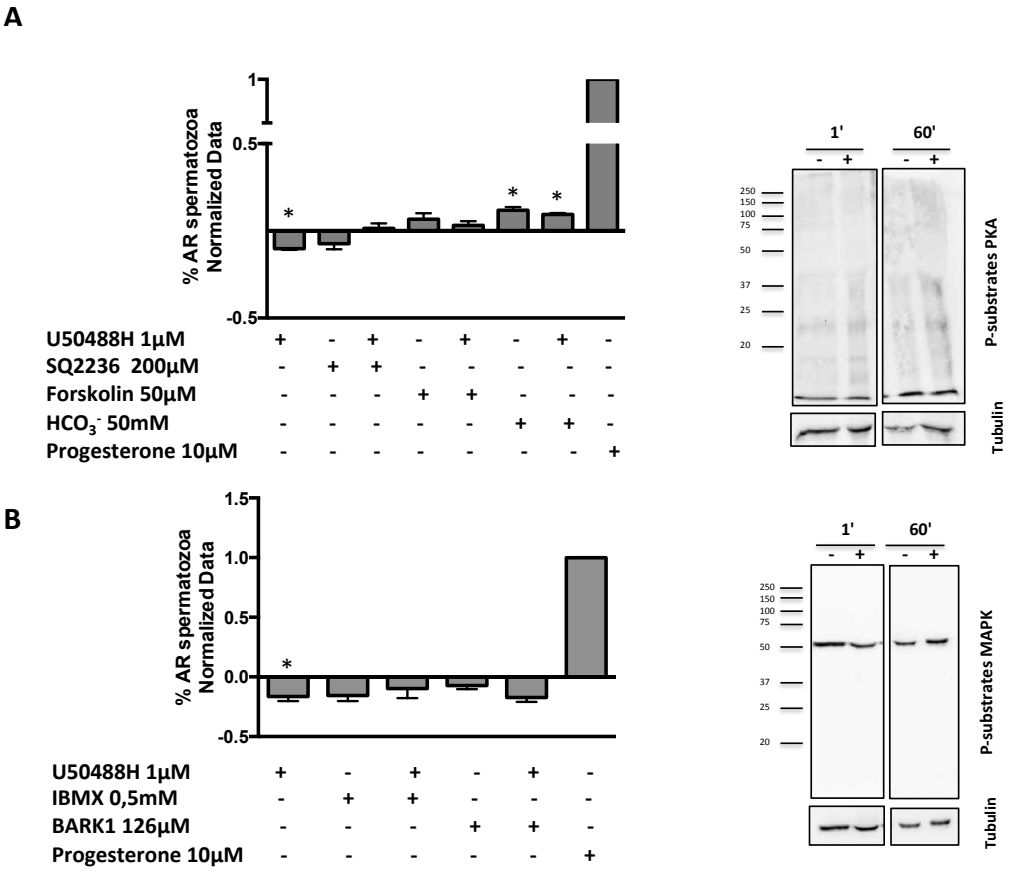
A



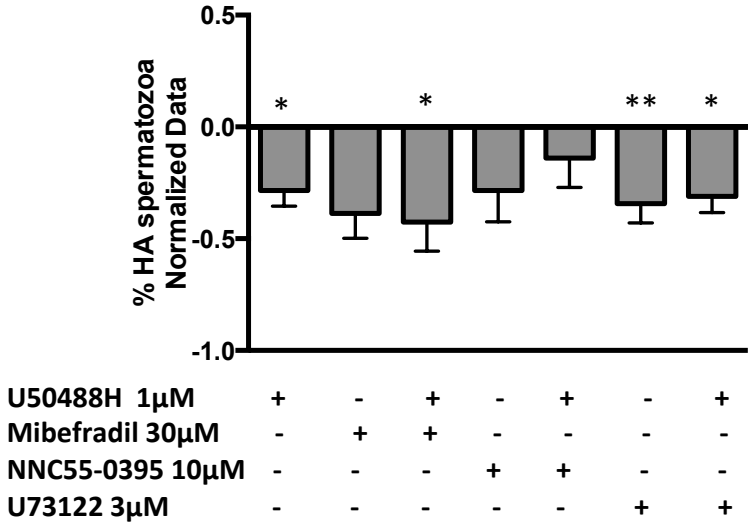
B

60 Minutes	
Soluble protein fraction	
Up-regulated proteins	---
Down-regulated proteins	KRT18 PPPGR3
Insoluble protein fraction	
Up-regulated proteins	GPRIN1 SENA3F IPO11 USP6NL FGL2
Down-regulated proteins	ENTPD3 C4orf17 DDX50 C5ORF49 TTC37 ARL14EP FBLN2 TBC1D1 SOGA1

Supplemental Figure S2. Effect of U50488H in the human sperm proteome. (A) Overall Log_2 U50488H/Control TMT fold change as a functional of –Log statistical significance of U50488H/Control (p value< 0.05) od the soluble and insoluble and insoluble protein fractions after 60 minute U50488H treatment. In blue are indicated the down-regulated proteins by U50488H. In red are represented the U50488H-up-regulated proteins. **(B)** Table containing the U50488H-regulated phosphosites following 60 minute treatment in the soluble and insoluble protein fractions. U50488H/Ctrl>1.5 in red and U50488H/Ctrl<0.67 in blue.



Supplemental Figure S3. Study the of the acrosome reaction downstream KOR in human spermatozoa. **(A)** Study of the acrosome reaction by 60 minute co-incubation of U50488H (1 µM) and: SQ2236 (200 µM), the tmAC inhibitor; Forskolin (50 µM), the tmAC activator, and HCO3-(50mM), the SACY activator. X axis shows the different treatments used for this study and the Y axis represents the normalized data of the % of acrosome reacted spermatozoa. The normalization was performed using the untreated samples and the acrosome reacted samples. N=6. (*p<0.05 vs Control). Immunoblotting assays showing the expression of the phosphorylated substrates of protein kinase A following 1 and 60 minute U50488H treatment **(B)** Study of the acrosome reaction by 60 minute co-incubation of U50488H (1 µM) and: IBMX (0,5mM), the phosphodiesterases inhibitor, and BARK1(126 µM), the GRK2 inhibitor. X axis shows the different treatments used for this study and the Y axis represents the normalized data of the % of acrosome reacted spermatozoa. The normalization was performed using the untreated samples and the acrosome reacted samples. N=6. (*p<0.05 vs Control). N=6. Immunoblotting assays showing the expression of the phosphorylated substrates of MAP kinases following 1 and 60 minute U50488H treatment. tmAC: Transmembrane Adenylate cyclase. SACY: soluble adenylate cyclase.

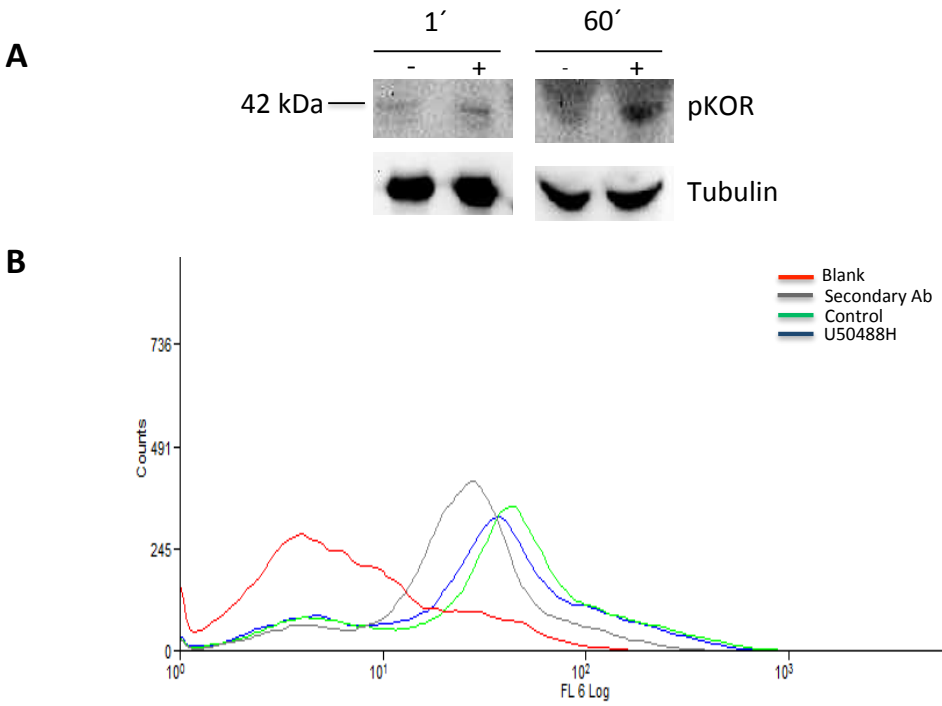


Supplemental Figure S4.Role of KOR in human sperm hyperactive motility at 60 minutes. Study of the hyperactive motility by 60 minute co-incubation of U50488H (1uM) and: NNC-55-0395 (10 µM), the Catsper inhibitor; Mibefradil (30 µM), a calcium channel activator, and U73122 (3 µM), the PLC inhibitor. X axis shows the different treatments used for this study and the Y axis represents the normalized data of the % of hyperactive spermatozoa. The normalization was performed using the untreated samples and the acrosome reacted samples. N=8. (*p<0.05 and **p<0.01 vs Control.

Eranskinak
Appendixes

I.Eranskina. U50488H agonistaren eragina KOR hartzailearen fosforilazio eta barneraketan

U50488H-k kappa-opioide hartzailearen aktibazioan duen eragina aztertzeko, horren fosforilazioa eta barneraketa ikasi ziren. Horretarako, espermatozoideak agonistarekin tratatu ziren 1 eta 60 minutuz. Hartzailearen fosforilazioaren ikasketarako Western Blotting teknika erabili zen eta hain zuzen ere, kappa-opioide hartzailearen fosforilazioa detektatzen duen antigorputza erabili zen (PA5-40216, Thermo Scientific). Hartzailearen barneraketaren analisirako ordea, fluxuzko zitometria erabili zen KOR-ren aurkako antigorputz espezifikoa erabiliz.

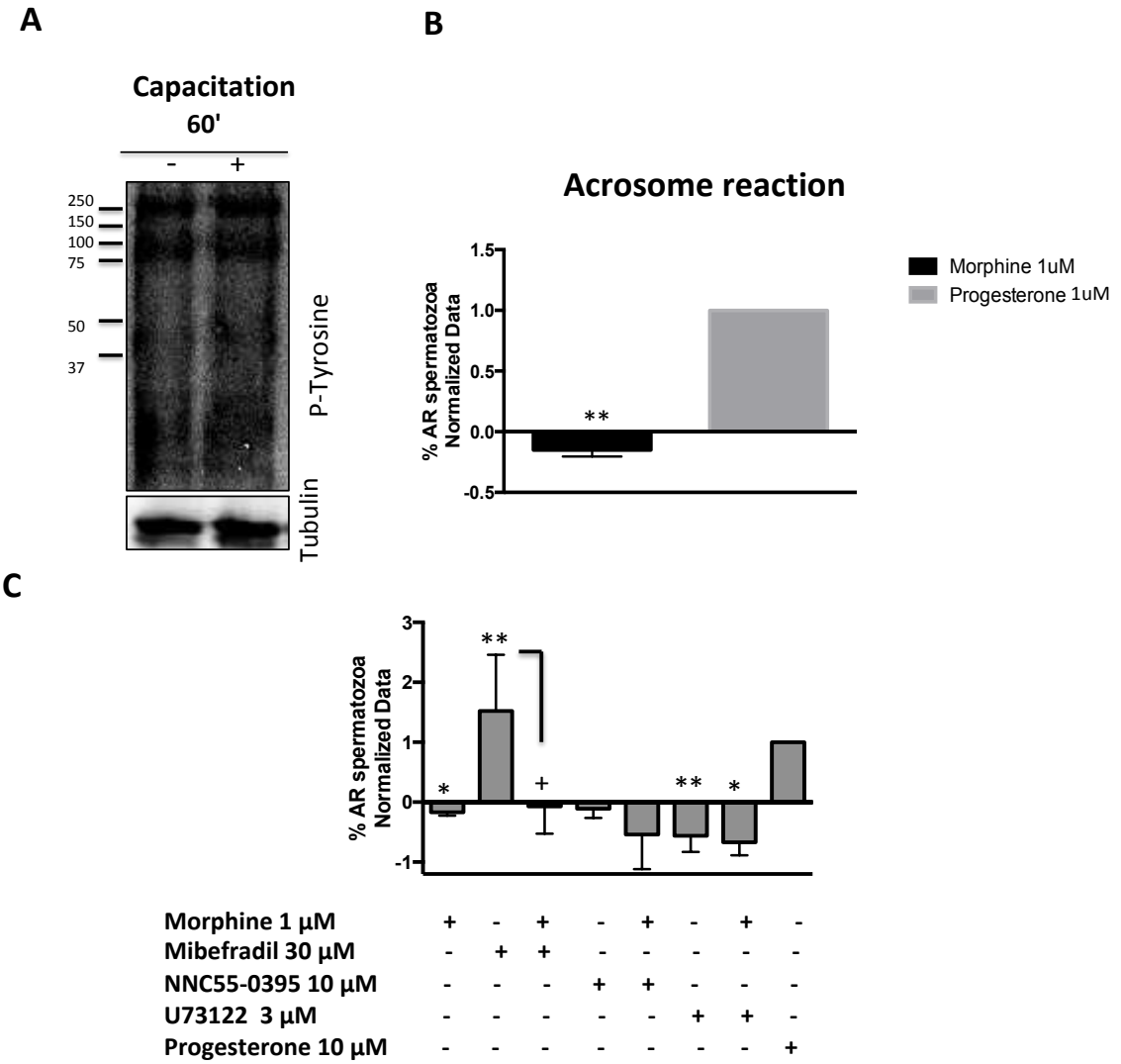


I.Eranskina. Kappa-opioide hartzailearen fosforilazioa eta barneraketa U50488H erabiliz. (A) Kappa-opioide hartzailearen fosforilazioaren ikasketa 1 eta 60 minutuz U50488H, kappa-opioide hartzailearen agonista espezifikoa, erabiliz. **(B)** Kappa-opioide hartzailearen barneraketaren efektuaren ikasketa 60 minutuz U50488H, kappa- hartzailearen agonista espezifikoa, erabiliz. Gorriz (Blank): espermatozoideak; Grisez: antigorputz sekundarioaren kontrol negatiboa; Urdinez: Kontrola; Berdez: U50488H. N=3. X ardatzak fluoroforoaren seinalearen balioari egiten dio erreferentzia eta Y ardatzak fluoroforoaren seinale horretarako positiboak diren zelula kopuruari buruzko informazioa ematen du.

1A irudian ikus daitekeenez, U50488H-k kappa-opioide hartzailearen fosforilazioa eragiten du 1 eta 60 minututan, azken hau nabarmenagoa izanik. Horrez gain, espermatozoideak agonistarekin 60 minutuz tratatu eta gero, hauen mintz zitoplasmatikoan agerian zeuden KOR hartzaileen %-a zenbatu zen eta tratatu gabeko laginekin konparatu zen. 1B irudian antzeman daitekeenez, KOR-en agonista espezifikoak, 60 minutura hartzailearen barneraketa eragiten du. Emaitza hauek kontuan hartuta, pentsa daiteke U50488H-k kappa-opioide hartzailea aktiba dezakeela hartzailearen fosforilazioa eta barneraketa sustatuz giza espermatozoideetan.

II.Eranskina. Mu-opioide hartzailearen funtzioa giza espermatozoideeen ahalmen ugalkorrean

Mu-opioide hartzailea giza espermatozoideetan adierazten den beste hartzaile opioide bat da. Agirregoitia eta lankideek 2006.urtean giza espermatozoideen mugikortasunean eragin inhibitzailea zuela ikusi bazuten ere, ez zuten kapazitazio eta erreakzio akrosomikoari buruzko ikasketarik egin. Hori dela eta atal honetan MOR hartzaileak giza espermatozoideen kapazitazioan eta erreakzio akrosomikoan duen funtzioa aztertu genuen. Horrez gain, MOR hartzailearen azpitik aktibatzen diren seinaliztapen bidezidorrak aztertu genituen KOR hartzailearen azpitik induzitzen direnekin konparatzeko.



II. Eranskina. Mu-opioide hartzailearen funtzioa giza espermatozoideen kapazitazioan eta erreakzio akrosomikoan. Morfinak (1 µM), 60 minutuan giza espermatozoideen **(A)** fosfotirosina mailetan (Ptyr) duen eragina Westen Blot bidez (N=3) eta **(B)** erreakzio akrosomikoan duen efektuaren azterketa fluxuzko zitometria bidez (N=10) (**p<0.01 vs Kontrola, t- student estatistikoa) **(C)** Fluxuzko zitometria bidezko CD46 proteinarako positiboak diren ehunekoaren azterketa giza espermatozoideen erreakzio akrosomikoaren ikasketarako, morfina (1 µM) eta kaltzio seinalizazio bidezidorraren proteina ituen aktibatzaile/inhibitzaile desberdinak 60 minutuz erabiliz: NNC-55-0395 (10 µM), Mibefradil (30 µM) eta U733122 (3µM) (N=7) (*p<0.05 and **p<0.01 vs Kontrola; +p<0.05 vs Mibefradil; ANOVA estatistikoa).

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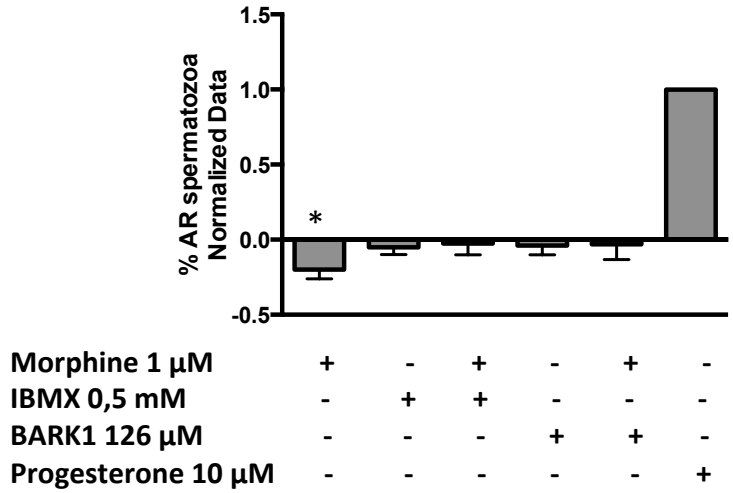

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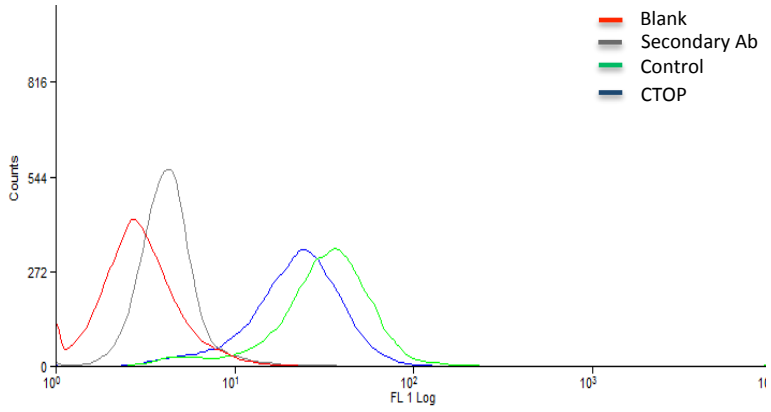


II. Eranskina. Mu-opioide hartzailearen funtzioa giza espermatozoideen ahalmen ugalkorrean. (D) Fluxuzko zitometria bidezko CD46 proteinarako positiboak diren ehunekoaren azterketa giza espermatozoideen erreakzio akrosomikoaren ikasketarako, morfina (1 µM) eta MAP kinasen seinalizazio bidezidorraren proteina ituen aktibatzaile/inhibitzaile desberdinak 60 minutuz erabiliz: IBMX (0,5 mM) eta BARK1 (126µM) (N=7) (*p<0.05 and **p<0.01 vs Kontrola; ANOVA estatistikoa). **(E)** Mu-opioide hartzailearen barneraketaren efektuaren ikasketa 60 minutuz CTOP 1 µM erabiliz. Gorriz (Blank): espermatozoideak; Grisez: antigorputz sekundarioaren kontrol negatiboa; Urdinez: Kontrola; Berdez: DPDPE. X ardatzak fluoroforoaren seinalearen balioari egiten dio erreferentzia eta Y ardatzak fluoroforoaren seinale horretarako positiboak diren zelula kopuruari buruzko informazioa ematen du. **(B)**, **(C)** eta **(D)** grafikoetarako X ardatzak esperimentuan erabilitako tratamenduei egiten dio erreferentzia eta Y ardatzak erreakzio akrosomikoa jasan duten espermatozoideen datu normalizatuen %-a adieratzen du. Normalizazioa ondorengo eran egin zen: [(Tratamendua-Kontrola)-(Progesterona-DMSO)].

Lehenengo eta behin, Mu-hartzaile opioideak (MOR) giza espermatozoideen ugalkortasunean betetzen duen funtzioa aztertu zen, kapazitazioan eta erreakzio akrosomikoan hain zuzen ere. Horretarako, Morfina (1 µM) agonista espezifikoa erabili zen eta espermatozoideak 60 minutuz tratatu ziren. Kapazitazioa ikasteko, Western Blot bidez, tirosinetan fosforilatutako proteinak (pTyr) aztertu ziren. 2A irudian ikus daitekeenez tratatutako eta tratatu gabeko espermatozoideen artean ez zen aldaketa esangarririk ikusi. Hori dela eta, morfinak 60

minutuan giza espermatozoideen kapazitazioan eraginik ez duela ondorioztatu zen. Ondoren, MOR hartzaileak giza espermatozoideen erreakzio akrosomikoan duen eragina aztertzeko espermatozoideak 60 minutuz morfinarekin tratatu ziren. 2B irudian ikus daitekeenez, morfinak espermatozoideen erreakzio akrosomikoa inhibitzen du 60 minutuan ($p<0.01$).

Morfinaren ondoriozko erreakzio akrosomikoaren inhibizioa kaltzioaren bidezko bidezidorraren bitartekoa zen aztertzeko, bidezidorraren proteina ituen aktibatzaile eta inhibitzaile desberdinak erabili ziren. 2C irudian beha daitekeenez, Mibefradil-ak (30 µM), kaltzio kanalen aktibatzaileak, espermatozoideen errekzio akrosomikoa aktibatzen du 60 minutura eta horren koinkubazioak morfinarekin efektuaren leheneratzea eragiten du ($p<0.05$). Bestalde, NNC55-0395-ak, Catsper espermatozoide-espezifikoa den kaltzio kanalaren inhibitzaileak eta horren koinkubazioak morfinarekin, erreakzio akrosomikoaren inhibizioa suertatzen du (estatistikoki ez-esanguratsua). Horrez gain, U73122-ak, phospholipasa C-aren (PLC) inhibitzaileak, erreakzio akrosomikoa pairatu duten espermatozoideen %-aren gutxitzea eragiten du ($p<0.01$). Morfinaren eta inhibitzailearen arteko koinkubazioak, inhibizioaren efektu sinergikoa sortzen du erreakzio akrosomikoan ($p<0.05$). Progesterona erreakzio akrosomikoaren kontrol positibotzat erabili zen. Emaitza hauen arabera, Morfinak giza espermatozoideen erreakzio akrosomikoa inhibitzen du kaltzio kanalen irekiera ekidituz eta PLC-aren funtzioa blokeatuz.

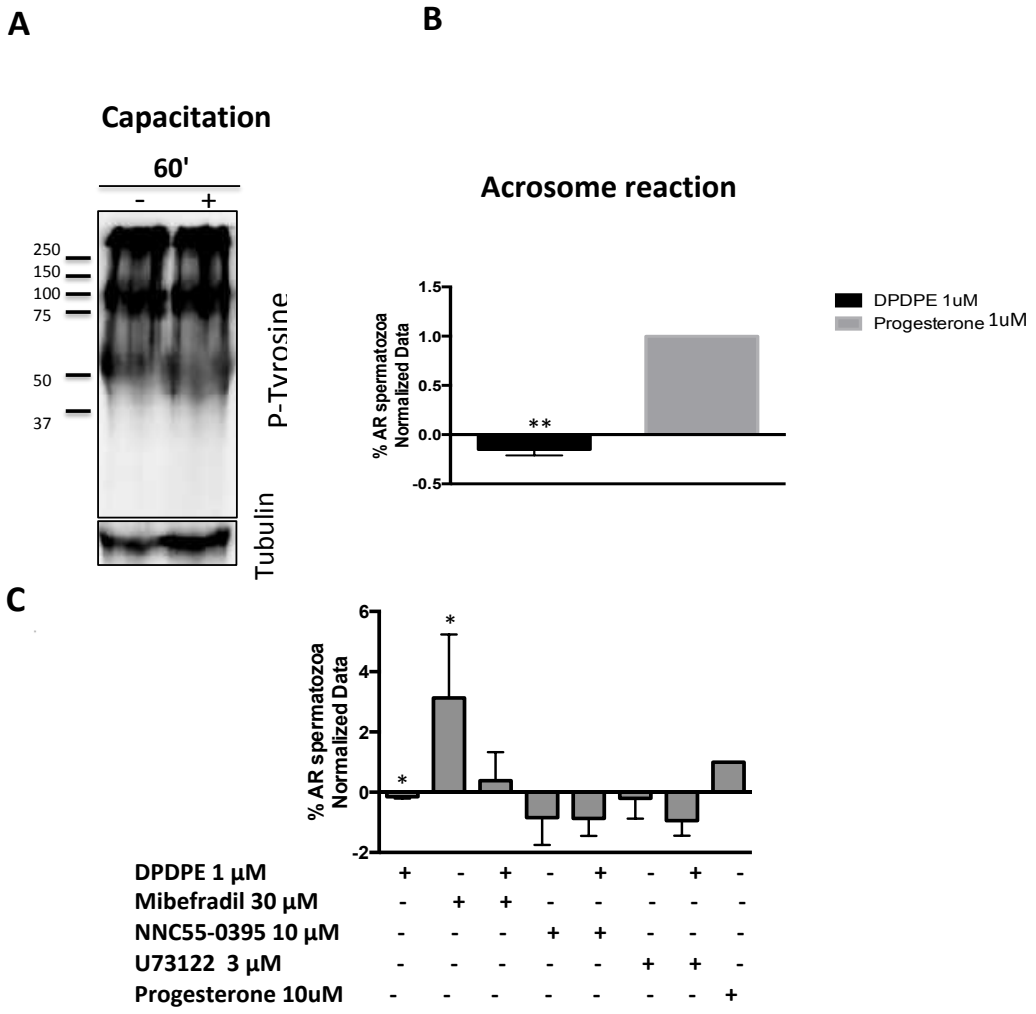
Morfinak MAPK-en seinaliztapen bidezidorrean zuen eragina aztertzeko fosfodiesterasen eta GRK-2-aren (GPCR hartzaileen kinasak) inhibitzaileak (IBMX eta BARK1, hurrenez hurren) erabili ziren. 2D irudian ikus daitekeenez, IBMX (0,5 mM) eta BARK1 (126 µM) inhibitzaileek espermatozoideen erreakzioa inhibitzen dute efektu hau estatistikoki esanguratsua ez delarik. Morfinaren eta inhibitzaileen arteko koikubazioek emaitza berdinak erakusten dituzte, izan ere, ez dira aldaketa estatistiko esanguratsuak lortzen. Emaitza horien arabera, badirudi morfina bidezko erreakzio akrosomikoaren inhibizioa 60 minutuan ez litzatekeela fosfodiesterasen eta GRK-2-aren menpekoa izango.

Morfina bidezko erreakzio akrosomikoaren inhibizioa MAPK-en bidezkoa ez zela ikusi genuen arren, MOR hartzailearen aktibazioa aztertu nahi izan genuen. Horretarako, mu-opioide hartzailearen barneraketa aztertu zen fluxuzko zitometria bitartez. Helburu hori lortzeko, CTOP ([3H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2] ([3H]CTOP) erabili zen 60 minutuz, mu-opioide hartzailearen agonista espezifikotzat, izan ere morfinarekin konparatuta CTOP-k efektu eraginkorragoa sorrarazten du. 2E irudian ikus daitekeenez, CTOP-k mu-opioide hartzailearen barneraketa eragiten du 60 minutuan.

Laburtuz, emaitza hauek aztertuta badirudi Morfinak, U50488H-ak bezala, erreakzio akrosomikoa inhibitzen duela kaltzio kanalen eta PLC entzimaren modulazioaren bidez. Efektu hori MAPK seinaliztapen bidezidorraren menpekoa ez bada ere, CTOP bidezko tratamenduak MOR hartzailearen barneraketa eragiten du. Hori dela eta, etorkizunean interesgarria litzateke hartzailearen barneraketa kaltzioaren seinaliztapen intrazelularrararekin eta ondoriozko erreakzio akrosomikoaren inhibizioarekin zelan erlazionatzen den aztertzea.

III. Eranskina. Delta-opioide hartzailearen funtzioa giza espermatozoideeen ahalmen ugalkorrean

Delta-opioide hartzailea giza espermatozoideetan adierazten den beste hartzaile opioide bat da. Agirregoitia eta lankideek 2006.urtean giza espermatozoideen mugikortasunean eraginik ez zuela ikusi bazuten ere, ez zuten kapazitazio eta erreakzio akrosomikoari buruzko ikasketarik egin. Hori dela eta atal honetan DOR hartzaileak giza espermatozoideen kapazitazioan eta erreakzio akrosomikoan duen funtzioa aztertu genuen. Horrez gain, DOR hartzailearen azpitik aktibatzen diren seinaliztapen bidezidorrak aztertu genituen KOR eta MOR hartzaileen azpitik induzitzen direnekin konparatzeko.



III. Eranskina. Delta-opioide hartzailearen funtzioa giza espermatozoideen ahalmen ugalkorrean. DPDPE-k (1 µM), 60 minutuan giza espermatozoideen **(A)** fosfotirosina mailetan (Ptyr) duen eragina Westen Blot bidez (N=3) (N=3) eta **(B)** erreakzio akrosomikoan duen efektuaren azterketa fluxuzko zitometria bidez (N=10). (**p<0.01 vs Kontrola, t- student estatistikoa) **(C)** Fluxuzko zitometria bidezko CD46 proteinarako positiboak diren ehunekoaren azterketa giza espermatozoideen erreakzio akrosomikoaren ikasketarako, DPDPE (1 µM) eta kaltzio seinalizazio bidezidorraren proteína ituen aktibatzaile/inhibitzaile desberdinak 60 minutuz erabiliz: NNC-55-0395 (10 µM), Mibefradil (30 µM) eta U733122 (3µM) (N=7) (*p<0.05 and **p<0.01 vs Kontrola; +p<0.05 vs Mibefradil; ANOVA estatistikoa).

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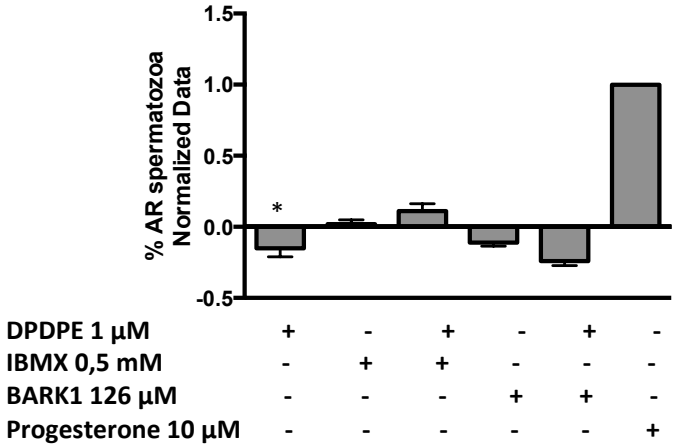

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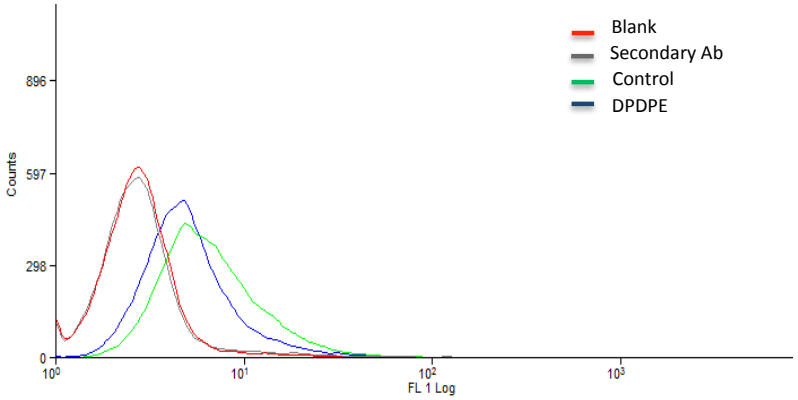

III. Eranskina. Delta-opioide hartzailearen funtzioa giza espermatozoideen ahalmen ugalkorrean. (D) Fluxuzko zitometria bidezko CD46 proteinarako positiboak diren ehunekoaren azterketa giza espermatozoideen erreakzio akrosomikoaren ikasketarako, DPDPE (1 µM) eta MAP kinasen seinalizazio bidezidorraren proteina ituen aktibatzaile/inhibitzaile desberdinak 60 minutuz erabiliz: IBMX (0,5 mM) eta BARK1 (126µM) (N=7) (*p<0.05 and **p<0.01 vs Kontrola; ANOVA estatistikoa). **(E)** Delta-opioide hartzailearen barneraketaren efektuaren ikasketa 60 minutuz DPDPE 1 µM erabiliz. Gorriz (Blank): espermatozoideak; Grisez: antigorputz sekundarioaren kontrol negatiboa; Urdinez: Kontrola; Berdez: DPDPE. X ardatzak fluoroforoaren seinalearen balioari egiten dio erreferentzia eta Y ardatzak fluoroforoaren seinale horretarako positiboak diren zelula kopuruari buruzko informazioa ematen du. **(B)**, **(C)** eta **(D)** grafikoetarako X ardatzak esperimentuan erabilitako tratamenduei egiten dio erreferentzia eta Y ardatzak erreakzio akrosomikoa jasan duten espermatozoideen datu normalizatuen %-a adieratzen du. Normalizazioa ondorengo eran egin zen: [(Tratamendua-Kontrola)-(Progesterona-DMSO)].

Delta-hartzaile opioideak (DOR) giza espermatozoideen ahalmen ugalkorrean betetzen duen funtzioa aztertzeko, kapazitazioa eta erreakzio akrosomikoa aztertu ziren. Horretarako, DPDPE (1 µM) agonista espezifikoa erabili zen eta espermatozoidean 60 minutuz tratatu ziren. Ondoren Western Blot bidez, tirosinetan fosforilatutako proteinak (pTyr) aztertu ziren. 3A irudian ikus daitekeenez tratatutako eta tratatu gabeko espermatozoideen artean ez zen

aldaketa esangarririk ikusi. Hori dela eta, DPDPE-k 60 minutuan giza espermatozoideen kapazitazioan eraginik ez duela ondorioztatu zen. Ondoren, DOR hartzaileak giza espermatozoideen erreakzio akrosomikoan duen eragina aztertzeko espermatozoideak 60 minutuz DPDPE agonista espezifikoarekin tratatu ziren. 3B irudian ikus daitekeenez, DPDPE-k espermatozoideen erreakzio akrosomikoa inhibitzen du 60 minutuan ($p<0.01$).

DPDPE tratamenduaren ondoriozko erreakzio akrosomikoaren inhibizioa kaltzioaren bidezko bidezidorraren bitartekoa zen aztertzeko, bidezidorraren proteina ituen aktibatzaile eta inhibitzaile desberdinak erabili ziren. 3C irudian beha daitekeenez, Mibefradil-ak (30 µM), kaltzio kanalen aktibatzaileak, espermatozoideen errekzio akrosomikoa aktibatzen du 60 minutura ($p<0.05$) eta horren koinkubazioak DPDPE-rekin efektuaren leheneratzea eragiten du. Bestalde, NNC55-0395-ak, Catsper espermatozoide-espezifikoa den kaltzio kanalaren inhibitzaileak eta horren koinkubazioak morfinarekin, erreakzio akrosomikoaren inhibizioa suertatzen du (estatistikoki ez-esanguratsua). Horrez gain, U73122-ak, phospholipasa C-aren (PLC) inhibitzaileak, erreakzio akrosomikoa pairatu duten espermatozoideen %-aren gutxitzea eragiten du (estatistikoki ez esanguratsua). DPDPE-aren eta inhibitzailearen arteko koinkubazioak, inhibizioaren efektu sinergikoa sortzen du erreakzio akrosomikoan (estatistikoki ez esanguratsua). Progesterona erreakzio akrosomikoaren kontrol positibotzat erabili zen. Emaitza hauen arabera, DPDPE-k giza espermatozoideen erreakzio akrosomikoa inhibitzen du. DPDPE eta inhibitzaileen arteko estatistikoki esanguratsuak ez diren emaitzak lortu ez badira ere, badirudi erreakzio akrosomikoaren inhibizioa kaltzio kanalen eta PLC-aren modulazioaren bitartekoa izan litekeela. Izan ere, KOR eta MOR hartzaileen agonista espezifikoek kaltzioaren bidezko seinaliztapen bidezidorrean duten efektua ikusiz eta III. Eranskin honetan aurkezten diren emaitzetan ikusten den joera dela eta, hipotesi hori proposatzeko ebidentzia nahikoa dago.

DPDPE-ak MAPK-en seinaliztapen bidezidorrean zuen eragina aztertzeko fosfodiesterasen eta GRK-2-aren (GPCR hartzaileen kinasak) inhibitzaileak (IBMX eta BARK1, hurrenez hurren) erabili ziren. 3D eranskinean ikus daitekeenez, IBMX (0,5mM) eta BARK1 (126 µM) inhibitzaileek espermatozoideen erreakzioa inhibitzen dute efektu hau estatistikoki esanguratsua ez delarik. DPDPE-ren eta inhibitzaileen arteko koikubazioak emaitza berdinak erakusten dituzte, izan ere, ez dira aldaketa estatistiko esanguratsuak lortzen. Emaitza horien arabera, badirudi DPDPE bidezko erreakzio akrosomikoaren inhibizioa 60 minutuan ez litzatekeela fosfodiesterasen eta GRK-2-aren menpekoa izango.

DPDPE bidezko erreakzio akrosomikoaren inhibizioa MAPK-en bidezkoa ez zela ikusi genuen arren, DOR hartzailearen aktibazioa aztertu nahi izan genuen. Horretarako, delta-opioide hartzailearen internalizazioa aztertu zen fluxuzko zitometria bitartez. Helburu hori lortzeko giza espermatozoideak DPDPE-rekin tratatu ziren 60 minutuz. 3E irudian ikus daitekeenez, DPDPE-ak delta-opioide hartzailearen barneraketa eragiten du 60 minutuan.

Laburtuz, emaitza hauek aztertuta badirudi DPDPE-ak, U50488H eta morfinarekin gertatzen den bezala, erreakzio akrosomikoa inhibitzen duela 60 minutuan kaltzio kanalen eta PLC entzimaren modulazioaren bidez. Efektu hori MAPK seinaliztapen bidezidorraren menpekoa ez bada ere, DPDPE bidezko tratamenduak DOR hartzailearen barneraketa eragiten du. Hori dela

eta, etorkizunean interesgarria litzateke hartzailearen barneraketa kaltzioaren seinaliztapen intrazelularrararekin eta ondoriozko erreakzio akrosomikoaren inhibizioarekin zelan erlazionatzen den aztertzea.

3.Kapitulua

Chapter 3

4.3. CHAPTER 3

Functional characterization of SPANX-A/D family in human spermatozoa and melanoma

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Summary

The SPANX-A/D protein family is a multi-gene family mapped on the X chromosome whose expression is limited to the testis and spermatozoa and to a wide variety of tumors. By combining proteomics and molecular approaches we found that SPANX-A/D is a multifunctional protein family, with a physiological role in nuclear envelope, sperm motility and metabolism in human spermatozoa, while in melanoma has a pathological role in migration of tumour cells. In fact, acting as a scaffold protein we suggest the role of SPANX-A/D in the formation of protein complexes into the nuclear envelope, promoting its physiological and pathological function by regulating nuclear processes. A better comprehension of the role of this family could be essential not only for the identification of therapeutic targets for treating male infertility and skin tumors but also in the development of safe male contraceptives and tumor-directed pharmacological drugs.

INTRODUCTION

The SPANX familiy (Sperm Protein Associated with the Nucleus mapped to the X chromosome) is a multigene family mapped on the X chromosome. SPANX genes code for proteins that belong to the so-called “Cancer testis antigens” (CTA) family, a group of proteins whose expression is limited to the testis and spermatozoa in normal tissues and to a wide variety of tumors in nongametic cells [1, 2]. SPANX proteins, as other CTAs, are exclusively expressed in post-meiotic haploid cells localized on the adluminal side of the hemato-testicular barrier, an immune-privileged site [1, 3, 4]. Due to its immunological characteristics, CTAs are considered one of the most promising candidates for cancer immunotherapy. However, the physiological as well as physiopathological role of SPANX protein family still remains poorly understood.

The SPANX family includes two subfamilies in humans. The SPANX-N subfamily is composed of five members that share a high level of sequence homology [5]. The

SPANX-A/D subfamily, on the other hand, includes SPANX-A1, -A2, -B, -C and D. SPANX-A1, -A2, -C and -D share a 90-98% sequence homology among themselves, and a 75-80% homology with SPANX-B [2, 6]. All SPANX proteins exhibit a similar postmeiotical expression pattern during spermatogenesis, appearing for the first time in the haploid spermatids [1, 6, 7]. However, the SPANX-N and A/D subfamilies localize differently in human spermatozoa. While the SPANX-N proteins are localized over the acrosome [5], SPANX-A/D proteins are expressed as dots in the cytoplasm and nuclear craters [1]. Their different localization could imply different roles for each subfamily in mature spermatozoa. In this regard, while SPANX-N proteins may be involved in acrosome reaction [5] the biological function of SPANX-A/D family is still largely unknown.

As other CTAs, SPANX-A/D proteins are overexpressed in several tumors, such as, myeloma, melanoma, and carcinomas of the breast, bladder and prostate [8–10]. SPANX-A/D have been detected in metastatic melanoma [9] and increased SPANX-A/D expression correlates with liver metastasis in colorectal cancer patients [11]. Thus, it has been suggested that these proteins may be involved in the invasion and/or metastasis of some tumors. However, whether the expression of SPANX-A/D proteins is causal in supporting metastasis remains unclear [12]. Combining proteomics and molecular approaches, we reveal for the first time a multi-functional role for SPANX-A/D in human spermatozoa and the molecular mechanisms underlying SPANX-A/D protein family in melanoma.

EXPERIMENTAL APPROACHES

Samples and isolation of spermatozoa

Ethical approval for this study was obtained from the Ethics Committee of the University of the Basque Country (CEISH-UPV/EHU (M10/2016/254)). Freshly ejaculated semen was collected from patients undergoing routine semen analysis at the Cruces Universitary Hospital (Bilbao, Spain). The donors had normal sperm parameters according to World Health Organization standards (WHO, 2010). Semen samples were obtained by masturbation after 3–4 days of sexual abstinence and processed immediately upon liquefaction (at 37 °C for 30 min). Spermatozoa were capacitated by the *swim-up* procedure and resuspended in G-IVF (Vitrolife, Goteborg, Sweden) supplemented with 1% bovine serum albumin for 3 h, at 37 °C under 5% CO_2.

Plasmids, cloning procedures and mutagenesis

To generate the plasmids encoding YFP–SPANX-A (WT), YFP-SPANX-B, YFP-SPANX-A-NLS Deleted (NLS^{DEL}), YFP-NLS^{MET}, YFP-NLS^{LEU}, YFP-NLS^{PM}, YFP-$NLS^{N\text{-}PM}$, YFP-$SPANXA^{PM}$ and YFP-$SPANXA^{N\text{-}PM}$ each cDNA was synthetized as HindIII/BamHI fragments (gBlocks Gene Fragments, Integrated DNA Technologies, CA, USA) and subcloned into pEYFP-C1 (Clontech, Mountain View, CA, USA) plasmid. All of the constructs generated were subjected to DNA sequencing (Stabvida, Caparica, Portugal), and the absence of any unwanted mutation was confirmed. The sequences of the oligonucleotides used in

cloning and site-directed mutagenesis are available upon request. The amino acid sequences coding each YFP-protein are detailed below in Figure 2A.

Transfections and confocal microscopy

A375, HEK293T and HeLa cells were grown as it is described above and twenty-four hours before transfection, cells were seeded onto 12-well or 6-well tissue culture plates. HEK293T and HeLa cell lines plasmid transfections were carried out with XtremeGENE 9 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) and A375 transfections using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Massachusetts, USA), according to the each manufacturer's protocol. To evaluate the transfection efficiency, cells, growing onto sterile coverslips, were fixed with 3.7% formaldehyde in PBS for 30 min and then assembled onto slides using the Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, USA). To avoid bias in the quantification of YFP fluorescence, slides were encoded, and images were taken and examined unaware of the identity of the samples. Image analysis with IMAGEJ software was used to quantify the intensity of YFP fluorescence.

RT-qPCR assays

Total RNA was extracted using PureLink® RNA Mini kit (Life Technologies Inc.), according to the manufacturer's instructions. RNAse-free DNase I was used to prevent DNA contamination. RNA concentration and purity was assessed by absorbance at 260 nm using the Synergy HT spectrophotometer (Bio-Tek, Winooski, VT, USA). Reverse transcription (RT) was performed in a 20 µl reaction volume with 1 µg of total RNA through iScriptTM cDNA Synthesis Kit (Bio-Rad) to synthesize first-strand cDNA, following manufacturer´s guidelines. Afterwards, RT-qPCR was carried out to check the relative expression level of SPANX-A/C/D using Tubulin as an internal control. Gene-specific amplification was performed using CFX96 Real-Time System (Bio-Rad). The quantification was performed using cDNA samples from three separate RNA isolations. The reactions were performed in a total volume of 10 µl that contained the following: 50 ng cDNA that was synthesized as described above, 5 µl of SYBR Green master mix (BioRad) and 200 nM of each pair of oligonucleotide primers. The following primers for RT-qPCR analyses were used: SPANX-A/C/D: forward: AACGAGATGATGCCGGAGAC; reverse: TTTGGAGGGGGTTGATTCTG.

Protein extraction

For protein expression analyses, isolated sperm cells were lysed using ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium pervanadate, 5 mM beta-glycerophosphate, 5 mM NaF, complete protease inhibitor cocktail (Complete tablets, Roche)). After protein homogenization and sonication (20% amplitude, 10 pulses, three times), proteins were separated both in soluble and insoluble fractions by centrifugation at 13000 g, 4 °C, 15 min.

At the same time, A375, Mel-Ho, HS294T, Colo-800 and HT144 human melanoma cells were lysed using the same ice-cold RIPA buffer to further separate soluble and insoluble protein fractions by centrifugation. Protein concentration was

measured by the Bicinchonic Acid (BCA) assay method.

Western blotting

Protein extracts were diluted in Laemmly sample buffer containing Dithiothreitol (DTT) and boiled for 5 min. 30 µg of each insoluble and soluble protein fractions from human spermatozoa were loaded onto 12% resolving gels and separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The same was performed with the insoluble protein fraction of the melanoma cell lines.

Proteins were then transferred to polyvinylidene fluoride membranes using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, the membranes were blocked with Blotto (20 mM TrisHCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100) containing 5% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h at room temperature and then incubated with a dilution of polyclonal rabbit anti-SPANX antibody (1:500) 5% BSA (ab119280, Abcam), the polyclonal rabbit anti-Lamin A/C (A0249, Abclonal) (1:1000), and the monoclonal mouse anti-FH (sc-393992, Santa Cruz Biotechnology) (1:200). After washing in Blotto buffer, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000) (Goat anti-rabbit IgG HRP, sc-2004, Santa Cruz Biotechnology) or peroxidase-conjugated donkey anti-mouse IgG antibody (1:2000) (Donkey anti-mouse IgG, HRP, sc-2314, Santa Cruz Biotechnology).

Indirect immunofluorescence and confocal microscopy

Isolated human sperm cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min and blocked for 30 min with 10% (v/v) fetal bovine serum (FBS) in PBS. For HEMn-LP, HEMn-DP, HEMn-MP, A375, Mel-Ho, MCF-7, A2780, HCT-8, SW480, SMS-KCNR and HeLa cell lines, however, the permeabilization was performed using 0.2% Triton X-100 for 10 min and blocking for 30 min with 10% (v/v) FBS in PBS. After, samples were incubated overnight at 4 °C with: the polyclonal rabbit anti-SPANX (ab119280, Abcam)(1:500), the polyclonal rabbit anti-Lamin A/C (A0249, Abclonal) (1:50), the monoclonal mouse anti-NUP98 (sc-74578, Santa Cruz Biotechnology) (1:50), the monoclonal mouse anti-TEKT1 (sc-398507, Santa Cruz Biotechnology) (1:50) and the monoclonal mouse anti-FH antibody (sc-393992, Santa Cruz Biotechnology) (1:50). Secondary antibody incubations included Alexa Fluor 488 donkey anti-rabbit IgG (1:2000) (Molecular Probes, Oregon USA), Alexa Fluor 555 goat anti-mouse IgG (1:1000) (Thermo Scientific) and Alexa Fluor 663 goat anti rabbit IgG (1:1000) (Thermo Scientific). At the same time, controls for the specificity of the secondary antisera were performed by omitting the primary antiserum before addition of the secondary antisera. Nuclei were stained with Hoechst 33258 at 10 µg/mL and slides were assembled with Fluoromount G (Molecular Probes). Finally, the samples were examined using confocal microscopy (Zeiss Apotome 2, Jena, Germany) at the High Resolution Microscopy Facility (SGIKER UPV/EHU). The

image analysis was conducted using the ImageJ software.

In-gel digestion

For study of SPANX isoforms in human spermatozoa and A375 cell line, the soluble and insoluble protein fractions were loaded onto a precast gradient NuPAGE 4-12% Bis-Tris Protein gel (Invitrogen) for a further visualization with Colloidal Blue (Invitrogen). Whole gel lines were cut into slices and subjected to reduction with 10 mM Dithiothreitol (DTT), alkylation by 55 mM chloroacetamide (CAA) and protein digestion by incubating with trypsin overnight at 37 °C.

Resulting tryptic peptides were extracted from the gel by serial incubations with 100% Acetonitrile (ACN) and 30% ACN/3% Trifluoroacetic acid (TFA). Finally, the solutions obtained in all the incubations were pooled and dried down in a vacuum centrifuge. Peptides derived from slices from each lane were concentrated and desalted using C18 stage tips (made in house using Empore disc –C18 Agilent Life Science) to further analyze by LC-MS/MS.

Immunoprecipitation and in-solution digestion

For SPANX-A/D interactome in human spermatozoa, protein lysates (quadruplicate) were incubated for 4 hours at 4 °C with magnetic-beads (PureProteome Protein A Magnetic Beads, Millipore) conjugated to anti-SPANX antibody (ab119280, Abcam). As negative control rabbit IgGs (X0903, DAKO) were used. In A375, the YFP-SPANX-A was pulled down in cadruplicates, using the Magnetic GFP-Trap Beads antibody (Chromotek) following manufacturer's instructions. As negative control, the empty YFP-vector was used. In both cases, immune complexes were independently recovered and washed. Elution of the immunocomplexes was carried out with Guanidinium hydrochloride 8 M, pH 8, 70 °C for 15 minutes. The proteins were then reduced and alkylated followed by in-solution digestion with LysC/Trypsin. Peptides derived from each sample were concentrated and desalted using C18 stage tips (made in house using Empore disc –C18 Agilent Life Science) for analysis by LC-MS/MS.

LC-MS/MS

Acidified peptide mixtures were separated by online C18- reverse-phase nanoscale liquid chromatography and analyzed by tandem mass spectrometry (LC-MS/MS). MS analysis was performed on an Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany) connected to an EASY-nanoLC 1000 System (Thermo) using a nanoelectrospray ion source (Proxeon Biosystems, Odense, DK). Survey full-scan MS spectra (m/z range, 200–2000; resolution 60,000 at m/z 400) were acquired in the Orbitrap followed by the fragmentation of the twelve most intense multiply charged ions. Ions selected for MS/MS were placed on a dynamic exclusion list for 45 s. To improve mass accuracy, internal real time lock mass calibration was enabled. Additional mass spectrometric parameters included a spray voltage of 2.3 kV, no sheath and auxiliary gas flow, and the temperature of the heated capillary was 275 °C. All raw files were searched against combined human database 2015.08 UniProt (with 42122 sequence entries) and TrEMBL (with 49496

sequence entries) using MaxQuant platform versions 1.5.2.8 and 1.5.3.30 with an Andromeda search engine. Precursor and fragment tolerances were 4.5 and 20 ppm, respectively. A peak list was generated using the Quant element of MaxQuant using the following parameters: A maximum of 2 missed cleavages were allowed and enzyme specificity was set to trypsin, allowing for cleavage N-terminal to proline and between aspartic acid and proline. In addition, carbamidomethyl (C) was chosen as fixed modification and variable modifications included oxidation (M), deamidation (NQ) and Phospho_STY (STY). The peptide and protein FDR 0.01; site FDR 0.01; max. peptide PEP, 1; min. peptide length 7; min unique peptides and peptides, 1. For protein quantitation, only unmodified peptides and peptides modified by acetyl (protein N-terminus), oxidation (Met) and deamidation (NQ) were used. According to the protein group assignment done by MaxQuant, the identified proteins were determined after removing the contaminants, reverse hits and proteins identified only by site. Moreover, we took into account those proteins with ≥2 identified peptides and ≥1 unique peptides. On the other hand, the phosphopeptide data was filtered by FDR < 1% and only the phosphosites displaying a localization probability above 0.75 were considered as confident phosphorylated sites (Class I sites). Finally, the output data was analyzed in Perseus 1.6.0.7 bioinformatics analysis program.

Bioinformatic Analysis

The Perseus software (v.1.6.0.7) was employed for the calculation of the statistical significance between the SPANX pulldown samples and the negative control in human spermatozoa and A375 cells. In the case of human spermatozoa, we filtered the protein list accepting as putative interactors those proteins identified with at least two peptides and containing a minimum of three unique peptides. In both cases the study was performed in quadruplicate. Moreover, we only accepted as candidates those proteins that appeared in all four replicas of the SPANX-A/D immunoprecipitation samples but not in any of the negative controls. In the case of A375 melanoma cell line, we only accepted as possible interactors those proteins that appeared in at least three replicas of the YFP-SPANX-A immunoprecipitation samples and maximum in one of the negative controls (vector).

The PANTHER (v13.1) functional annotation tool (http://geneontology.org/) was used to detect the overrepresented gene ontology (GO) term “biological process” within the possible SPANX interactors in both human spermatozoa. The Cytoscape computational tool (v.3.5.1) was used for the visualization of the SPANX interactome in human spermatozoa and A375 cell line.

SPANX-A/D deficient stable cell line generation

For the generation of stable SPANX-A/D deficient cell line, A375 human melanoma cells (ATCC, Manassas, VA, USA) were infected with Scramble or SPANX-A/D shRNA lentiviral particles containing puromycin resistance gene and GFP, purchased from OriGene (Rockville, MD, USA). Transfections were carried out using polybrene (4 ng/ml) as transfection reagent, which facilitates the introduction

of lentiviral particles into the cells. To further select transfected cells, cells with expressing medium-high GFP were selected through cell sorting using BD FACSJazz (2B/4YG) cell sorter (BD Bioscience, San Jose, CA, USA) and maintained with 5 µg/ml puromycin (Sigma-Aldrich, San Luis, MO, USA). Efficiency of SPANX-A/D knockdown was periodically checked by Western blot.

The information regarding the three different shRNAs is detailed below:

SPANX-A/D shRNA 1:
CCAAATGGAGGAGGAGGAATTCAT
SPANX-A/D shRNA 2:
AAGAACATCTCCAGAGGAACTG
SPANX-A/D shRNA 3:
CTAGTGGTTCGCTACAGGAGGAAC

Cell viability assay

To study the effect of SPANX-A/D knockdown in A375 melanoma cells, Scramble-Sh and SPANX-A/D Sh 5 x 10^3 cells were seeded into 96 well plates in complete medium (DMEM supplemented with L-Glutamine and 10% FBS (Sigma-Aldrich)) and incubated overnight. After 18 hours, cells were considered to be in T=0. After further incubation for 24 hours, the viability was measured by means of Prestoblue Viability Reagent (Life Technologies, Thermo Scientific, MA, USA) following manufacturer´s indications. Briefly, the medium was replaced with Prestoblue diluted 1/10 in complete medium and the viability was measured after 2 hours of incubation. The proliferation of SPANX-A/D Sh A375 cells was compared to that of Scramble Sh A375 cells. The obtained results are the mean of three different experiments.

Wound Healing Assay

We analyzed the involvement of SPANX-A/D in the migration potential of A375 melanoma cells through wound healing assay. A375 cells were plated in 24 well plates and incubated for 18 hours. Next, the medium was changed for fresh complete medium supplemented with Mytomicin (5 µg/ml) (Sigma-Aldrich) and incubated for 1h 30 minutes. Afterwards, a wound was made in each well using 200 µl tip and the medium was again changed for fresh complete medium, after extensive wash of the scratched wells. Photographs were taken at T=0 to use them as initial wound area and after 24 hours of incubation (T=24h), using inverted light microscope (Axioscope). The closed area was calculated using the Image J software by means of initial wound area at T=0 – T=24. The results are the mean of three independent replicates.

Transwell migration assay

In order to examine the role of SPANX-A/D during tumor cell migration, we carried out transwell migration assay. 1.5 x 10^4 A375 cells were seeded on 8 µm pore membrane inserts (Falcon) in complete medium. Cells were allowed to migrate overnight for 20 hours. Next, inserts were processed by means of fixation in PFA 4%, rehydrated in PBS and stained using Crystal Violet (Sigma-Aldrich) 0.5 % in PBS for 15 minutes for their visualization under light microscope. For cell migration quantification, six different fields were randomly counted in each well. The results are expressed as the average of total number of migrated cells in 20x field. The obtained results are the mean of three different experiments.

RESULTS

Identification and characterization of novel SPANX-A/D phosphosites.

The SPANX multigene family has been previously well characterized at the gene level [5, 9]. To shed light on the role of SPANX-A/D subfamily in human spermatozoa, we first carried out its characterization at the protein level. Using immunoblotting, we evaluated the expression of endogenous SPANX-A/D in both soluble and insoluble protein fractions of human spermatozoa. The anti-SPANX polyclonal antibody labeled two bands (37 kDa and 20 kDa) in the soluble fraction and three bands (50kDa, 37kDa and 20 kDa) in the insoluble fraction (Figure 1A). No immunoreactivity was observed when the primary antibody was omitted (data not shown). Next, we determined the localization of SPANX-A/D in normal human spermatozoa by immunofluorescence. These studies confirmed the presence of SPANX-A/D proteins in human sperm cells (Figure 1B). Strong immunoreactivity was observed at the neck of spermatozoa (60% of the SPANX-A/D-positive cells), over the acrosome (30% of the SPANX-A/D-positive cells) and in the nucleus (10% of the SPANX-A/D-positive cells), specifically in nuclear craters of spermatozoa (Figure 1B). In addition, immunoreactivity was detected along the tail in 100% of the spermatozoa. No fluorescent staining was observed when the primary antibody was omitted. Due to the high amino acid sequence homology among members of SPANX-A/D the family [1] we were not able to distinguish the different SPANX-A/D isoforms by using polyclonal antibodies. To circumvent this limitation, the presence of SPANX-A/D isoforms was evaluated by mass spectrometry (LC/MS-MS). In addition to common peptides shared by all isoforms, unique peptides belonging to SPANX -A, -B, -C and -D were identified by LC-MS/MS (Figure 1C), indicating that all SPANX-A/D family members are expressed in human. Of note, one of the common peptides identified (TSESSTILVVRYR) overlaps a putative nuclear localization signal (NLS) that has been previously described for the protein subfamily [1, 13]. Additionally, LC-MS/MS analyses were performed to search for phosphorylation sites on SPANX-A/D family members. Following the strict criteria described in Material and Methods, we detected a total of 7 unique phosphorylation sites on SPANX-A/D family members. According to PhosphositePlus database [14], this is the first report showing that those residues are phosphorylated on SPANX-A/D protein subfamily *in vivo* in human spermatozoa (Figure 1D). We identified seven phosphorylated residues in SPANX-A (Ser^{5}, Ser^{13}, Ser^{18}, Thr^{28}, Ser^{47}, Ser^{48} and Thr^{49}), six in SPANX-B (Ser^{5}, Ser^{13}, Ser^{18}, Ser^{53}, Ser^{54} and Thr^{55}) and four in SPANX-C/D (Ser^{5}, Ser^{47}, Ser^{48} and Thr^{49}). Intriguingly, the Ser^{47}, Ser^{48}, and Thr^{49} (Ser^{53}, Ser^{54} and Thr^{55} for SPANX-B) residues are located within the NLS.

Next, we used site-directed mutagenesis and transfection of YFP-tagged proteins to determine whether the phosphorylation state of SPANX-A/D had an influence on nuclear translocation of SPANX-A/D (Figure 2A). Due to the impossibility of performing transfections in human spermatozoa, we performed these experiments in HeLa and HEK293T cell lines. We first evaluated the functionality of the putative NLS using NLS-null SPANX mutants (YFP-SPANX-$A^{DEL/NLS}$)

(Figure 2B and figure supplement 1A). YFP-SPANX-A exhibited a prominent nuclear localization, accumulating in nuclear dots and in the periphery of the nucleus. In contrast, YFP-SPANX-A$^{DEL/NLS}$ mutant was diffusely localized throughout the cell (Figure 2B and figure supplement 1A), indicating that the NLS is required for the nuclear accumulation of SPANX-A. In addition, we generated a number of YFP-SPANX mutants to assess whether the differences in the sequence of SPANX family members affect the functionality of the NLS. The NLS sequence of SPANX-A/-B and SPANX-C/-D differs at a single amino-acid position. To evaluate if this amino acid difference affects NLS function, we designed two different constructs encoding the NLS with either a leucine (YFP-NLS^{LEU}) or a methionine (YFP-NLS^{MET}) residue (Figure 2A). Both NLS variants led to similar nuclear localization of YFP (Figure 2C and figure supplement 1B), suggesting that the leucine/methionine sequence variation does not significantly affect NLS function. Then, we aimed to evaluate if the difference in length between SPANX-A and SPANX-B, being 6 aminoacid longer, correlates with a different subcellular distribution, we designed a construct encoding YFP-SPANX-B. Both YFP-SPANX-A and YFP-SPANX-B exhibited a similar nuclear localization and accumulated in nuclear dots in both cell lines (Figure 2D and figure supplement 1C). In fact, a 3D reconstruction (Supplementary Figure 2) revealed that both proteins are organized in nuclear dots of variable size: larger dots localized inside the nucleus and smaller dots showing epinuclear localization along the nuclear envelope. Finally, site-directed mutagenesis was used to replace the three residues inside the NLS found to be phosphorylated (Ser^{47}, Ser^{48} and Thr^{49}) with glutamate (D) or alanine (A), mimicking the phosphorylated (YFP^{NLS-PM}) and non-phosphorylated (YFP$^{NLS-N-PM}$) states of the NLS, respectively. We detected no significant difference in the localization of the WT, NLS^{PM} and NLS^{N-PM} constructs (Figure 2E and figure supplement 1D). We next replaced all the phosphosites identified in SPANX-A/D (Ser^{5}, Ser^{13}, Ser^{18}, Thr^{28}, Ser^{47}, Ser^{48} and Thr^{49}) by glutamate or alanine to mimick the phosphorylated (YFP-SPANX-A^{PM}) and non-phosphorylated (YFP-SPANX-A^{N-PM}) version of the protein (Figure 2A). Both constructs were able to translocate into the nucleus of HeLa (Figure 2F) and HEK293T (Figure supplement 1E) cells and accumulated on nuclear dots, indicating that the phosphorylation state is not a crucial determinant of the nuclear translocation of the protein. Nevertheless, phosphomimetic YFP-SPANX-A^{PM} mutant showed larger and more intense dots inside the nucleus of HeLa and HEK293T cells compared to both WT and the non-phosphorylable mutant (Figure 2F and figure supplement 1E), suggesting that the phosphorylation state of the protein has an influence on SPANX-A/D protein complex stabilization.

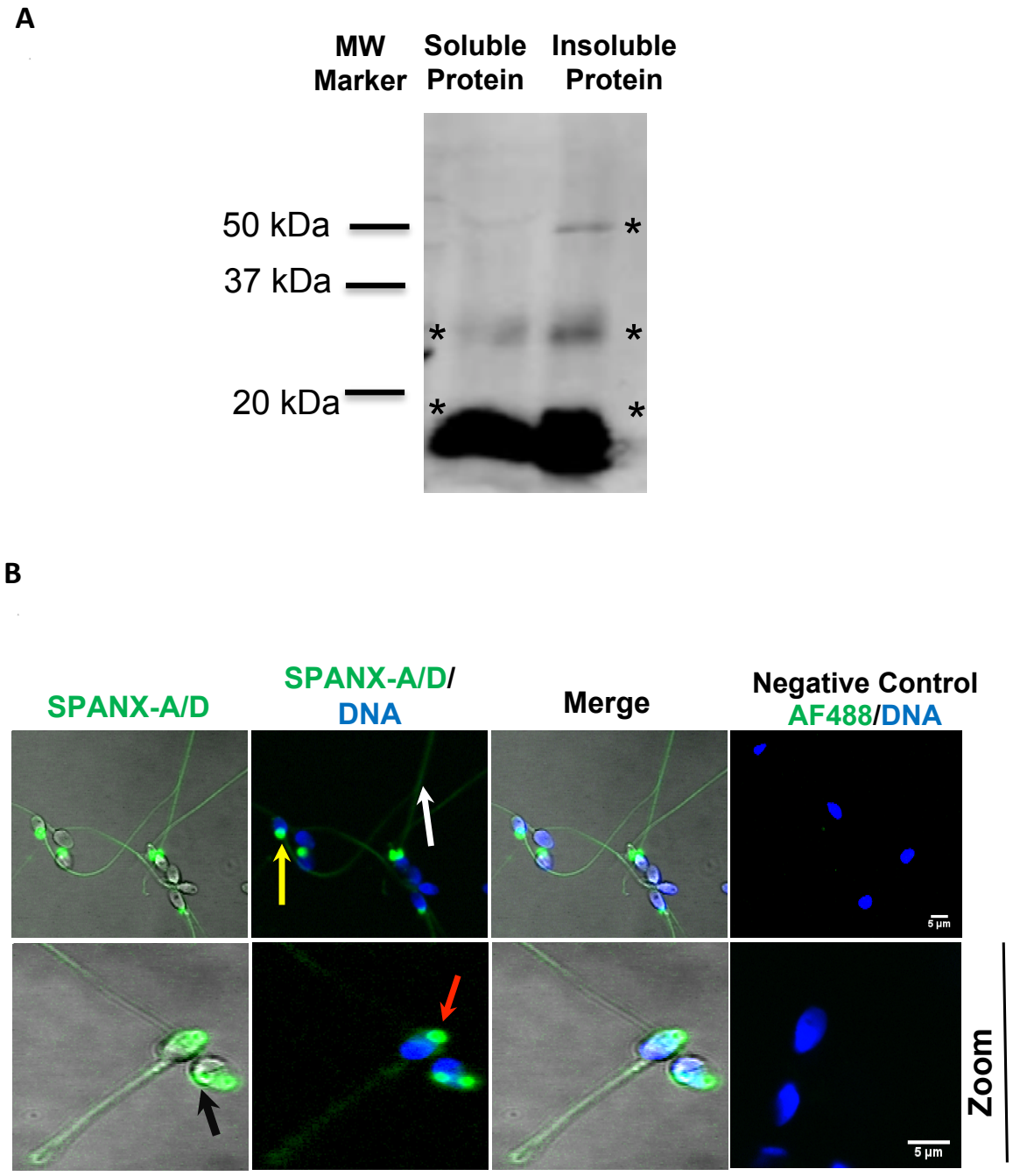

Figure 1. Characterization of SPANX-A/D in human spermatozoa (A) by Western blotting, in soluble and insoluble protein fractions showing immunoreactivity at 50, 37 and 20 kDa. * indicate the different bands of SPANX-A/D (N=5) **(B)** by immufluorescence, in the midpiece (yellow arrow) (x63, zoom 1.5), tail (white arrow) (x63, zoom 1.5), acrosome (red arrow) (x63, zoom 2.5) and nuclear craters (black arrow) (x63, zoom 2.5). The nuclei were stained with Hoechst and are represented in blue. (N=4). Scale bar 5 µm. **(C)** Table containing the unique peptides corresponding to each SPANX-A/D isoforms found by LC-MS/MS in human spermatozoa. Comparison of each SPANX isoform sequences, each color corresponding to the different peptides found by mass spectrometry **(D)** Phosphorylation sites of each SPANX-A/D isoform found by LC-MS/MS are represented in yellow. Three overlapping consensus nuclear localization signals are indicated in boxes.

C

	SPANX isoform	Unique peptides
Human spermatozoa	SPANX-A	RSVPCDSNEANEMMPETPTGDSDPQPAPKK
	SPANX-B	ENRINPDQMEEEEFIEITTERPKK
		LKRSVPCESNEANEANEANK
		TMPETPTGDSDPQPAPK
	SPANX-C	RTSPEELVNDHARENR
	SPANX-D	KNRINPLQMEEEEFMEIMVEIPAK
		RTSPEELVNDHARK

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SPANX-A MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
SPANX-B MGQQSSVRRLKRSVPCESNEANEANEANKTMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNVKRTSPEELLNDHARENRINPDQMEEEEFIEITTERPKK
SPANX-C MDKQSSAGGVKRSVPCESNEA------NEMMPETSSGYSDPQPAPKKLKTSESSTILVVRYRRNVKRTSPEELVNDHARENRINPLQMEEEEFMEIMVEIPAK
SPANX-D MDKQSSAGGVKRSVPCDSNEA------NEMMPETSSGYSDPQPAPKKLKTSESSTILVVRYRRNFKRTSPEELVNDHARKNRINPLQMEEEEFMEIMVEIPAK
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D

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            5          13     18                       28                      47 48 49
SPANX-A MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
SPANX-B MGQQSSVRRLKRSVPCESNEANEANEANKTMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNVKRTSPEELLNDHARENRINPDQMEEEEFIEITTERPKK
SPANX-C MDKQSSAGGVKRSVPCESNEA------NEMMPETSSGYSDPQPAPKKLKTSESSTILVVRYRRNVKRTSPEELVNDHARENRINPLQMEEEEFMEIMVEIPAK
SPANX-D MDKQSSAGGVKRSVPCDSNEA------NEMMPETSSGYSDPQPAPKKLKTSESSTILVVRYRRNFKRTSPEELVNDHARKNRINPLQMEEEEFMEIMVEIPAK
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Functional characterization of SPANX-A/D protein family in human spermatozoa.

In order to uncover the physiological role of SPANX-A/D subfamily in human spermatozoa, we studied the interactome by using a label free quantitative proteomics approach. Due to the fact that approximately 30% of the proteins expressed in human spermatozoa are unique, and not found in any other cell types, we looked for SPANX interactors in human spermatozoa using endogenous SPANX as bait. SPANX and co-immunoprecipitating proteins were digested in solution with trypsin and analyzed by LC-MS/MS in 4 independent biological replicates. A non-specific rabbit IgG that does not bind SPANX-A/D was used as a negative control in parallel pull-down assays. A total of 307 potential SPANX-A/D interactors were identified. We considered as potential SPANX interactors those proteins that were confidently detected in the four SPANX pulldowns, but in none of the negative control pulldowns (supplemental table S1). Gene ontology analysis (Figure 3B) revealed that potential SPANX-A/D interactors are mainly involved in nuclear pore organization (23.65-fold enrichment), cilium or flagellum-dependent motility (23.65-fold enrichment), assembly of the axonemal dynein complex (16.02-fold enrichment) and mitochondrial electron transport (11.58-fold enrichment). These results suggest that SPANX-A/D protein subfamily may exert a multifunctional role in human spermatozoa. A more in depth analysis (Figure 3C and 3D) revealed that several nuclear proteins such as Histone1, chromatin regulator SETD9 protein and nucleoporins (NUPs) co-precipitated with SPANX-A/D. Specifically, NUP98 co-localized perfectly with SPANX-A/D in the neck of the spermatozoon (Figure 3E). On the other hand, the structural component of ciliary and flagellar microtubules such as dyneins and tektins also co-precipitated with SPANX-A/D and TEKT1 co-localized with them in the neck of the spermatozoa and along the tail (Figure 3E). Finally, the mitochondrial metabolic proteins such as Fumarate Hydratase (FH), assembly factors related to NADH deshidrogenase subcomplex like NDUFAF2, and ATP5E (Figure 3C and 3D) were also identified as potential SPANX-A/D interactors. In this group, FH co-localized with SPANX-A/D in the neck on the spermatozoa (Figure 3E). Importantly, immunoblot analyses confirmed that FH, the protein showing highest MS intensity (Figure 3E), interacted with SPANX-A/D (Figure 3F) suggesting that SPANX-A/D and FH may associate at the neck of human spermatozoa, and hence exert a physiological function.

A
NLS
YFP-SPANX-A (WT)
YFP MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
YFP-SPANX-B
YFP MGQQSSVRRLKRSVPCESNEANEANEANKTMPETPTGDSDPQPAPKKMKTSESSTILVVRYRRNVKRTSPEELLNDHARENRINPDQMEEEEFIEITTERPKK
YFP-SPANX-A NLS/DEL
YFP MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDS----------TSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
YFP-NLS MET
YFP DPQPAPKKMKTSESSTILVVRYRRNFKR
YFP-NLS LEU
YFP DPQPAPKKLKTSESSTILVVRYRRNFKR
YFP NLS-PM
YFP MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDSDPQPAPKKMKTSEDDDILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
YFP NLS-N-PM
YFP MDKQSSAGGVKRSVPCDSNEA------NEMMPETPTGDSDPQPAPKKMKTSEAAAILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
YFP-SPANX-A PM
YFP MDKQDSAGGVKRDVPCDDNEA------NEMMPEDPTGDSDPQPAPKKMKTSEDDDILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK
YFP-SPANX-A N-PM
YFP MDKQASAGGVKRAVPCDANEA------NEMMPEAPTGDSDPQPAPKKMKTSEAAAILVVRYRRNFKRTSPEELLNDHARENRINPLQMEEEEFMEIMVEIPAK

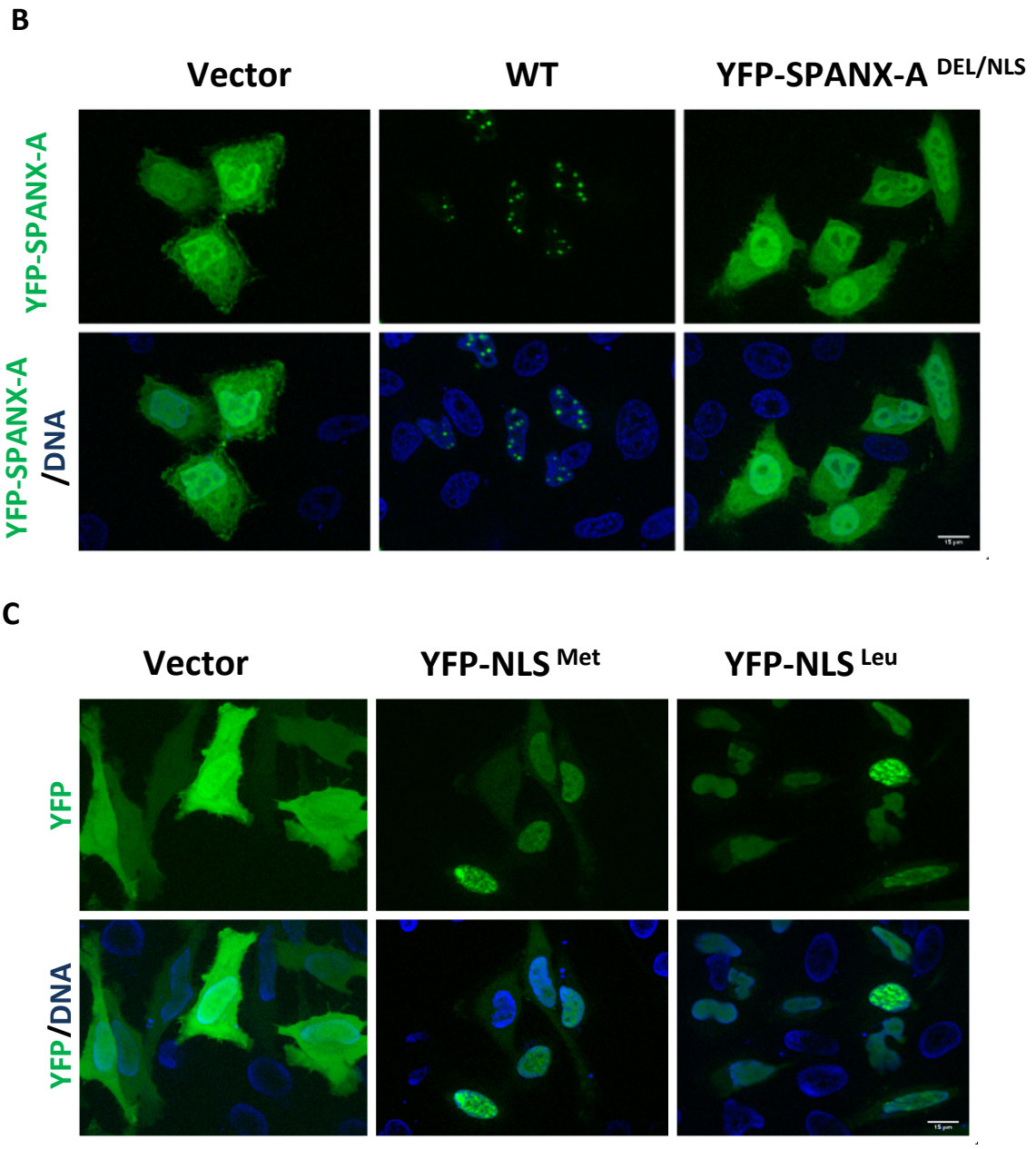


Figure 2. **Mutagenesis studies of SPANX-A/D protein subfamily in HeLa cells. (A)** Schematic representation of all mutants transfected in both HeLa and HEK293T cells. All expression plasmids were expressed with a YFP tail. YFP-SPANX-A was used as wild type sequence. The NLS appears drawn in yellow within the sequence of each YFP-mutant. Confocal microscopy images showing representative examples of HeLa cells transfected with expression plasmids encoding **(B)** YFP (vector), WT and WT mutant with deleted NLS (YFP-SPANX-A$^{NLS/DEL}$) (x63). DAPI was used to counterstain nucleus (DNA panels) (N=3) **(C)** YFP, the NLS of WT with Methionine (YFP-NLS^{MET}) and Leucine (YFP-NLS^{LEU}) at the 42nd positions of the sequence (x63). (N=3).

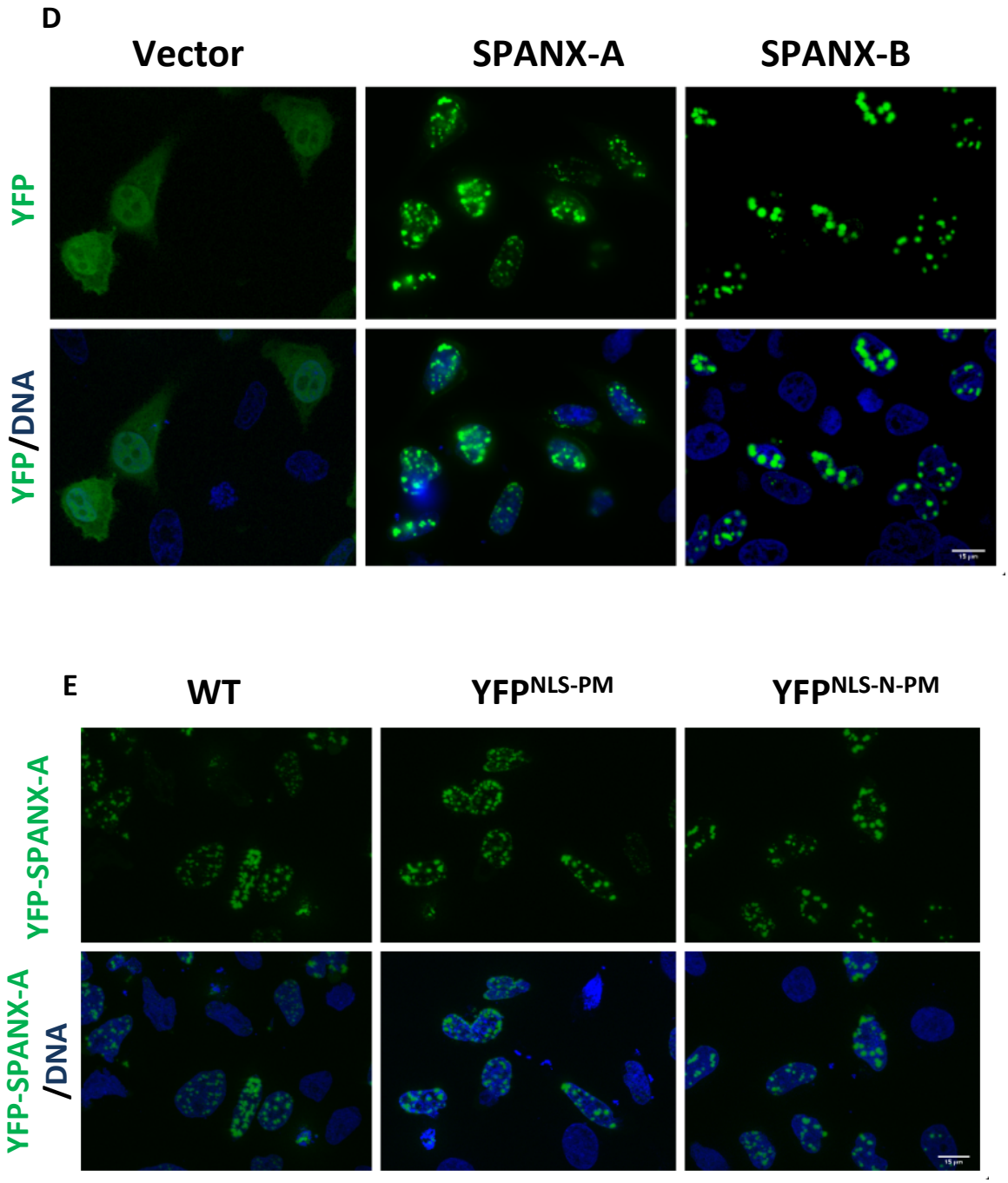


Figure 2. **Mutagenesis studies of SPANX-A/D protein subfamily in HeLa cells.** Confocal microscopy images showing representative examples of HeLa cells transfected with expression plasmids encoding (**D)** YFP, SPANX-A (WT) and SPANX-B (x63). (N=3) **(E)** WT and the phospho-mimetic (YFP-NLS^{PM}) and dephospho-mimetic mutants (YFP-NLS$^{N\text{-}PM}$) at the 47th, 48th and 49th positions of the NLS (x63). (N=3).

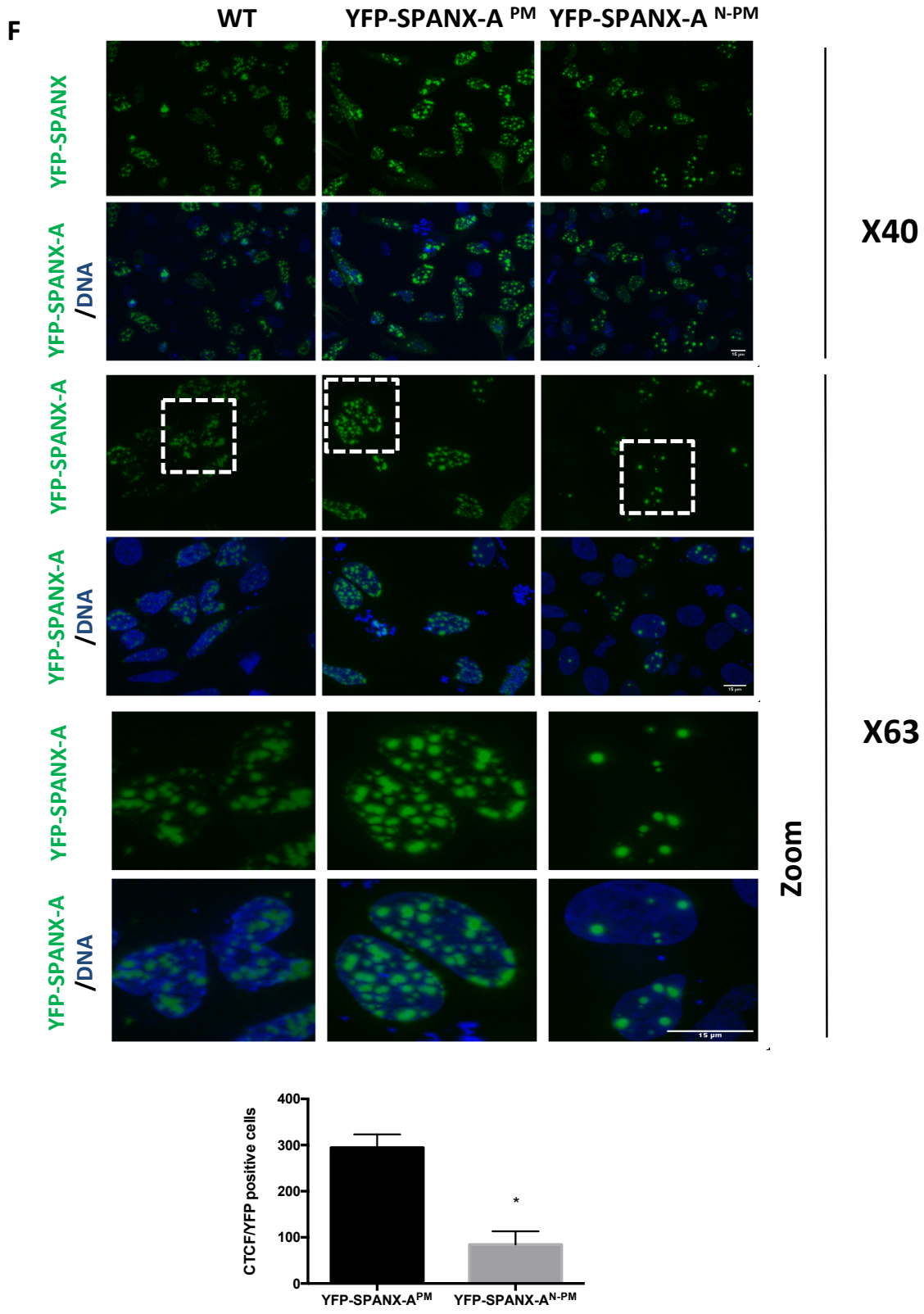


Figure 2. **Mutagenesis studies of SPANX-A/D protein subfamily in HeLa cells.** Confocal microscopy images showing representative examples of HeLa cells transfected with expression plasmids encoding **(F)** WT and the phospho-mimetic (YFP-SPANX-A^{PM}) and dephospho-mimetic mutants (YFP-SPANX-A^{N-PM}) at 5th, 13th , 18th, 28th, 47th, 48th and 49th positions of the WT sequence (x40 and x63). The zoomed section appears framed by a dotted line Scale bar: 15 µm. (N=3) Graph showing the CTCF intensity/YFP positive cells of the YFP-SPANX-A^{PM} vs SPANX-A^{N-PM} mutants. The data shown in the graph correspond to the mean of three independent experiments, and error bars indicate the SEM. *P<0.05 (Students T-test).

Characterization of SPANX-A/D protein expression, phosphorylation and localization in human melanoma.

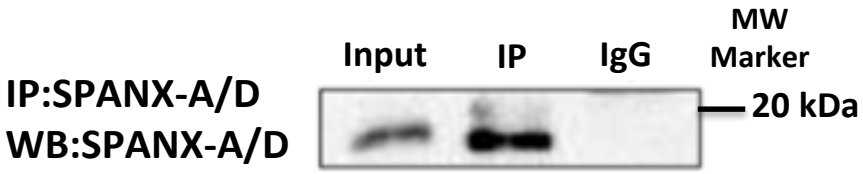
As described above, SPANX-A/D proteins are so-called cancer testis antigens (CTAs), proteins whose normal expression occurs predominantly in male germ cells, but is aberrantly re-activated in cancer cells [1, 15]. Expression of SPANX-A/D has been associated with more aggressive skin tumors, particularly in distant, nonlymphatic metastatic melanomas [9]. As shown in Figure 4 and supplement figure 2, immunofluorescence analysis revealed expression of SPANX-A/D in several cell lines derived from various types of cancer, including colorectal cancer (SW480 and HCT-8), cervical cancer (HeLa), neuroblastoma (SMS-KCNR), breast cancer (MCF-7) and melanoma (A375, MelHo, Colo800, HS294T and HT144) . All the melanoma cell lines showed a strong staining in the nucleus (Figure 4B). The presence of SPANX-A/D in melanoma cell lines was subsequently confirmed by immunoblot, in which the polyclonal anti-SPANX antibody labeled a unique band around 11kDa (Figure 4A). We further evaluated the expression of the SPANX-A/D subfamily in melanoma cells by LC-MS/MS but unfortunately we did not detect any isoform-specific peptide and the detection of the shared peptide TSESSTILVVR overlaps with in the NLS (Figure 4C). In contrast to our findings in spermatozoa, we neither found any phosphorylated residues within this peptide in A375 cells, further supporting our view that the phosphorylation state of the NLS is not determinant on the nuclear translocation of SPANX-A/D.

Functional characterization of SPANX-A/D protein family in human melanoma.

We and other have shown that SPANX-A/D proteins are present in metastatic melanoma cells [9], but the molecular mechanisms underlying their potential contribution to metastatic development remains unknown. Given the potential significance of SPANX-A/-C/-D in cell invasion [12], we further investigated the function of SPANX-A/D in melanoma cells. To this end, we generated a stable A375 human melanoma cell line with reduced expression of SPANX-A/D using lentiviral transduction of shRNAs targeting SPANX-A/D. Depletion of SPANX-A/D was confirmed using qRT-PCR, immunoblot and immunofluorescence, where in comparison to the control a reduction in expression of 70% was measured in SPANX-A/D-silenced A375 cells (Figure 5A, 5B, 5C).

We first analyzed cell proliferation with Prestoblue Assay (Thermo Scientific). SPANX-A/D-silenced cells exhibited a 35% reduction in cell proliferation compared to scramble shRNA-transduced control cells at 24 hours post-transduction. Next we analyzed the ability of SPANX-A/D to modulate A375 melanoma cell migration and invasion. Wound healing assay revealed that SPANX-A/D-silenced cells have a reduced ability to migrate towards the inside of the wound (Figure 5D). Scramble shRNA-transduced cells closed 45% of the wound, while SPANX-A/D shRNA-transduced cells closed 35% of the scratch. Therefore, SPANX-A/D deficiency led to 20% attenuated wound closure compared to control cells. Finally, we carried out a transwell migration assay to evaluate the of SPANX-A/D in cell

A



B

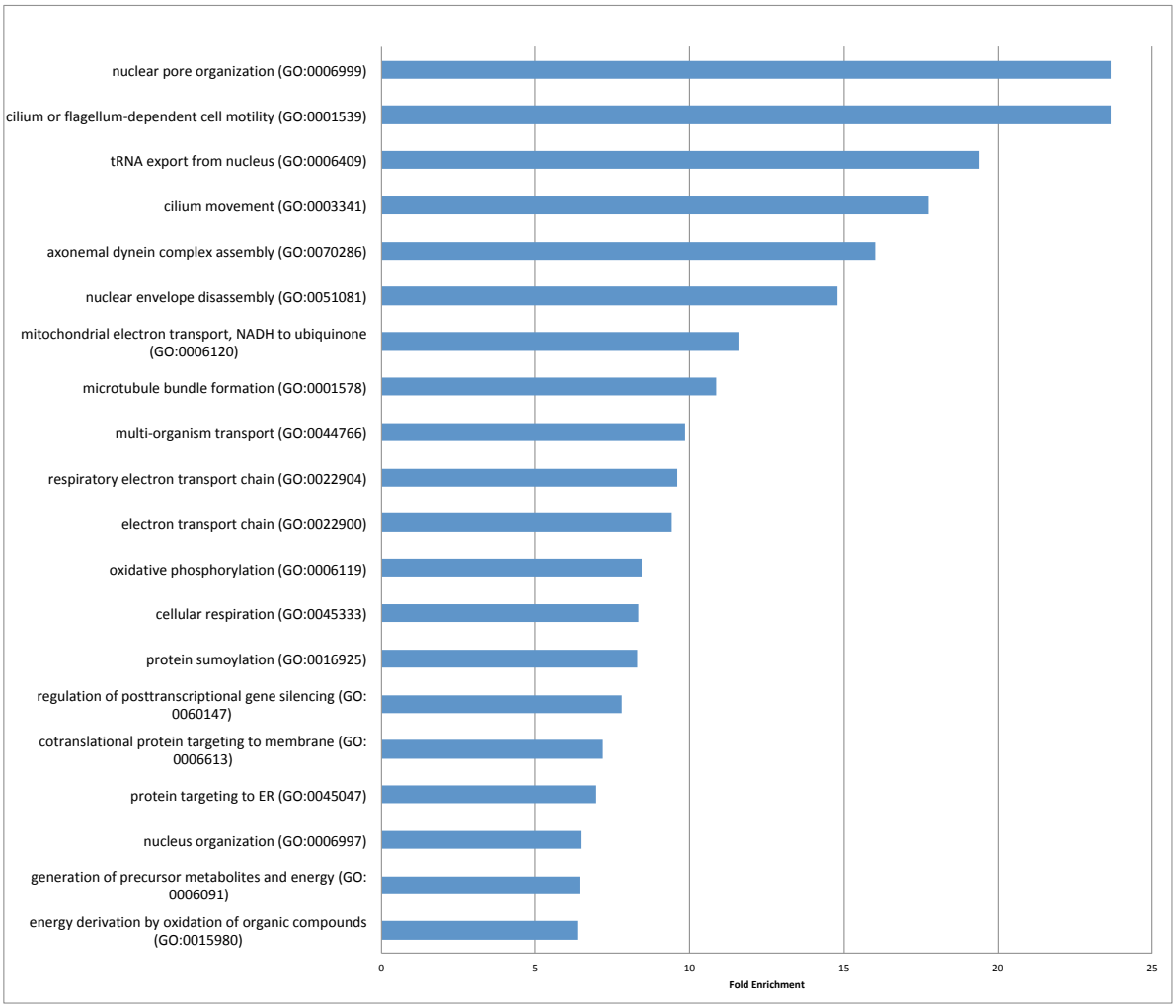
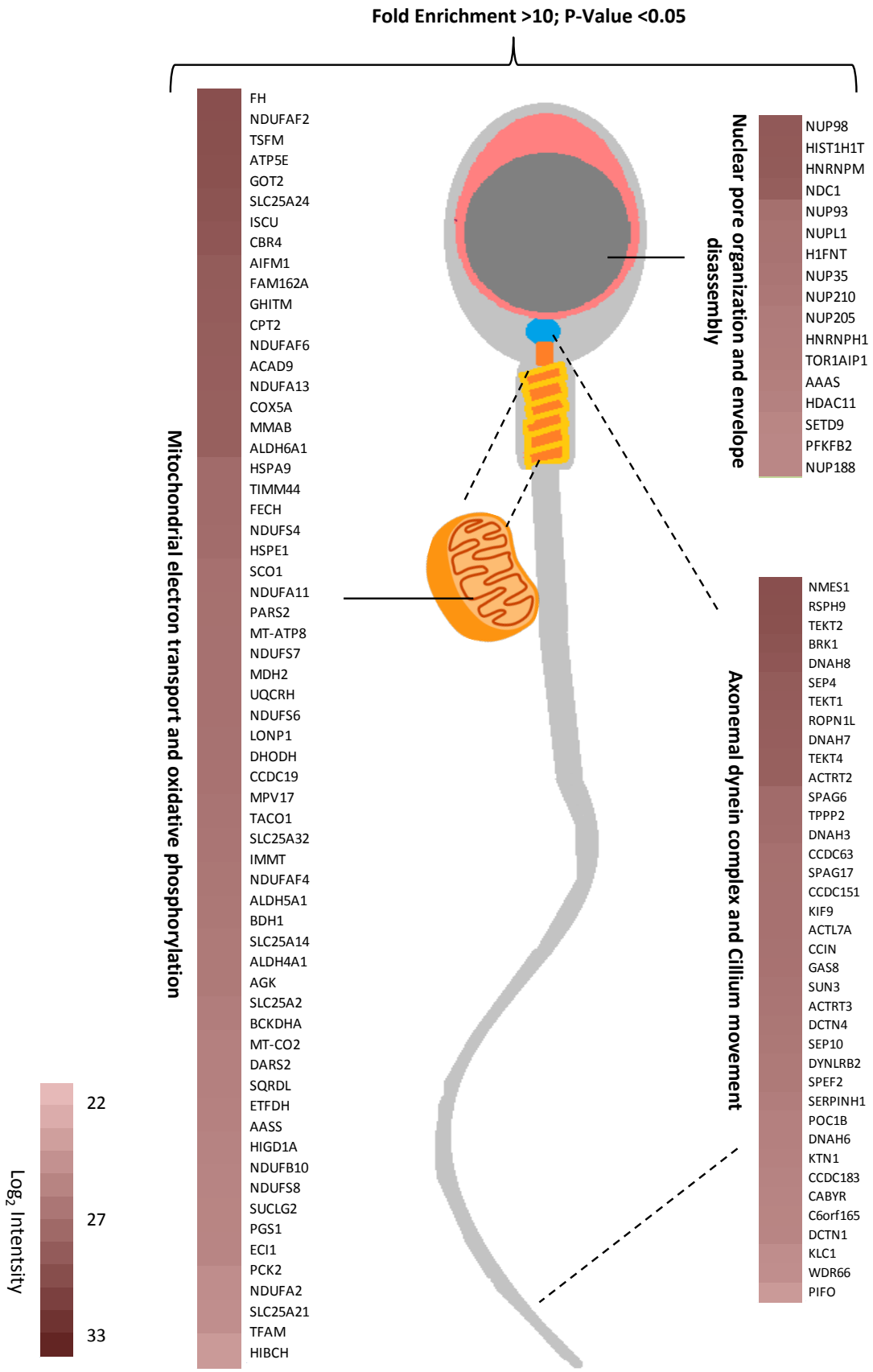


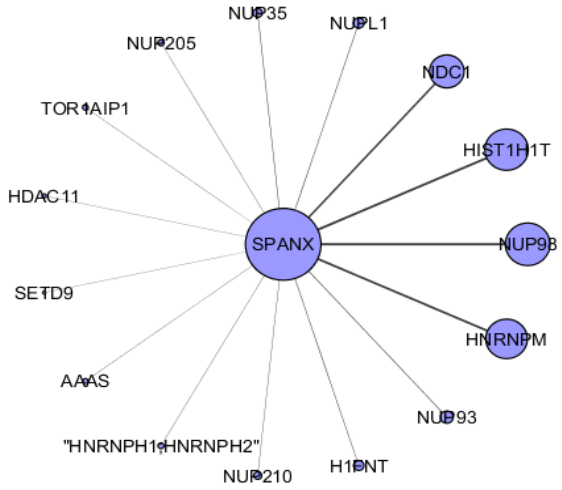
Figure 3. Study of the SPANX-A/D interactome in human spermatozoa. (A) Immunoprecipitation of SPANX-A/D in human spermatozoa (N=3) **(B)** Gene ontology analysis based on the biological functions of the SPANX-A/D possible interactors in human spermatozoa. The twenty most enriched processes are shown. $p<0,05$.

C

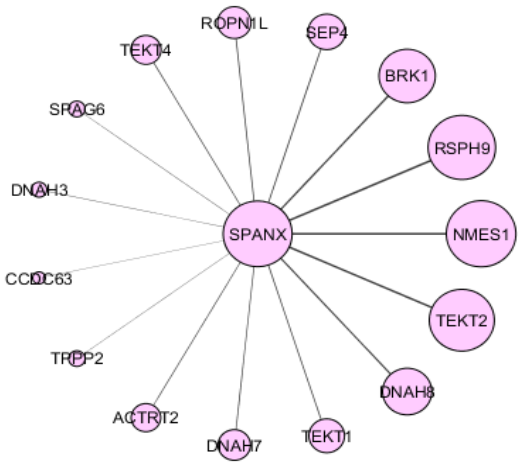

Fold Enrichment >10; P-Value <0.05
Mitochondrial electron transport and oxidative phosphorylation
FH
NDUFAF2
TSFM
ATP5E
GOT2
SLC25A24
ISCU
CBR4
AIFM1
FAM162A
GHITM
CPT2
NDUFAF6
ACAD9
NDUFA13
COX5A
MMAB
ALDH6A1
HSPA9
TIMM44
FECH
NDUFS4
HSPE1
SCO1
NDUFA11
PARS2
MT-ATP8
NDUFS7
MDH2
UQCRH
NDUFS6
LONP1
DHODH
CCDC19
MPV17
TACO1
SLC25A32
IMMT
NDUFAF4
ALDH5A1
BDH1
SLC25A14
ALDH4A1
AGK
SLC25A2
BCKDHA
MT-CO2
DARS2
SQRDL
ETFDH
AASS
HIGD1A
NDUFB10
NDUFS8
SUCLG2
PGS1
ECI1
PCK2
NDUFA2
SLC25A21
TFAM
HIBCH
Nuclear pore organization and envelope disassembly
NUP98
HIST1H1T
HNRNPM
NDC1
NUP93
NUPL1
H1FNT
NUP35
NUP210
NUP205
HNRNPH1
TOR1AIP1
AAAS
HDAC11
SETD9
PFKFB2
NUP188
Axonemal dynein complex and Cilium movement
NMES1
RSPH9
TEKT2
BRK1
DNAH8
SEP4
TEKT1
ROPN1L
DNAH7
TEKT4
ACTRT2
SPAG6
TPPP2
DNAH3
CCDC63
SPAG17
CCDC151
KIF9
ACTL7A
CCIN
GAS8
SUN3
ACTRT3
DCTN4
SEP10
DYNLRB2
SPEF2
SERPINH1
POC1B
DNAH6
KTN1
CCDC183
CABYR
C6orf165
DCTN1
KLC1
WDR66
PIFO
Log2 Intentsity
22
27
33

D

Nuclear pore organization and envelope dissasembly



Axonemal dynein complex and cillium movement



Mitochondrial electron transport and oxidative phosphorylation

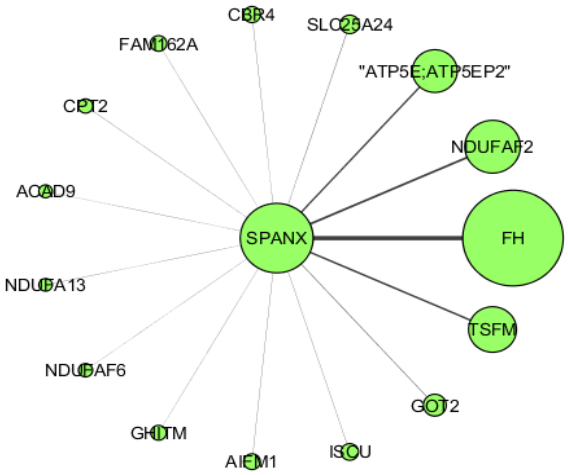


Figure 3. Study of the SPANX-A/D interactome in human spermatozoa. (C) Representative scheme of principal interactors of SPANX-A/D based on their localization in human spermatozoa and biological function. Only the 45 most intense interactors with no enriched biological function are shown. **(D)** Schematic representation of the 15 most intense interactors of each biological process. The size of the nodes and the thickness of the edges represent the intensity of each potential interactor.

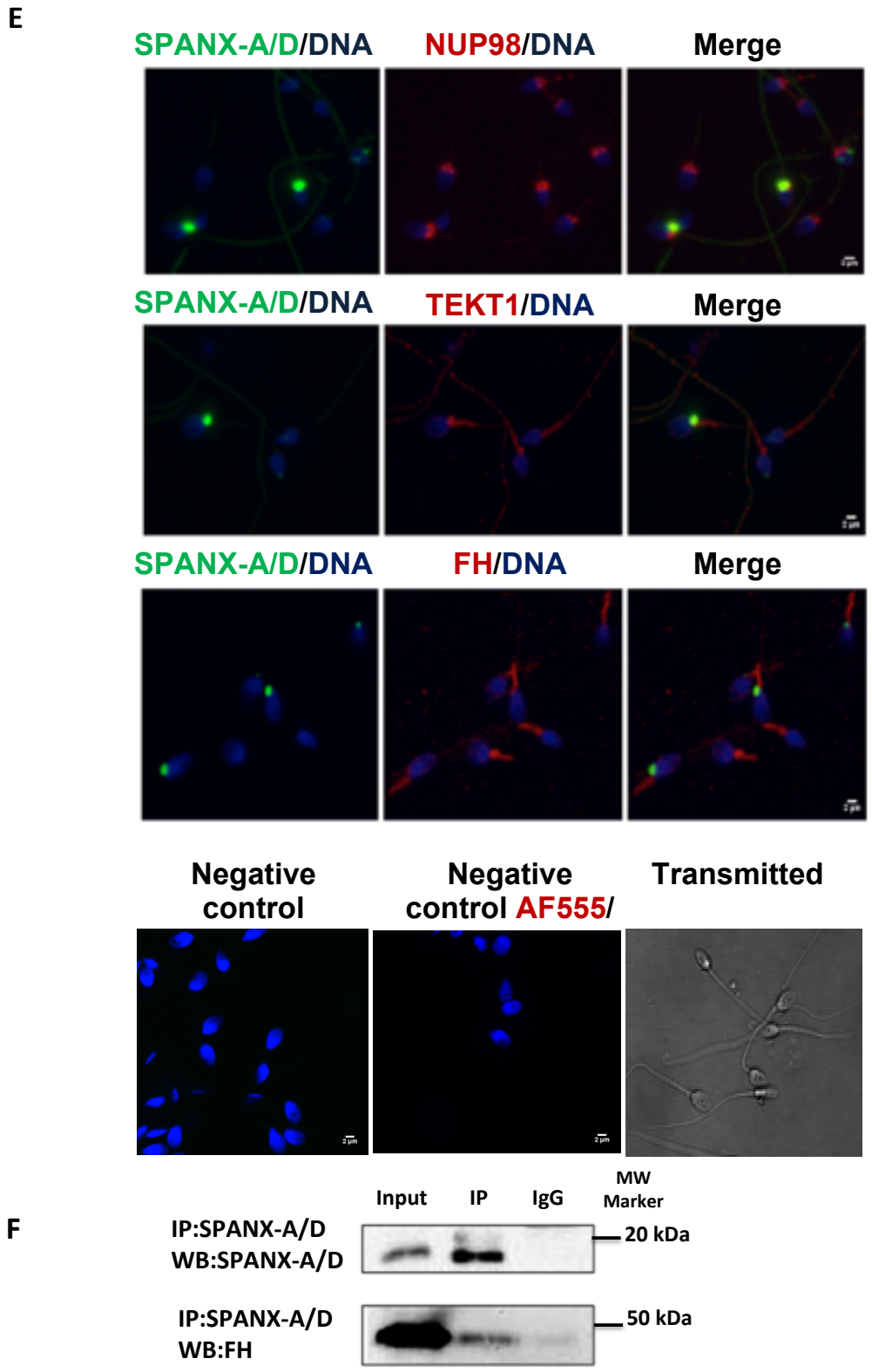


Figure 3. Study of the SPANX-A/D interactome in human spermatozoa. (E) Colocalization assays by immunocitochemistry of SPANX-A/D and NUP98, TEKT1 and FH. SPANX-A/D is represented in green, while the NUP98, TEKT1, and FH are shown in red. For the specificity of the secondary antisera, the primary antibodies were omitted. The nuclei were stained with Hoechst and are represented in blue. The transmitted image of human sperm is included to show the integrity of the sample. Scale bar 2 µm. **(F)** Validation of the interaction between SPANX-A/D and FH by coimmunoprecipitation. As negative control the non-specific IgGs were used.

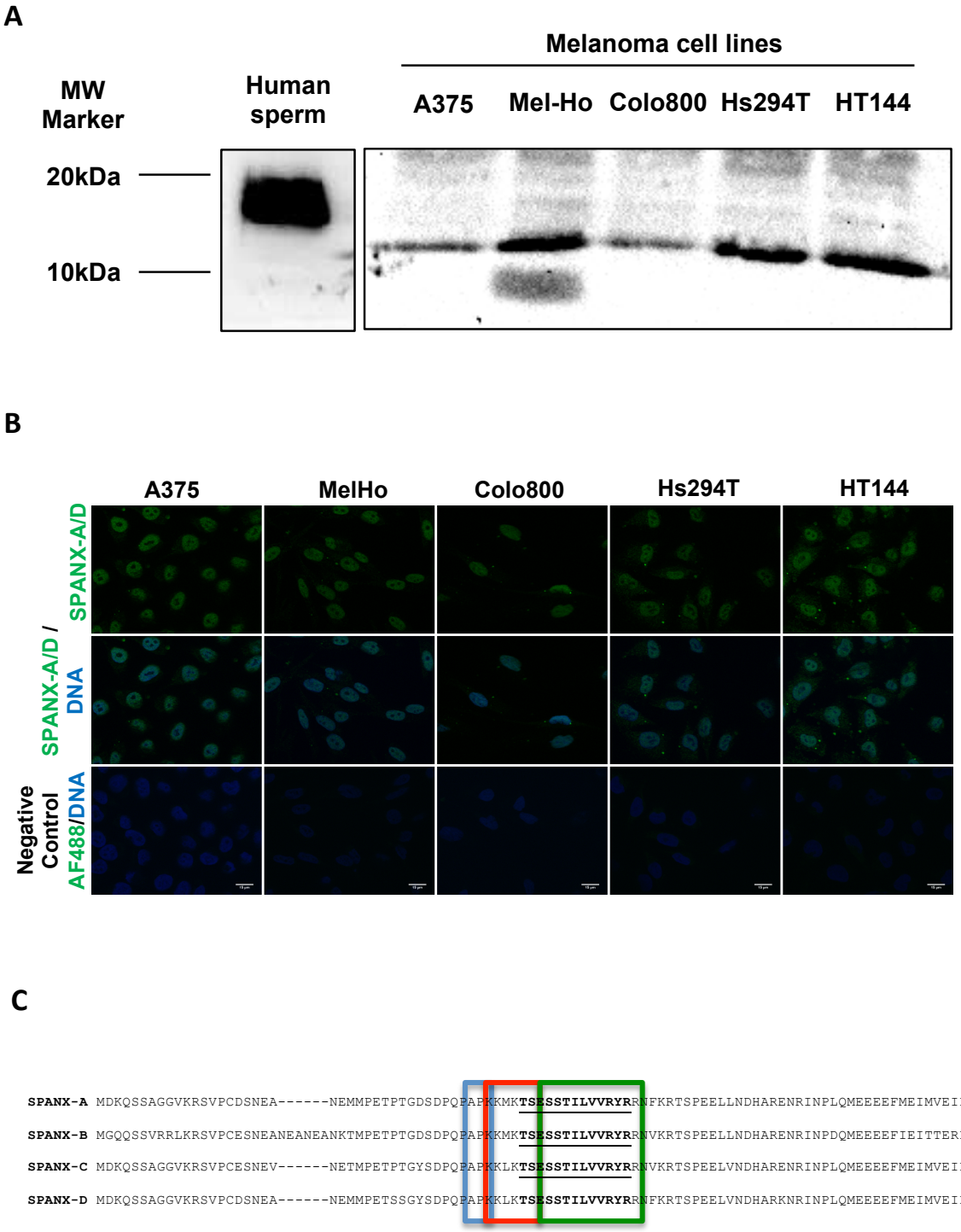


Figure 4. Characterization of SPANX-A/D in human melanoma cancer cells. **(A)** by Western blotting technique. A375, Mel-Ho, Colo800, Hs294T and HT144 human melanoma cancer cell lines were used. Human sperm lysate was used as positive control. (N=3) **(B)** by immunocytochemistry. For the specificity of the secondary antiserum, the primary antibody was omitted. The nuclei were stained with Hoechst and are represented in blue. Scale bar 15 µm. (N=4) **(C)** Comparison of each SPANX isoform sequences with the three overlapping consensus nuclear localization signals indicated with boxes. The peptide found by LC-MS/MS is in bold and underlined for all isoforms.

migration (Figure 5E). We observed that, on average, 90 and 149 cells per field (10x) migrated through the transwell pores in SPANX-A/D-silenced and control condition, respectively, indicating that SPANX-A/D silencing reduced by up to 35% the potential of A375 melanoma cells to migrate. Altogether, these findings reveal that SPANX-A/D play a role in essential processes important for *in vitro* metastatic development. Aiming to elucidate the mechanisms that may underlie the role of SPANX-A/D in melanoma cell migration, we looked for SPANX interacting proteins in A375 melanoma cells. For that purpose, we over-expressed YFP-SPANX-A in A375 cells and carried out a MS-based proteomics approach in 4 independent biological replicates. The efficiency of all the pulldowns performed was evaluated by immunobloting. To consider a protein as a putative binding partner of SPANX in melanoma, it should be detected at least in three out of the four YFP-SPANX-A pulldowns respect to the control ones. Overall, four proteins were identified followed this criteria: Histone H4 (HIST1H4A), Lamin A/C (LMNA), 60S ribosomal protein L8 (RPL38) and Heat shock cognate 71kDa protein (HSPA8) (Figure 6A, 6B and supplemental table S2). The interaction between SPANX-A and Lamin A/C was subsequently confirmed by direct immunoblotting Figure 6C), and both proteins co-localized in the nuclear envelope of A375 melanoma cells (Figure 6D).

To evaluate a potential relationship among putative SPANX interactors in melanoma, we generated an interactor network using the biological network integration platform GeneMANIA in which only physical interactions and co-expressed protein relationship were analyzed (Figure 6E). This analysis did not reveal a direct physical interaction between the identified proteins. However, LMNA showed a strong physical interaction with the chaperone DNAJB1 which, in turn, is known to associate with HSPA8 [16]. Additionally, the network identified a physical interaction between LMNA and COL8A2, a recognized binding partner of HIST1H4A [17]. Of note, the interactor network linked predominantly all those proteins with the exon junction protein complex (EJC), an RNA-binding protein complex that binds to exon–exon junctions during splicing [18]. The EJC is composed by four core components, the eukaryotic initiation factor 4A-III (EIF4A3), Protein mago nashi homolog (MAGOH), RNA-binding protein 8A (RBM8A), and Barentsz (BTZ) [18]. As seen in Figure 6E, SPANX-A interactors identified in our analysis, HIST1H4A, HSPA8, LMNA and RPL38, are connected to EIF4A3 and MAGOH proteins either directly or indirectly.

Although further analyses are necessary, our results suggest that SPANX-A/D interactors may provide insight into how regulatory functions in the nucleus promote invasion and metastasis in human melanoma.

DISCUSSION

The presence of SPANX-A/D subfamily has been widely studied in a broad variety of cancer types over the last twenty years [1, 9, 10], in an attempt to clarify the potential role of this protein subfamily in tumorigenesis. In 2001, these proteins were described for the first time in testis and spermatozoa [1], being their role completely unknown. Therefore, elucidating the physiological function of SPANX-A/D proteins in male fertility can help to understand more in deep their role

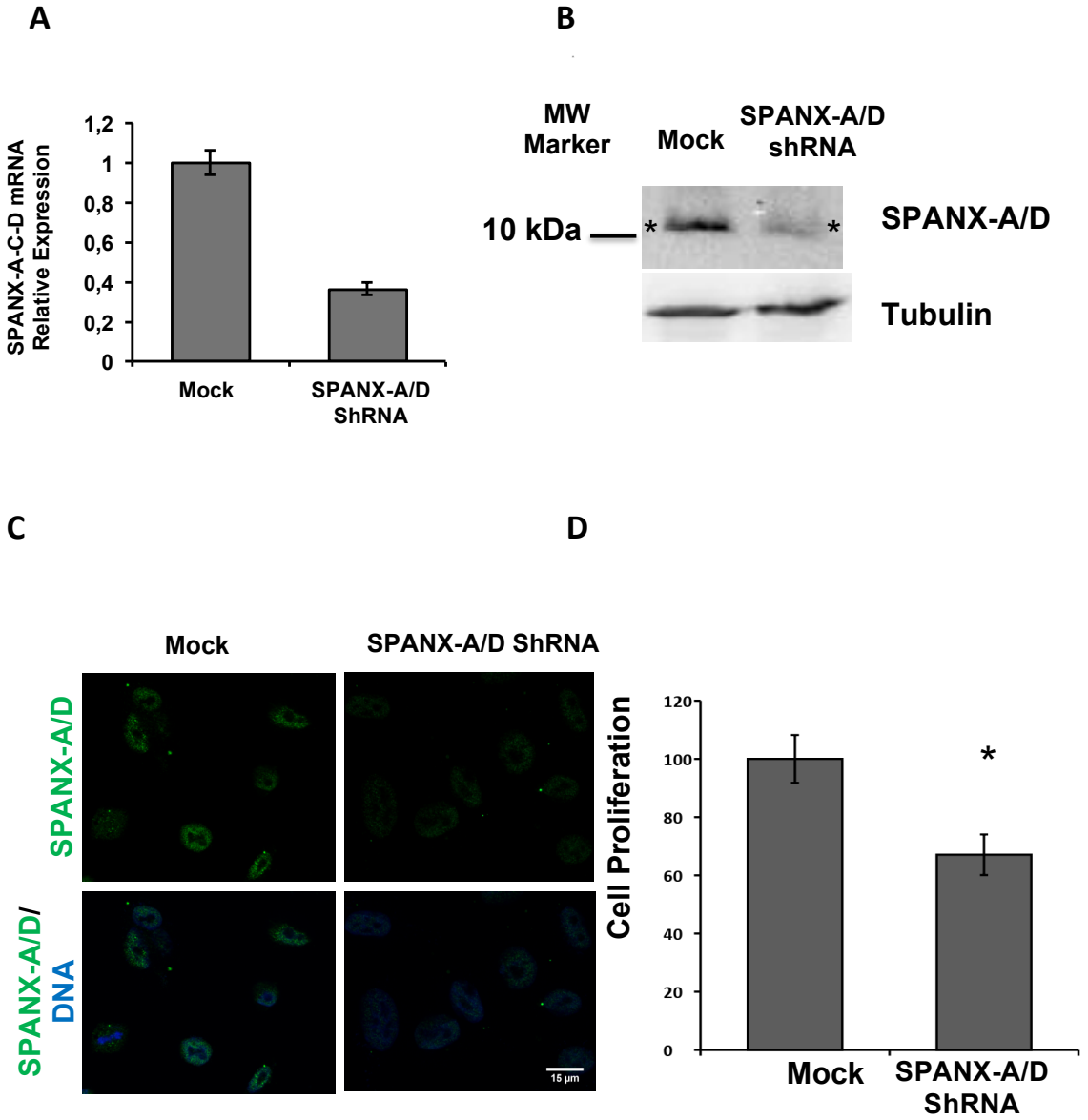


Figure 5. Effect of the SPANX-A/D reduction of proliferation and migration of A375 human melanoma cell line. Silencing of SPANX-A/D **(A)** at mRNA level by RT-qPCR assays **(B)** at protein level by Western blotting. * indicate the bands of the SPANX-A/D protein family and **(C)** immunocytochemistry assays after transfecting A375 cells with shRNA targeting SPANX-A/D. Mock were transfected with scramble shRNA. Nuclei were stained with Hoechst and are represented in blue. Scale bar 15 µm. (N=3). Effect of A375 cells transfected with shRNA SPANX-A/D on **(D)** Cell proliferation. Results are presented as percent proliferation comparing scamble shRNA vs shRNA SPANX-A/D cells

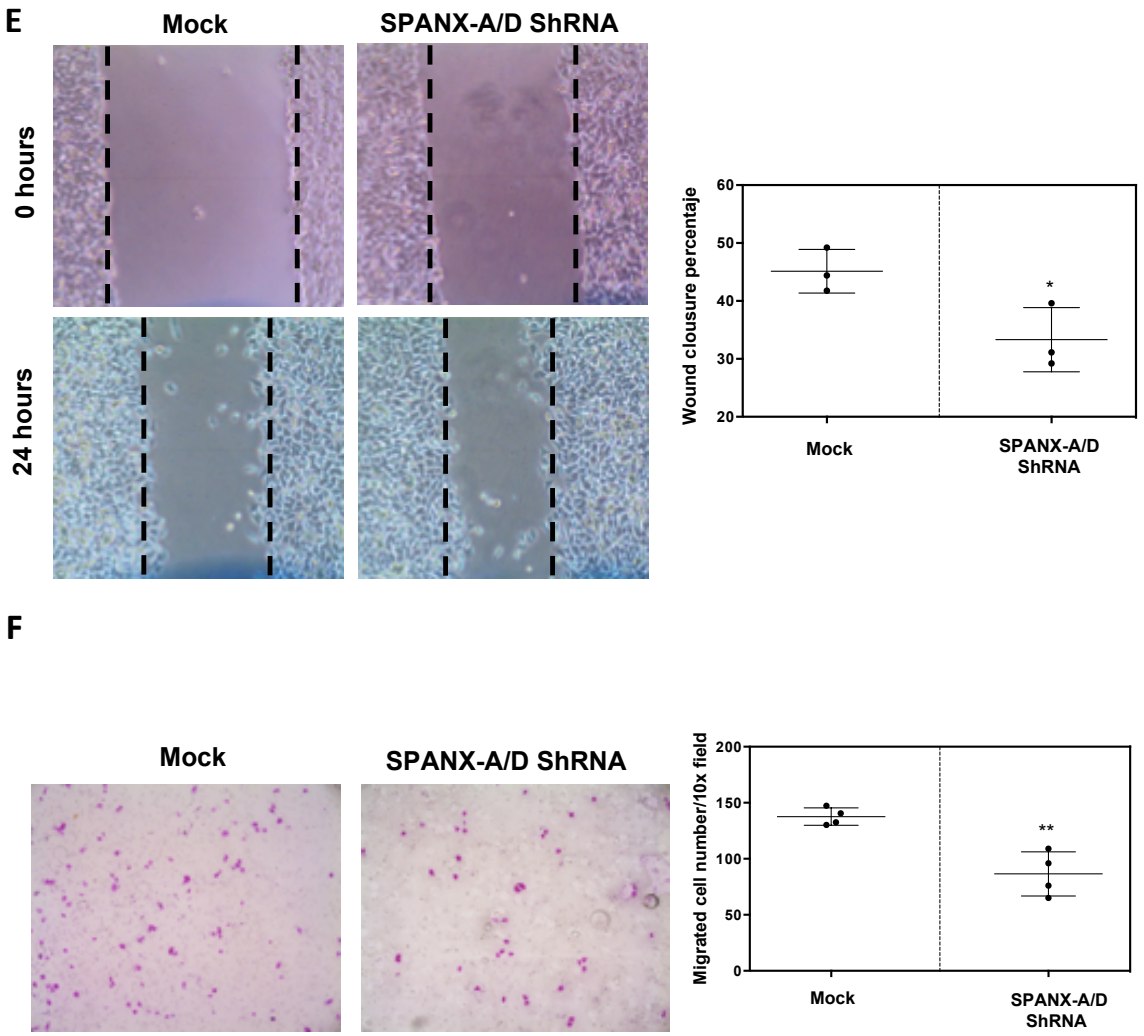
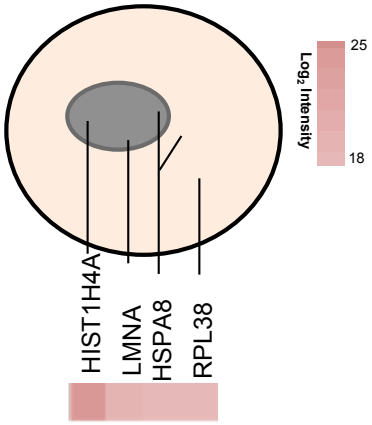


Figure 5. Effect of the SPANX-A/D reduction of proliferation and migration of A375 human melanoma cell line. Effect of A375 cells transfected with shRNA SPANX-A/D on **(E)** Cell migration. Photographs were taken at T=0 to use them as initial wound area and after 24 hours of incubation (T=24h), using inverted light microscope. Results are presented in a graph as wound closure percentage comparing scamble shRNA vs shRNA SPANX-A/D cells. The closed area was calculated using the Image J software by means of initial wound area at T=0 – T=24. **(F)** Transwell migration. Results are expressed as the average of total number of migrated cells in 20x field and compared scamble shRNA vs shRNA SPANX-A/D cells. The number of invading cells was quantified by counting stained cells in random fields of the membrane. The data shown in graphs (D), (E) and (F) correspond to the mean of three independent experiments, and error bars indicate the SEM. *P<0.05; **P<0.01 (Students T-test).

A

Protein name	Gene name	Localization	Function
Histone H4	HIST1H4A	Nucleus	Chromatin Organization
Lamin A/C	LMNA	Nucleus	Nuclear assembly, Chromatin Organization, Nuclear Membrane and Telomere Dynamics
Heat shock cognate 71 kDa protein	HSPA8	Nucleus and Cytoplasm	Protein transport and folding
60S ribosomal protein	RPL38	Cytoplasm	Protein translation

B



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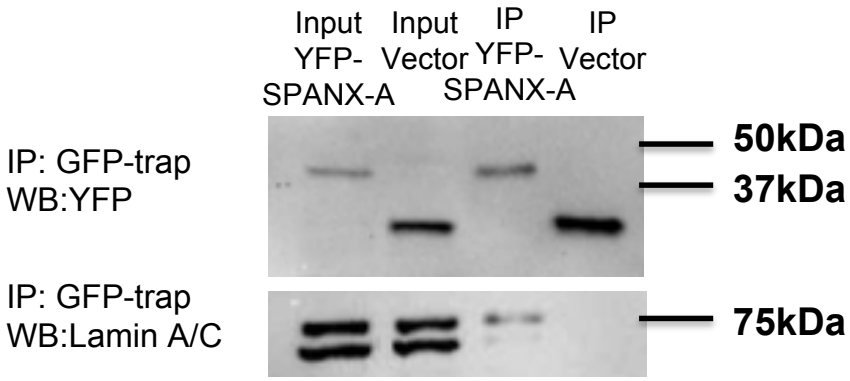

Figure 6. Figure 6. Study of the YFP-SPANX-A interactome A375 human melanoma cell line. (A) Table containing the protein name, gene name, localization within the cell and biological function of the putative interactors of YFP-SPANX-A in A375 melanoma cell line. **(B)** Representative scheme of the possible interactors of SPANX-A based on their localization and intensities in A375 cell line. **(C)** Validation of interaction betwenn YFP-SPANX-A and Lamin A/C by coimmunoprecipitation and Western Blotting (N=3).

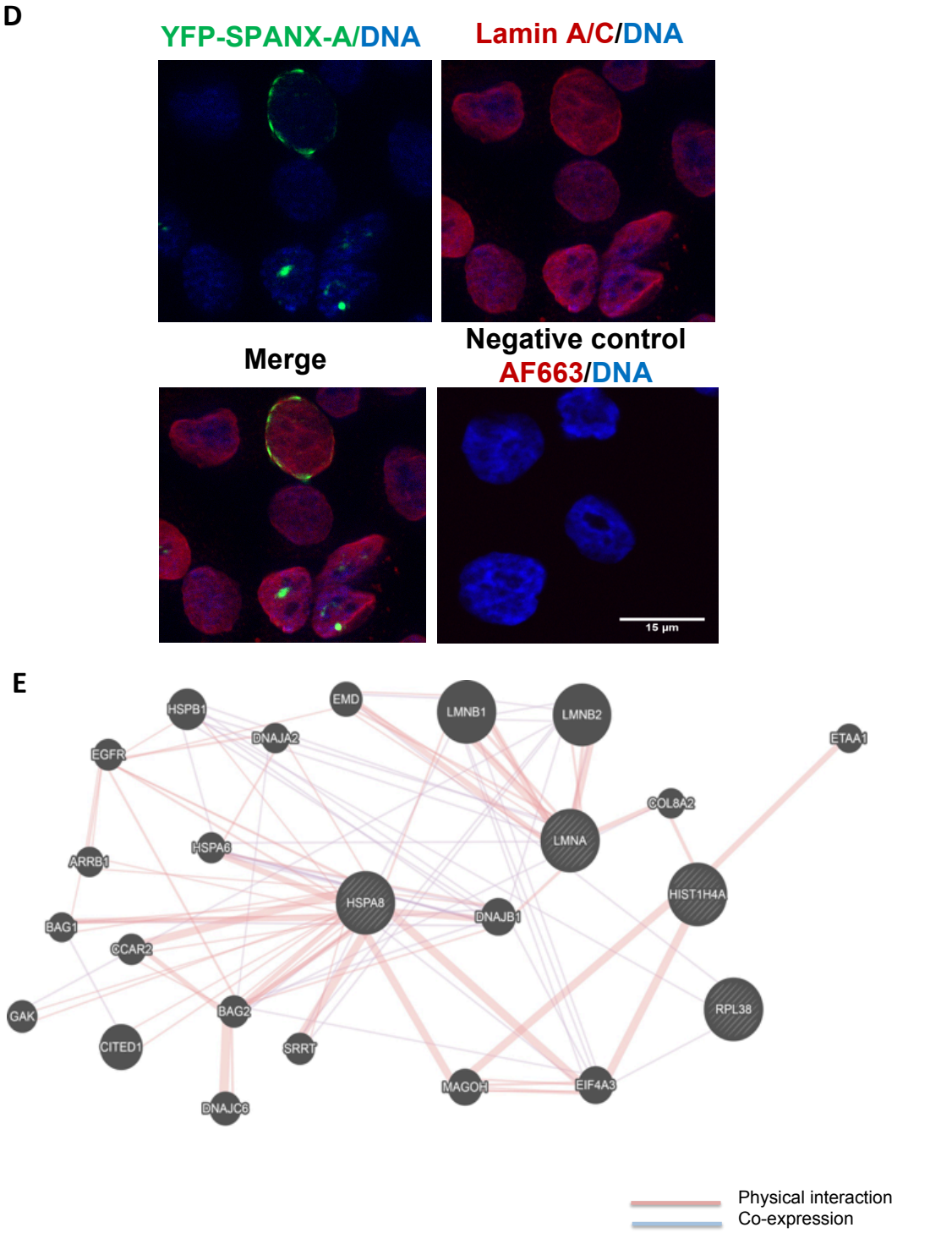

Figure 6. Study of the YFP-SPANX-A interactome A375 human melanoma cell line. (D) Validation of the colocalization between YFP-SPANX-A and Lamin A/C by inmunofluorescence. YFP-SPANX-A is represented in green and Lamin A/C is shown in red. For the specificity of the secondary antiserum, the primary antibody was omitted. The nuclei were stained with Hoechst and are represented in blue. Scale bar 15 µm. (N=3) **(E)** Representation of working model of protein interactions belonging to the putative interactors of YFP-SPANX-A in A375 human melanoma cell line. Physical interactions between different proteins are shown in pink, while data regarding co-expression are colored in purple. Putative protein interactors are indicated in stripped circles. The presented model is created using the interactive functional association network tool GENEMANIA (http://genemania.org/).

in tumorigenesis. Through a combination of proteomic analysis, site-directed mutagenesis and function testing, we found that SPANX-A/D is a mutifunctional protein family that plays different roles in human spermatozoa and melanoma cells, being essential for nuclear envelope function in both physiological and physiopathological processes (Figure 7).

Physiologically, SPANX-A/D is a multigene family mapped on the X chromosome exhibiting, among normal tissues, testis-specific expression and mRNA/protein localization exclusively in post-meiotic spermatids [2, 13]. Consistent with previous studies [1, 13], we have also reported the expression of SPANX-A/D family in human spermatozoa, confirming its nuclear localization at the nuclear craters which are known to be related to genome instability and sperm fertility [19]. We also corroborate here the presence of this protein family at the neck of the spermatozoon and over the acrosome [1, 7] and we add extra information about its expression all over the flagellum. Proteomic analyses confirmed the expression of all isoforms of SPANX-A/D subfamily in human spermatozoa and seven novel phosphosites along the SPANX-A/D protein sequences were described (Ser^{5}, Ser^{13}, Ser^{18}, Thr^{28}, Ser^{47}, Ser^{48}, and Thr^{49}. Although some SPANX phosphosites have been previously reported in different cancers cell types (https://www.phosphosite.org/siteAction.action?id=11076738, 11076741, 9161501, 50832232, 11076732, 11076735), this is the first study reporting SPANX phosphorylated residues in human spermatozoa. Three of these phosphosites lie within the NLS. However, site-directed mutagenesis analyses showed that phosphorylation state is not a critical determinant on SPANX-A/D nuclear translocation. Nevertheless, phosphorylation-mimicking mutations appeared to promote the stabilization of the SPANX-A complex into nucleoplasm, suggesting that this post-translational modification can potentially influence the nuclear function of SPANX-A/D.

Taking into account their widespread localization in human spermatozoa, SPANX-A/D proteins could play different roles in sperm differentiation and fertility acquisition. Since the expression of SPANX-A/D subfamily appears to be restricted to hominoids [6], knockout mice are not useful models for elucidating its biological function. Importantly, human spermatozoa present a unique proteome with at least a 30% of sperm-specific proteins [20]. Therefore, to gain a better understanding of SPANX–A/D physiology, we carried out an interactome analysis of the endogenous protein in human spermatozoa. Our proteomic analysis confirmed a multifunctional role of SPANX-A/D protein family in human spermatozoa. We identified 307 proteins that co-immunoprecipitated with SPANX-A/D, reporting the most comprehensive analysis of the endogenous protein interactome to date. Our findings indicate that SPANX-A/D is involved in nuclear envelope disassembly and nuclear pore organization (Figure 7A). The immunohistochemical localization of SPANX-A/D has previously suggested a role in nuclear pore organization [1]. Our results confirm this hypothesis since we found that SPANX-A/D co-precipitates with several nuclear pore proteins such, as NUP98, NUP93, NUP35 and NUP188.

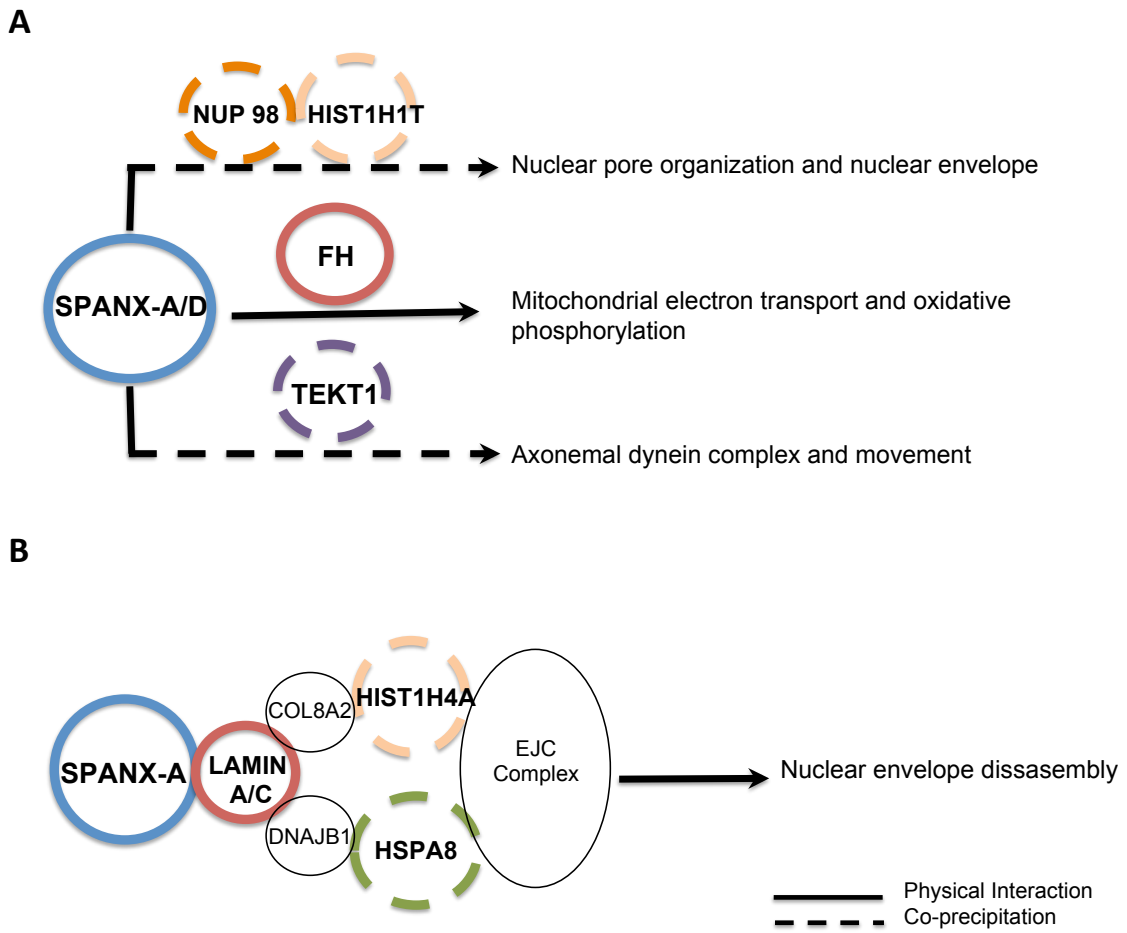

Figure 7. Role of SPANX-A/D in human spermatozoa and melanoma. Schematic representation of molecular mechanisms in which SPANX-A/D is involved **(A)** in human spermatozoa and **(B)** A375 melanoma cell line. Physical interactions are represented with a continuous line while the co-expression is shown with a discontinuous line.

SPANX-A/D and NUP98 co-localized at the neck of the sperm cells, which is found to be a redundant nuclear envelope in mature spermatozoa [21]. Nuclear pore complexes (NPCs) regulate the transport of macromolecules between the nucleus and cytoplasm and are known to directly interact with chromatin and to influence transcription [21]. Consistent with this, we found that SPANX-A/D co-precipitated not only with several NPC proteins but also with chromatin regulators such as SETD9 and histones (HIST1H1T). Together with the fact that SPANX-A/D proteins are reported to be basic proteins able to interact with nucleotide acids [1], these findings, suggest that SPANX-A/D can be a scaffold protein in the regulation of chromatin organization, suggesting a molecular mechanism that may underlie the role of this protein family in sperm fertility.

The interactome analysis also indicates that SPANX-A/D can regulate novel physiological functions related to mitochondrial metabolism, cilium organization and flagellum-dependent motility (Figure 7A). The contribution of metabolic/energy pathways to sperm motility has been previously reported,

since it is known that the beat frequency of the flagellum is directly related to the production rate of energy from ATP [22–25]. The axonemal dyneins generate force through the hydrolisis of the ATP created in the mitochondria and bind to the structural components of the microtubules like tektins, to actively participate in the flagellar movement [26]. Consistent with the fact that low expression of SPANX-A/D was reported in patients with sperm motility deficiency (asthenozoospermic patients) [27, 28], our findings suggest that SPANX-A/D proteins could be essential in human sperm to meet energy demands for sperm motility. In fact, comparative proteomic analysis carried out for asthenozoospermic patients highlighted this association. A down-regulated expression of proteins related to flagellum organization, and cell energy production, such as FH and TEKT1 has been reported [29], that we found co-precipitated with SPANX A/D in human spermatozoa.

Like other CTAs, SPANX-A/D proteins, are emerging as strong candidates for cancer immunotherapy [9, 11, 12]. In this regard, a more detailed understanding of the role of this protein family in skin tumorigenesis is required to fulfil its therapeutic potential. It is possible that SPANX-A/D proteins may have implications well beyond immunotherapy and may represent previously unrecognized functions for tumour cell biology. After evaluating its physiological role in human spermatozoa, our aim was to elucidate if the pathological role of SPANX-A/D in melanoma is related to nuclear envelopment and pore organization, cellular movement or cellular metabolism. Our results indicate that SPANX-A/D can also promote cell migration in melanoma cells by regulating nuclear functions. Functional analyses have reported a role of SPANX-A/D in different pro-tumoral processes such as proliferation, migration and invasion, as has been previously described in breast and lung cancer [12, 30]. To further analyse the molecular mechanism underlying SPANX-A/D proteins in human melanoma, we performed an interactome analysis, identifying four proteins (HIST1H4A, Lamin A/C, the chaperon HSPA8 and RPL38) that co-precipitated together with SPANX-A. Consistent with previous studies performed with SPANX-C [12], SPANX-A localized at the nuclear envelope and interacts with Lamin A/C. Lamin A/C are components of a meshwork of nuclear lamina proteins that underlay the inner nuclear membrane and provide structural stability to the nucleus (Dahl, 2004; Swift and Dischер, 2014; Berk et al., 2013). In this regard, the complex Lamin A/C-SPANX A can modulate chromatin accessibility in melanoma cell lines. Additionally, we demonstrated that SPANX-A is able to co-precipitate with the histone HIST1H4A (Figure 7B). Furthermore, the chaperon Heat shock cognate 71 kDa protein (HSPA8) that co-precipitates with SPANX-A, is known to be part of the spliceosome complex [32]. HSPA8 together with its chaperonin DNAJB1 is involved in mRNA splicing (Stricher et al., 2013, Stepanov et al., 2013), interacting with the EJC complex (Figure 7B). A potential SPANX A-Lamin A/C-DNAJB1-HSPA8 complex, therefore, could also interact with EJC complex that is the complex responsible for the catalytic activity of the spliceosome [18]. Although further analyses are required, our results suggest that SPANX-A/D

protein family, by scaffolding the formation a multi-protein complex, could be part of the spliceosome pathway, playing a role in mRNA splicing in melanoma cells.

In summary, our findings suggest that SPANX-A/D is a multifunctional protein family, with a physiological role in nuclear envelope, sperm motility and metabolism in human spermatozoa; and with a pathological role in migration of melanoma cells. Being a scaffold protein to form protein complexes into nuclear envelope, SPANX-A/D can promote both its physiological and pathological roles by regulating nuclear functions such as chromatin accessibility. A more in deep knowledge of the physiological function of SPANX-A/D family in human spermatozoa would be essential for understanding not only sperm fertility but also the pathological role of these proteins in male infertility and tumorgenesis, allowing to identify new therapeutic targets for male infertility and skin tumors as well as to develop of safe male contraceptives and tumor-directed pharmacological drugs.

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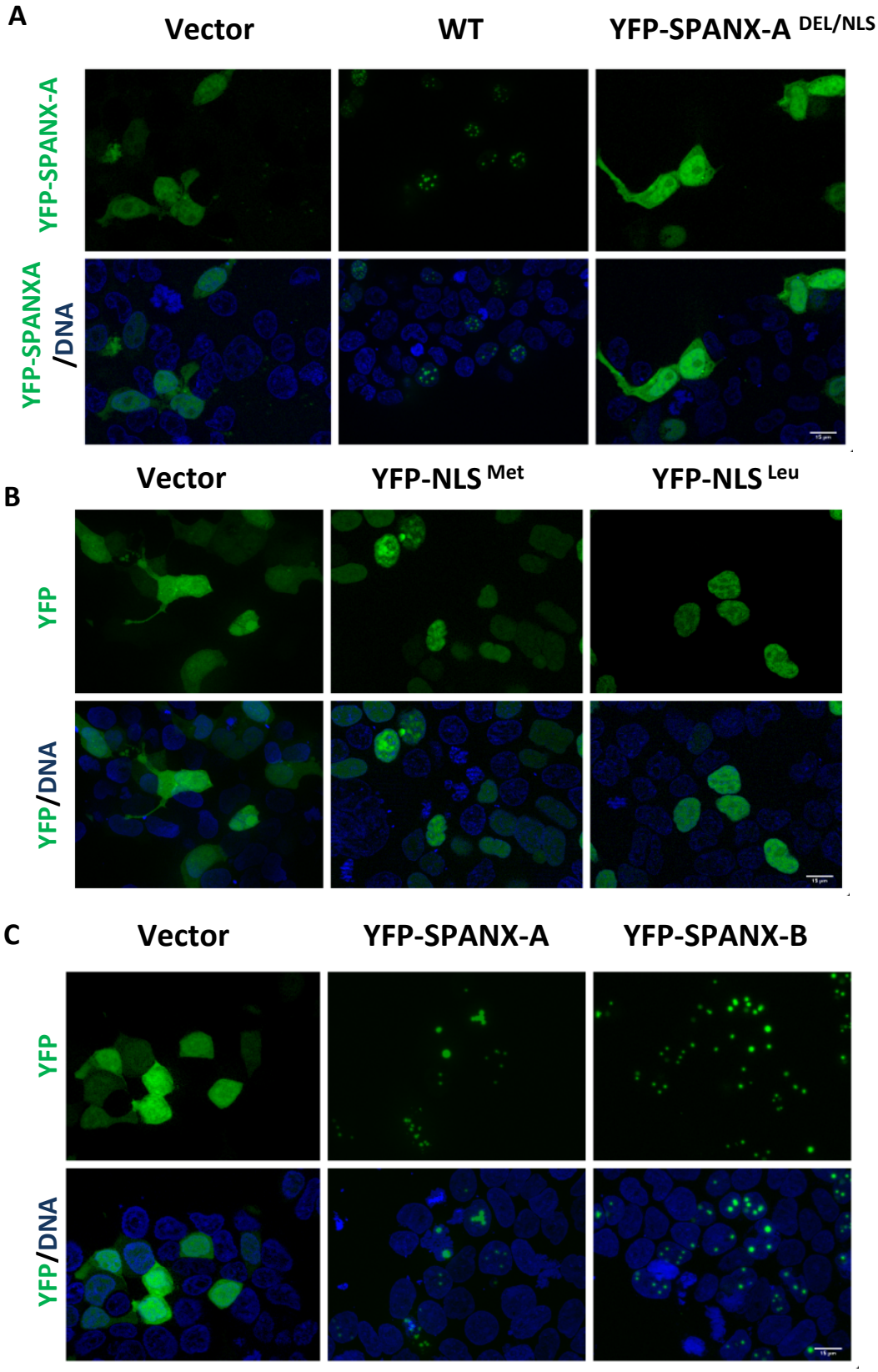
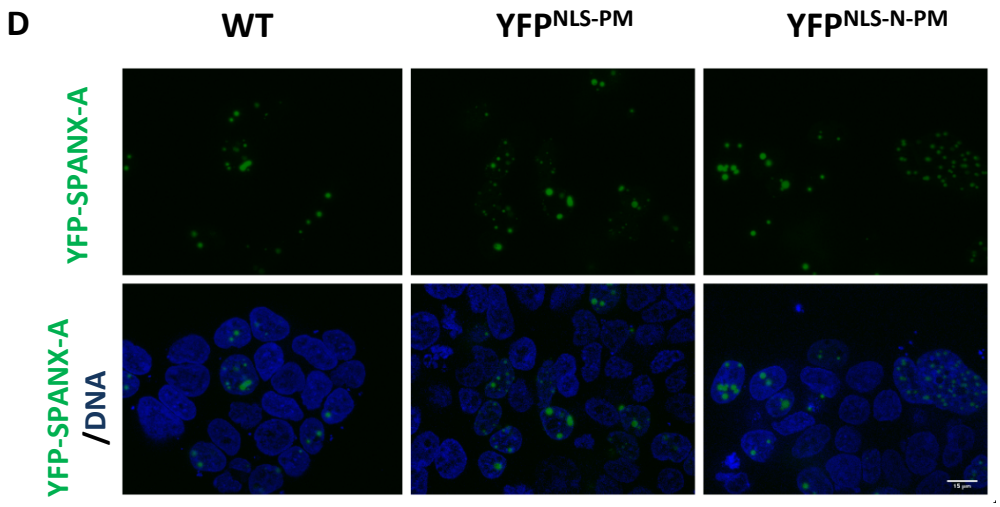
Supplemental Figures

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WT
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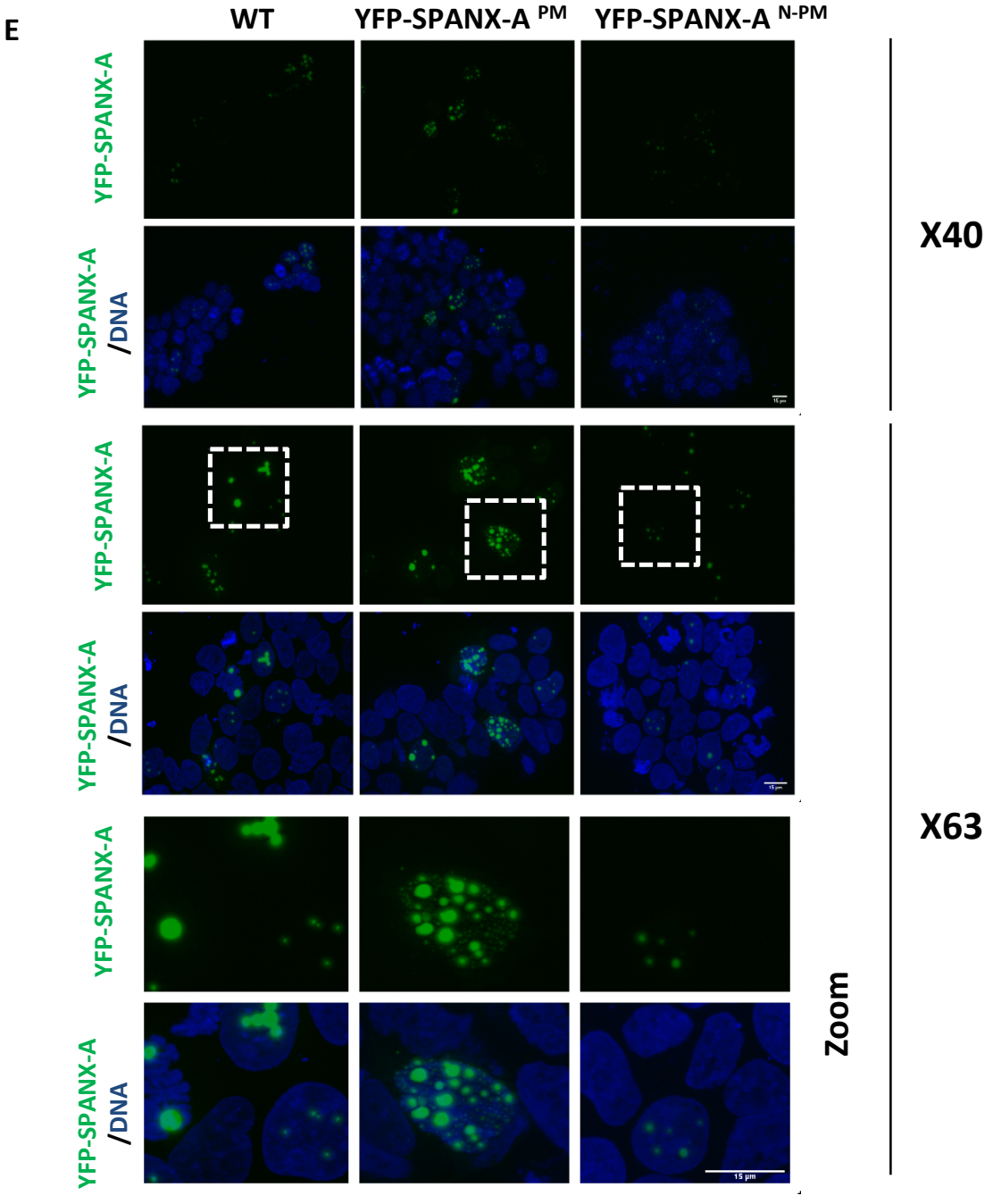
YFP^NLS-N-PM
YFP-SPANX-A
YFP-SPANX-A /DNA


E
WT
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YFP-SPANX-A /DNA
X40
X63
Zoom
15 µm

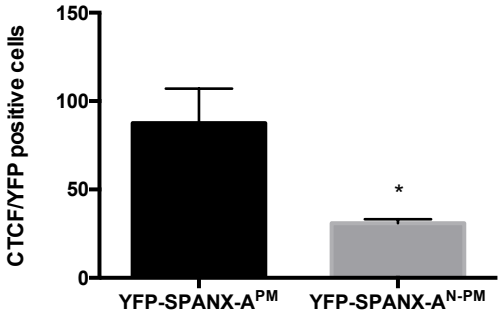


Figure supplement 1. Mutagenesis studies of SPANX-A/D protein subfamily in HEK293T cells. Confocal microscopy images showing representative examples of HEK293T cells transfected with expression plasmids enconding **(A)** YFP (vector), WT and WT mutant with the deleted NLS (YFP-SPANX-$A^{NLS/DEL}$) (x63). DAPI was used to counterstain the nucleus (DNA panels) (N=3) **(B)** YFP, the NLS of the WT with Methionine (YFP-NLS^{MET}) and Leucine (YFP-NLS^{LEU}) at the 42^{nd} positions of the sequence (x63). (N=3) **(D)** YFP, SPANX-A (WT) and SPANX-B (x63). (N=3) **(C)** WT and the phospho-mimetic (YFP-NLS^{PM}) and dephospho-mimetic mutants (YFP-NLS^{N-PM}) at the 47^{th}, 48^{th} and 49^{th} positions of the NLS (x63). (N=3) **(D)** WT and the phospho-mimetic (YFP-SPANX-A^{PM}) and dephospho-mimetic mutants (YFP-SPANX-A^{N-PM}) of the 5^{th}, 13^{th} , 18^{th}, 28^{th}, 47^{th}, 48^{th} and 49^{th} positions of the WT sequence (x40 and x63). The zoomed section appears framed by a dotted line Scale bar: 15 µm. (N=3) Graph showing the CTCF intensity/YFP positive cells of the YFP-SPANX-A^{PM} vs SPANX-A^{N-PM} mutants. The data shown in the graph correspond to the mean of three independent experiments, and error bars indicate the SEM. *P<0.05 (Students T-test).

Figure supplement 2. 3D reconstruction of the localization YFP-SPANX-A and YFP-SPANX-B isoforms in HEK293T cells. Confocal microscopy images showing representative examples of HEK293T cells transfected with expression plasmids enconding **A)** YFP-SPANX-A and **B)** YFP-SPANX-B.

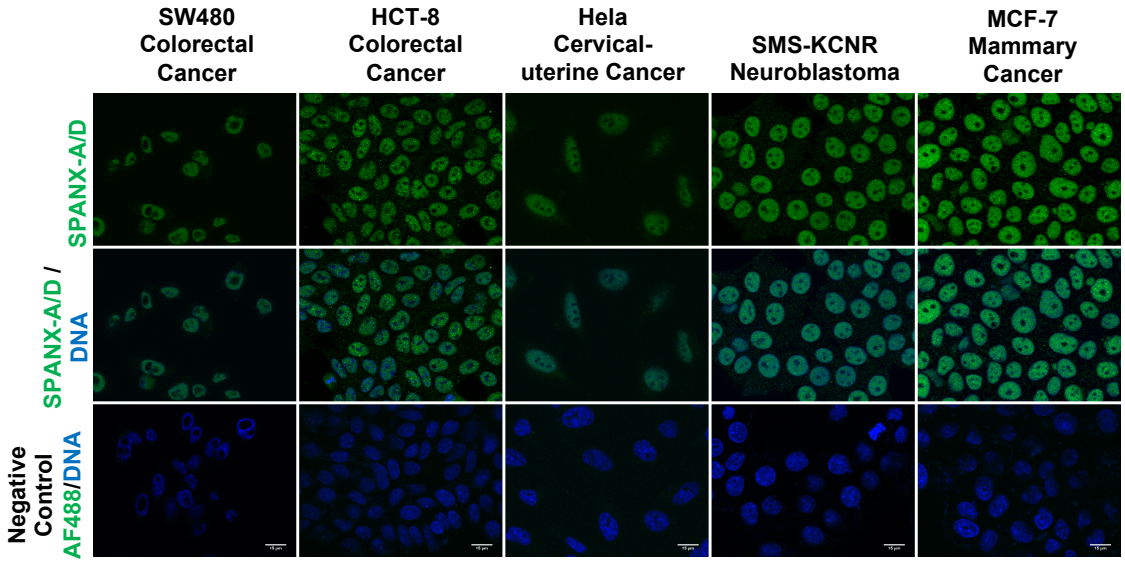


Figure supplement 3. Characterization of SPANX-A/D in cancer cells by immunocytochemistry. Colorectal cancer cell lines (SW480 and HCT-8), cervical uterine cancer cells (HeLa), neuroblastoma cells (SMS-KCNR) and mammary cancer cells (MCF-7) were immunostained with the anti-SPANX antibody and the staining was analyzed by confocal microscopy. For the specificity of the secondary antiserum, the primary antibody was omitted. The nuclei were stained with Hoechst and are represented in blue. Scale bar 15 µm. (N=3).

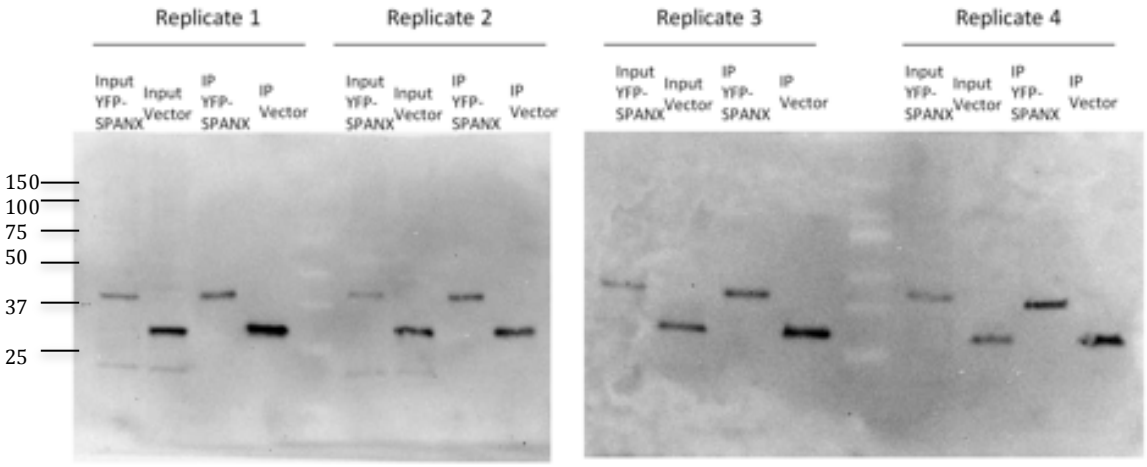


Figure supplement 4. YFP-SPANX-A immunoprecipitation in A375 melanoma cell line. A375 cells were transfected with the YFP vector and YFP-SPANX-A mutant. Immunoprecipitation was performed using the GFP-trap_MA antibody. Western blotting was performed using the anti-GFP antibody. Molecular weight of YFP-SPANX-A is around 37 kDa, whereas molecular weight of the vector is around 25 kDa. Input corresponds to total protein lysate.

Eranskinak
Appendixes

I.Eranskina. U50488H agonistaren efektua giza espermatozoideen SPANX-A/D proteina familian

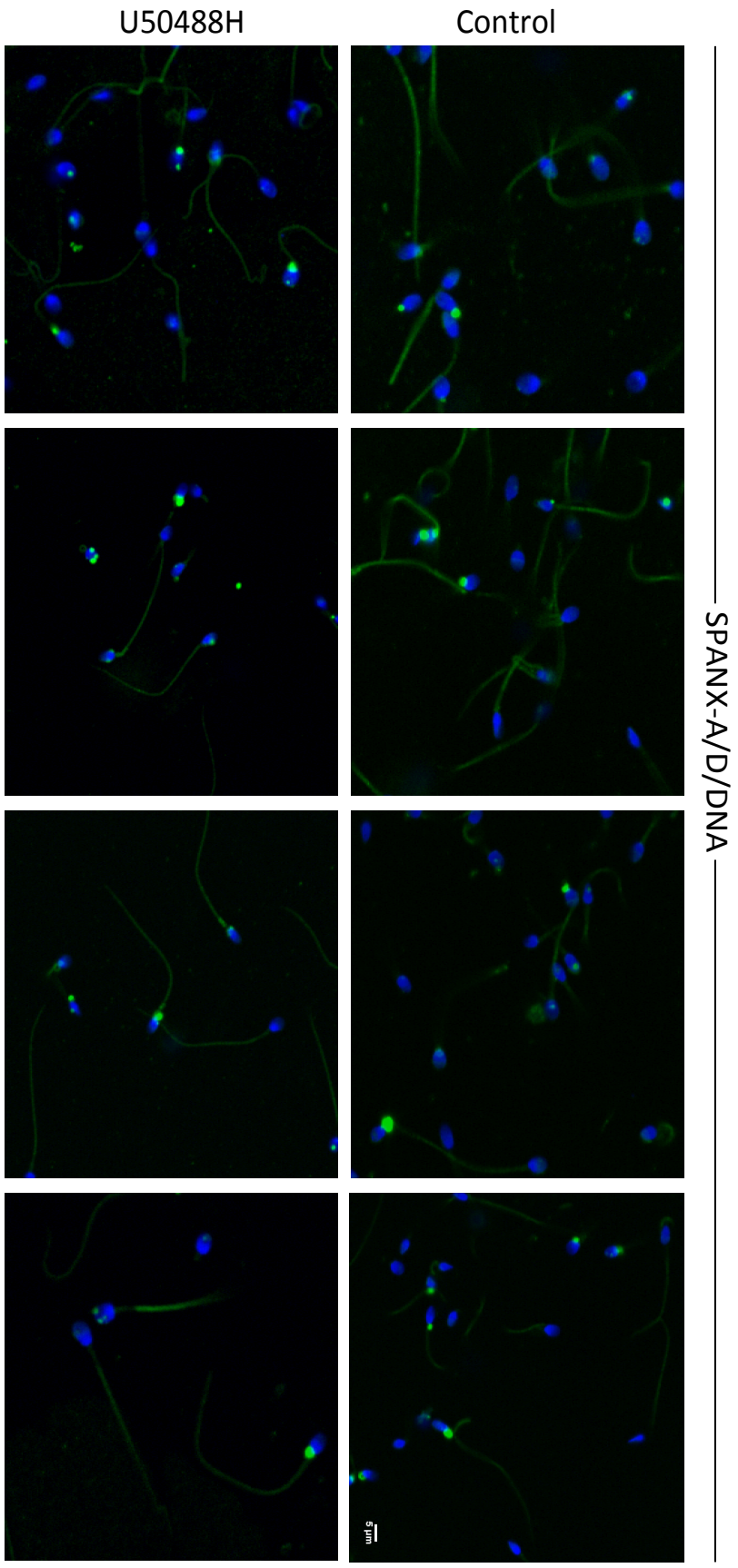
Tesi honetako 3.kapitulu honetan ikusi dugun bezala SPANX-A/D proteina familiak funtzio anitzak bete ditzazke giza espermatozoideetan. Horien artean SPANX-A/D proteina familia zilioen mugimenduarekin erlazionatzen dela deskribatu dugu. Gainera, ezaguna da mugikortasun baxua duten espermatozoideek SPANX-A/D proteina gutxiago adierazten dutela patologiarik gabeko espermatozoideekin alderatuta.

Beste alde batetik tesi honetako 2. kapituluan deskribatu dugun bezala, KOR hartzaileak mugikortasuna erregulatzen du giza espermatozoideetan. Lortutako emaitzetan oinarrituta, KOR hartzailearen bidezko mugikortasunaren erregulazioak SPANX-A/D proteina familiaren aktibazioa suposatzen duen aztertu genuen. Beraz, giza espermatozoideen KOR hartzailearen eta SPANX-A/D proteina familiaren artean erlaziorik ote zegoen aztertu genuen.

Lehenengo eta behin U50488H agonistak SPANX-A/D proteina familiaren lokalizazioan eraginik zuen aztertu zen inmufluoreszentzia bidez giza espermatozoideetan. Horretarako, espermatozoideak 1 µM agonista espezifikoarekin tratatu ziren 1 minutuz. Zelulak fixatu, permeabilizatu eta blokeatu ondoren, anti-SPANX antigorputza erabili zen tindaketarako. Esperimentua birritan egin zen eta estatistika egiteko gai izan ez baginen ere, emaitzek tendentzia argia erakusten zuten. Hain zuzen ere, U50488H gehitu eta gero, SPANX-A/D proteina familia akrosoma, flagelo eta nukleoa bezalako konpartimentuetan ugariagoa zen. U50488H tratamenduak ordea, erdiko piezan SPANX-A/D proteina gutxiago metatzea eragiten zuen (I.Irudia).

Horren arabera, badirudi KOR hartzailearen agonistak SPANX-A/D proteina familia endogenoaren lokalizazio aldaketa eragiten duela giza espermatozoideen zelula barneko konpartimentu desberdinetan. Emaitza horretan oinarrituta U50488H agonistak SPANX-A/D proteina familiaren interaktoreetan aldaketarik sortzen zuen aztertu genuen. Horren ikasketarako lehenengo eta behin espermatozoideak agonistarekin tratatu ziren 1 minutuz (1 µM) eta 3.3.5 atalean azaltzen den metodologia jarraitu zen ondoren laginak masa espektrometria bidez aztertzeko.

Lehenengo eta behin, tratamenduarekin eta hori gabe SPANX-A/D proteina familiaren interaktoreen artean agertzen edo desagertzen diren proteinak aztertu genituen. Horretarako, interaktore berdina baldintza batean bestean baino 3 kasu gehiagotan agertu behar zen gutxienez. I. Taulan eta II.irudian ikus daitekeenez, tratatu gabeko laginetan SPANX-A/D proteinak 9 koprezipitaturekin elkarrekingo luke. Tratamenduarekin ordea 9 proteina horien elkarrekintza desagertuko litzateke eta TSG101, RAB1A, EFHC2, WDR63 eta PARL bezalako proteinekin interakzio berriak emango lirateke. Hala ere, orokorrean bi baldintzetan agertzen eta desagertzen diren interaktore posibleen funtzioa eta zelulan barreneko lokalizazioari erreparatuta, antzeko rola betetzen duten proteinak daudela esan dezakegu (I.Taula). Beraz, emaitza hauekin momentuz, U50488H tratamenduarekin giza espermatozoideen SPANX-A/D proteina familiari lotzen zaizkion interaktore posibleak aldatzen direla soilik esan dezakegu.


U50488H
Control
SPANX-A/D/DNA

	Acrosome	Midpiece	Flagella	Nucleus
Control	17,5	22	40	6,5
U50488H	30	10,5	63	23,5

I. Irudia. U50488H agonistaren efektua giza espermatozoideen SPANX-A/D proteinaren lokalizazioan. **(A)** Immunofluoreszentzia bidezko azterketak U50488H (1 µM, 1 minutuz) agonistak SPANX-A/D proteinaren lokalizazioan duen eragina aztertzeko. Nukleoak (DNA) Hoescht markatzailea erabiliz tindatu ziren. Argazkiak Apotome mikroskopio konfokalarekin atera ziren. Handipena: x63. Eskala-barra: 5 µm. (N=2) **(B)** U50488H tratamenduak SPANX-A/D proteinaren zelulan barreneko konpartimentalizazioan duen eragina erakusten duen taula X ardatzak giza espermatozoideen konpartimentu desberdinak adierazten ditu eta Y ardatzak kontrol eta U50488H-ez tratatutako zeluletan konpartimentu bakoitzerako SPANX-A/D-rako positiboak ziren zelula kopurua adierazten du. Totalean 200 espermatozoide zenbatu ziren baldintza bakoitzerako.

Beste alde batetik, agonistarekin tratatutako eta tratatu gabeko espermatozoideen SPANX-A/D proteinen interaktore berdinen artean ugaritasuna aldatzen zen aztertzeko interaktore berdina tratatutako zein tratamendurik gabeko lau errepliketan derrigorrez agertu behar zen. Scatter plot itxurako grafikoan U50488H tratamenduarekin eta tratamendurik gabe 9 proteinek SPANX-A/D-rekin era ugariagoan elkarrekiten dute kontrolarekin konparatuta (II.Taula, III. Irudia). Alde batetik, zitoeskeletoarekin erlazionatutako ODF3, FAM154A, CCDC familia eta C4orf47 bezalako proteinak ugariagoak dira. Gauza bera gertatzen da mRNA splicing prozesuarekin erlazionatutako SNRNP70 proteinarekin, proteinen sintesirako beharrezkoa den EIF-3 konplexuaren osagai den EIF3L proteinarekin eta histonen metilazioan diharduen SETD9 proteina nuklearrarekin. Aitzitik, ubikinol-zitokromo erreduktasaren konplexuaren osagai den UQCRH proteina mitokondrialak ugariago elkarrekiten du SPANX-A/D proteina familiarekin trataturiko gabeko laginetan. Aipatzekoa da, tratamenduak SPANX-A/D proteinaren interaktoreen ugaritasunean eragiten dituen aldaketak ez direla proteina desberdinen sintesiaren, edo degradazioaren menpekoak (2. Kapitulua, Supplemental Figure S2).

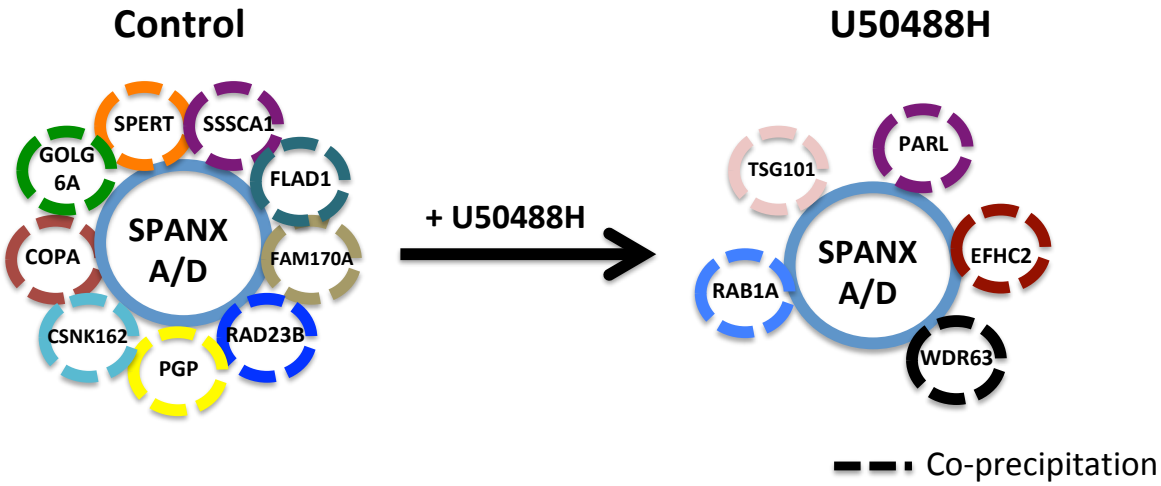
Hori horrela izanda, badirudi SPANX-A/D proteina familiak interaktore desberdinen bahiketan parte hartuko lukeela U50488H agonista gehitzerakoan. Horrela interaktore desberdinek duten funtzioa oztopatuta suertatuko litzateke. Esaterako, giza espermatozoideak tratatzerakoan zitoeskeletoarekin erlazionatutako proteinak SPANX-A/D proteina familiarekin era ugariagoan elkartuko lirateke. Emaitza hauek bat datoz 2.kapituluan deskribatzen diren emaitzekin, izan ere, U50488H-k giza espermatozoideen mugikortasuna inhibitzen du 1 minutuko tratamenduaren ostean. Gainera, agonistak SPANX-A/D proteina familiaren eta kaltzioaren seinaliztapen bidezidorrarekin erlazionatuta dagoen EFHC2 proteinaren erreklutazioa sustatzen du. Emaitza horiek bat datoz KOR hartzaileak kaltzioaren bidezidorra modulatu dezakeen emaitzekin.

I. Taula. U50488H tratamenduaren ondorioz desagertzen eta agertzen diren SPANX-A/D interaktore posibleak. Interaktore berdina baldintza batean bestean baino 3 kasu gehiagotan agertu behar zen gutxienez.

	Gene name	Protein name	Function	Localization
CONTROL	**FAM170A**	Protein FAM170A	Acts as a nuclear transport factor that positively regulates the expression of Heat shock genes	Nucleus
	RAD23B	UV excision repair protein RAD23 homolog B	Modulation of proteasomal degradation	Nucleus
	PGP	Glycerol-3-phosphate phosphatase	Lipid and energy metabolism	Mitochondria
	FLAD1	FAD synthase	Synthesis of FAD	Mitochondria
	CSNK1G2	Casein kinase I isoform gamma-2	Serine/threonine kinase	Cytoplasm
	GOLGA6A	Golgin subfamily A member 6A	Unknown	Golgi Apparatus
	COPA	Coater subunit alpha	Protein transport	Golgi Apparatus
	SSSCA1	Sjoegren síndrome/scleroderma autoantigen 1	Unknown	Centromere
	SPERT	Spermatid associated protein	Unknown	Unknown

	Gene name	Protein name	Function	Localization
U50488H	**TSG101**	Tumor susceptibility 101	Protein transport	Nucleus and endosomes
	PARL	Presenilins associated rhomboid like protein	Proteolitic activity	Mitochondrial
	RAB1A	RAS Related protein RAB1A	Protein transport	Cytoplasm Endoplasmic reticulum Golgi apparatus

	WDR63	WD repeat domain containing protein 63	Dynein binding	Cytoskeleton
	EFHC2	EF hand domain containing protein member C2	Calcium ion binding	Unknown



II. Irudia. U50488H tratamenduaren ondorioz desagertzen eta agertzen diren SPANX-A/D interaktore posibleen irudikapen eskematikoa. Eskema egiteko, interaktore berdina baldintza batean bestean baino 3 kasu gehiagotan agertu behar zen gutxienez.

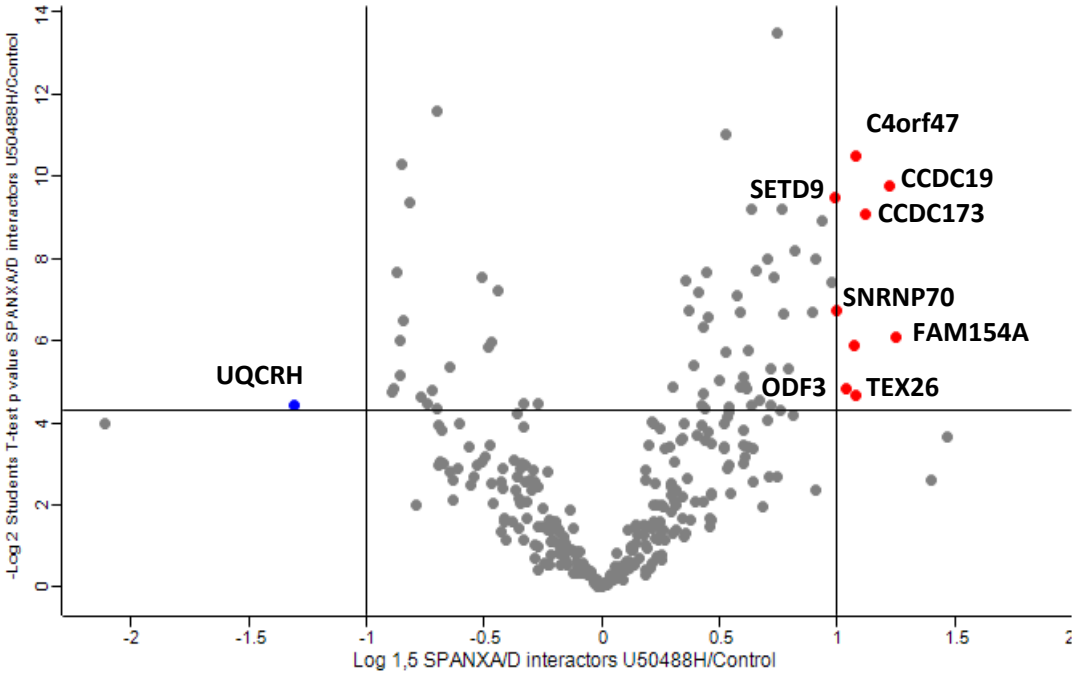
Bestalde, 3. Kapituluko II. Eranskinean aurkezten diren immunofluoreszentziako emaitzek ildo beretik jarraitzen dute, izan ere, agonista gehitzerakoan SPANX-A/D proteina akrosoma, flagelo eta nukleoa bezalako konpartimentuetan ugariagoa zen bertan kokatzen diren proteinekin era ugariagoan elkarrekinez. Aldiz, U50488H tratamenduak, erdiko piezan SPANX-A/D proteina gutxiago metatzea eragiten zuen. Ezaguna denez, giza espermatozoideek konpartimentu horretan mitokondrioak dituzte eta zelularen energia iturri garrantzitsuena dira. Emaitza hauek III. eranskineko III.Irudian lortutakoekin bateragarriak dira, izan ere, tratamendua gehitzerakoan UQCRH bezalako proteina mitokondrialak (Park et al.,2017) SPANX-A/D proteinarekin era murriztuagoan elkarrekiten du. Emaitza hauen arabera esan daiteke KOR hartzailea gai dela SPANX-A/D proteina familiaren elkarrekintzak erregulatzeko.

Horretan oinarrituta pentsa genezake KOR hartzailearen bidezko SPANX-A/D proteina familiaren interaktoreen aldaketa horren fosforilazioaren ondoriozkoa izan litekeela. Horregatik, U50488H agonistak giza espermatozoideen SPANX-A/D proteina familiaren isoforma desberdinen fosforilazioan eraginik zuen aztertzeko, fosfopeptido desberdinen ugaritasuna aztertu zen tratatu eta tratatu gabeko laginetan (1 µM, 1 eta 60 minutuz). III.Taulan ikus daitekeenez, fosfoproteoma azterketatik eratorritako SPANX-A/D proteina familiako isoforma desberdinei dagozkien emaitzak erakusten dira, identifikatutako fosfopeptido guztiek 0.75 baino gehiagoko lokalizazio probabilitatea daukatelarik.

II Taula. U50488H agonistaren eragina giza espermatozoideen SPANX-A/D proteinaren interaktoreetan. U50488H agonistarekin tratatutako zein tratamendurik gabeko lau errepliketan aldatzen diren interaktorei buruzko informazioa.

	Gene name	Protein name	Function	Localization
U50488H/ CONTROL>1,5	**SNRNP70**	U1 small nuclear ribonucleoprotein 70 kDa	Recognition of spliceosome and mRNA splicing	Nucleus
	SETD9	SET domain-containing protein 9	Methylation of Histones and other proteins	Nucleus
	EIF3L	Eukaryotic translation initiation factor 3 subunit L	Assembly of EIF-3 complex: Initiation of protein synthesis	Cytoplasm
	FAM154A (SAXO1)	Protein FAM154A (Stabilizer of axonemal microtubules 1)	Regulation of cillium lenght and axoneme stabilization	Cytoskeleton
	ODF3	Outer Dense Fiber 3 protein	Mantainance of the passive elastic structures and elastic recoil of the sperm tail. Cancer testis-Antigen in cancer	Cytoskeleton
	CCDC19 (CFAP45)	Coiled-coil domain-containing protein 19, mitochondrial (Cillia and flagella associated protein 45)	Tumor supressor in non-small cell lung cancers	Cytoskeleton
	C4orf47	Coiled-coil domain-containing protein 173	Component of the centrosome	Cytoskeleton
	CCDC173	Coiled-coil domain-containing protein 173	Unknown	Unknown
	TEX26	Testis expressed sequence 26	Unknown	Unknown

U50488H/ CONTROL<0,67	**UQCRH**	Cytochrome b-c1 complex subunit 6, mitochondrial	Component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain.	Mitochondria



III. Irudia. U50488H agonistaren eragina giza espermatozoideen SPANX-A/D proteinaren interaktoreetan. SPANX-A/D interaktore posibleen U50488H/Kontrol ratioaren Log $_{1,5}$ vs. U50488H/Kontrol arteko T Student estatistikoaren $-\text{Log}_2$ ($p<0.05$). Scatter plot itxurako grafiko hau egiteko interaktore berdina tratatutako zein tratamendurik gabeko lau errepliketan derrigorrez agertu behar zen.

Aldaketa esanguratsua kontsideratzeko ondorengo baldintzak finkatu ziren U50488H/Kontrol Ratioa >1.5 eta <0.67 (Log_2 >0,58 eta <-0,58, hurrenez hurren), eta p value <0.05 (Log_2>4,32). Emaitzei erreparatuz, ez genuen baldintza horiek betetzen zituen kasu esangarririk ikusi. Beraz, lortutako datuen arabera eta markaketan oinarritutako TMT estrategia fosfoproteomikoa jarraituz, U50488H agonistak ez du 1 eta 60 minutuetan SPANX-A/D familiako isoforma desberdinen fosforilazioan eragiten giza espermatozoideetan.

Eranskin honetan lortutako emaitzei guztiei erreparatuta badirudi KOR hartzaileak giza espermatozoideen mugikortasuna erregula dezakeela SPANX-A/D proteina familiarekin elkarrekiten duten interaktoreak aldatuz. Interaktore hauen aldaketa ordea, ez da SPANX-A/D proteina familiaren fosforilazio egoeraren menpekoa. Hala ere etorkizunean esperimentu gehiago egingo lirateke emaitza horiek berresteko.

III. Taula. U50488H-ren eragina giza espermatozoideen SPANX proteina familiaren isoformen fosforilazioan.

				Log2 Fold change U50488H/Control				-Log2 Students T-test p-value U50488H/Control			
Gene name	Position	Amino acid	Localization Probability	Soluble 1min	Soluble 1 hour	Insoluble 1 min	Insoluble 1 hour	Soluble 1min	Soluble 1 hour	Insoluble 1 min	Insoluble 1 hour
SPANXB1	13	S	1	0	0	-0,025115	0,4124763	0	0	0,0548961	4,475935
SPANXB1	18	S	1	0	0	-0,106204	0	0	0	0,3127995	0
SPANXB1	34	T	0,96485	0	0	0	-0,133832	0	0	0	0,9530729
SPANXB1	53	S	0,960769	-0,026254	0,5743328	0,4217419	0,2306211	0,2371816	1,794209	2,2695776	1,4854686
SPANXD	47	S	0,960769	0,071714	0,5140495	0,788049	0,1110608	0,5882393	1,7432542	1,6114174	0,7595689
SPANXA1	13	S	0,947883	0	0	0	-0,071774	0	0	0	0,8063736
SPANXB1	54	S	0,844546	0	0	0,236605	0,4148651	0	0	1,2260415	11,381345
SPANXD	48	S	0,844546	0	0	0,602912	0,3106418	0	0	1,3857114	4,7385775
SPANXA1	28	T	0,843021	0	0	0	-0,062082	0	0	0	1,3028177
SPANXB1	55	T	0,750828	0	0	0	0	0	0	0	0
SPANXD	49	T	0,750828	0	0	0	0	0	0	0	0

5. Eztabaida

Discussion

5. EZTABAIDA/DISCUSSION

Antzutasun eta ugalkortasun arazoak betidanik kezka mediko eta sozial garrantzitsuak direla onartu bada ere, azken urteotan arazo kliniko esanguratsuan bihurtu dira. Izan ere, mundu osoan zehar adin ugalkorrean dauden bikoteen %15ak pairatzen ditu gutxi gora behera (ESHRE.,2014; Mascarenhas et al., 2012). Normalean, antzutasunaren eragileak faktore maskulinoa (%40) eta femeninoa (%40) badira ere, kasu askotan arazoaren kausa ulertezina izan daiteke (Mascarenhas et al., 2012).

Giza espermatozoideei dagokienez, ezaguna da behin isuriak direnean ez direla obozito bat *in vivo* edota *in vitro* ernaltzeko gai. Hori dela eta, emakumezkoaren ugaltze-traktuan mugikortasun ahalmena, kapazitazioa eta erreakzio akrosomikoa jasan behar dituzte gameto emearekin bat egin ahal izateko eta zigotoa eratzeko (Visconti et al., 2011). Prozesu fisiologiko horien erregulatzaile nagusiak kanale ionikoak badira ere (Hille et al.,1992), badirudi azken 10 urteotan giza espermatozoideen gainazalean deskribatutako GPCR hartzaileek ere espermatozoidearen fisiologian zeregin garrantzitsua izango luketela (Aitken, 1985; Calogero et al., 1996; Morales et al., 1998; Spehr et al., 2004; Zitta et al., 2007).

Gaur egun, gero eta ikerketa gehiago giza espermatozoideen GPCR hartzaileen ikasketan oinarritzen badira ere, gutxi dira horien azpitik induzitzen diren mekanismo molekularrak aztertzen dituztenak. Hala ere, zelula somatikoekin konparatuta, giza espermatozoideek hartzaile hauen azpitik eta proteina espezifikoen bitartez, seinaliztapen bidezidor intrazelular bakar eta bereziak piztuko lituzkete. Bereziki, ezaguna da gizakiaren gameto arrak ez duela mintzarteko adenilato ziklasarik (tmAC) adierazten eta horren ordez, bikarbonatoaz aktibatzen den forma solugarria (SACY) erakusten duela (Rojas et al., 1992; Urizar-Arenaza et al., 2018a, errebisiopean). Kaltzioaren seinaliztapen bidezidorrari dagokionez, giza espermatozoideek CatSper izeneko kaltzio kanale espermatozoide-espezifikoa adierazten dute, mugikortasun hiperaktiboaren eta erreakzio akrosomikoaren erregulaziorako ezinbestekoa dena (Brenker et al., 2012). Horrez gain, transkripzio eta itzulpen prozesuak isilik badituzte ere, MAP kinasen seinaliztapen bidezidorrean parte hartzen duten zenbait proteinen presentzia deskribatu da (Almog et al., 2008). Hori guztia kontuan hartuta, zelakoak dira giza espermatozoideen GPCR hartzaileek pizten dituzten mekanismo molekularrak? Gizonezkoaren antzutasunaren kausak konplexuak eta faktore anitzen menpekoak badira ere, seinaliztapen bidezidor horien deskribapen sakonagoa antzutasunaren diagnostikorako erabilgarriak diren proteinen identifikazioan zein antisorgailuen garapenean lagungarria litzateke (Schlyer and Horuk, 2006). Izan ere, GPCR hartzaileak industria farmazeutikoan droga anitzen itu garrantzitsuenak direla onartzen dira (Schlyer and Horuk, 2006).

Sistema opioidea, fisiologian garrantzia handia duen zelulen komunikazio sistema da (Subirán et al., 2011) eta GPCR kontsideratzen diren hartzaile opioideen bitartez jarduten du. Klasikoki, Nerbio Sistema Zentralean minarekin eta plazerrarekin (Böttcher et al., 2017) erlazionatu bada ere, azken urteotan ugalkortasunarekin eta garapenarekin harremandu da (Subirán et al., 2011). Bereziki, gure ikerketa taldeak 2006. urtean (Agirregoitia et al., 2006) MOR, DOR eta

KOR opioide hartzaileen presentzia deskribatu zuen giza espermatozoideetan, fisiologiaren erregulazioan funtzio garrantzitsua bete zezaketela iradokituz. MOR eta DOR opioide hartzaileek giza espermatozoideen mugikortasunaren erregulazioan parte hartzen zutela ikusi bagenuen ere (Agirregoitia et al., 2006), KOR opioide hartzaileak giza espermatozoideen mugikortasunean zuen funtzioa ez zen argitu. Horrez gain, hiru opioide hartzaileek kapazitazioan eta erreakzio akrosomikoan duten rola zein horien azpitik dauden mekanismo molekularrak gaur egun aski ezezagunak dira.

Hartzaile opioideek giza espermatozoideen kapazitazioan eta erreakzio akrosomikoan betetzen zuten funtzioa aztertzeko, lehendabixi beta-endorfina peptido opioide endogenoa erabili zen. Bereziki, beta-endorfina ahalmen berdinarekin lotzen da MOR eta DOR hartzaileetara (Subirán et al., 2011). Emaitzek erakutsi zutenez, beta-endorfina giza espermatozoideen erreakzio akrosomikoa estimulatzen du PKC-seinaliztapen bidezidor intrazelularraren bitartez, beste induzitzaile batzuetarako deskribatu den bezala (O'Toole et al., 1996; Vigil et al., 2011). Gure emaitzek iradokitzen dutenez, badirudi PKC hori kaltzioarekiko independentea litzatekeela (Urizar-Arenaza et al., 2016), beta-endorfinak ez baitu kaltzio intrazelularren mailan eraginik sortzen. Hori dela, eta pentsa genezake beta-endorfinak giza espermatozoideen erreakzio akrosomikoa bultzatuko lukeela bere aktibaziorako kaltziorik behar ez duen PKC baten bitartez. Ezaguna denez, ζ eta λ/ι isoformak kaltzioarekiko eta DAG-rekiko independenteak dira eta azken urteotako ikerketek erakutsi dute giza espermatozoideez gain zenbait zelula somatikoek ere adierazten dituztela (Govekar and Zingde., 2001; Steinberg., 2008; Ferrer-Vaquer et al., 2016; Urizar-Arenaza et al., 2018a, errebisiopean). Hala ere, soilik emaitza horiek kontuan hartuta, ezin dugu aitortu giza espermatozoideek mekanismo molekular bereizgarriak aktibatzen dituztela GPCR hartzaileen azpitik.

Emaitza horietan oinarrituta, GPCR hartzaileek giza espermatozoideetan mekanismo molekular espezifikoak induzitu lezaketela pentsa genezake. Hori aztertzeko, KOR opioide hartzailearen azpitik induzitzen diren mekanismo molekularrak aztertzeari eta GPCR honek giza espermatozoideen ahalmen ugalkorrean duen funtzioa ikasteari ekin genion. Analisi fosfoproteomiko eta funtzionalek erakutsi zutenez, KOR hartzaileak giza espermatozoideen mugikortasun hiperaktiboa eta erreakzio akrosomikoa inhibitzen ditu kaltzio-kanaleen modulazioaren eta espermatozoide-espezifikoak diren proteinen fosforilazio aldaketen bidez (Urizar-Arenaza., 2018a, errebisiopean). Bereziki, KOR hartzailearen ligando espezifikoak, fosforilazio aldaketak sortzen ditu AKAP3 eta CABYR bezalako proteina espermatozoide-espezifikoetan zein PKC-ren sustratuetan (Urizar-Arenaza., 2018a, errebisiopean). Jakina denez, AKAP3 eta CABYR proteinek fisikoki elkarrekiten dute giza espermatozoideetan (Li et al., 2011). Beraz KOR-ek espermatozoidearen mugikortasuna eta erreakzio akrosomikoa erregulatu ditzake AKAP/PKA eta kaltzioaren seinaliztapen bidezidor intrazelularren arteko seinaliztapenen *crosstalk*-a ahalbidetuz. (Urizar-Arenaza et al.,2018a, errebisiopean). Emaitza hauei erreparatuta, esan daiteke KOR hartzailearen azpitik espermatozoide-espezifikoak diren mekanismo molekularrak induzitzen direla ahalmen ugalkorra erregulatzeko. Emaitza hauetan oinarrituta, etorkizunean beharrezkoa litzateke giza espermatozoideen beste GPCR hartzaile batzuen azpitik induzitzen diren mekanismo molekularrak aztertzea.

Giza espermatozoideen fosfoproteoman arreta handiagoa jarriz, aipatzekoa da lan honetan gaur egun arte gameto arrean egindako fosfoproteomarik osatuena deskribatzen dela (Urizar-Arenaza et al., 2018a, errebisiopean), identifikatutako fosforilazio lekuetatik %60a baino gehiago berriak izanik. Identifikatutako fosfopeptido gehienak testikuluetako espezifikoak diren proteinetatik eratorriak dira eta hauen artean SPANX-A/D proteina familia aurkitzen da.

SPANX-A/D (Sperm Protein Associated with the Nucleus mapped to the X chromosome) familia X kromosoman kodetzen den familia multigenikoa da. SPANX geneek proteina desberdinak kodetzen dituzte eta "Cancer testis antigens" (CTA) bezala ezagutzen diren familiaren barruan kokatzen dira. Izan ere, hauen adierazpena testikuluetara eta espermatozoideetara mugatuta badago ere, zenbait minbizi motetan kasuen %40an aktibatu eta agertzen dira (Chen et al., 1997; Mirandola et al., 2011; Scanlan et al., 2004).

Fisiologikoki, SPANX-A/D familiak adierazpen postmeiotikoa azaltzen du espermatogenesian (Kouprina et al., 2004; Salemi et al., 2004; Westbrook et al., 2001) eta giza espermatozoideetan krater nuklearretan, tanta zitoplasmatikoan eta flageloan adierazten da (Westbrook et al., 2001, Urizar-Arenaza et al., 2018b errebisiopean). SPANX superfamiliaren funtzio biologikoa ezezaguna bada ere, espermatogenesi prozesuan eta giza espermatozoidearen ernaltzeko gaitasunean funtzio garrantzitsua bete dezakeela uste da (Kouprina et al., 2004; Westbrook et al., 2001). SPANX-A/D proteina familia endogenoaren interaktoma analisiak erakutsi zuenez, proteina familiak funtzio anitzak izan ditzazke giza espermatozoideen nukleoaren antolakuntza, mugikortasuna eta metabolismoa erregulatuz (Urizar-Arenaza et al, 2018b, errebisiopean). Izan ere, gure emaitzen arabera SPANX-A/D proteina familiak kromatinaren antolakuntzan jarduten duten kromatina erregulatzaileekin zein histonekin koprezipitatzen du. Horrez gain, SPANX-A/D familia espermatozoidearen mugikortasunerako beharrezkoa den energiaren lorpenean garrantzitsua litzateke, mitokondrioen metabolismoarekin zein zilio eta flageloko proteinekin elkarrekinez.

Beste alde batetik, jakina da SPANX-A/D familia muga hematostikularraren barruan kokatzen diren eta meiosia jasan duten zelula haploideetan adierazten dela, minbiziaren tratamendurako inmunoterapian itu garrantzitsuak kontsideratzen direlarik. Aitzitik, badirudi SPANX-A/D proteina familiak inmunoterapiarako baliogarriak diren ezaugarriez haratago ezezagunak diren funtzioak izan ditzakeela tumorearen biologia zelularrari dagokionez. Jakina denez, SPANX-A/D proteina familia zenbait tumoretan deskribatu da, melanoman besteak beste (Almanzar et al., 2010; Wang et al., 2003; Westbrook et al., 2004; Zendman et al., 1999, Urizar-Arenaza et al., 2018b, errebisiopean). SPANX-A/D proteina familia melanoma zelula lerro desberdinetan adierazten bada ere (Urizar-Arenaza et al., 2018b, errebisiopean), lerro primarioetatik lerro metastatikoetara arte (Westebrook et al., 2004) agertuz, gaur egun minbizian duen funtzio fisiopatalogikoa ez da oraindik argitu. Horrez gain, aipatzekoa da SPANX-A/D esklusiboki maila nuklearrean adierazten da, baina deskribatu dugunez proteina familiaren fosforilazio mailak ez du nukleoaranzko translokazioan eraginik (Urizar-Arenaza et al., 2018b, errebisiopean).

Funtzionalki, SPANX-A/D proteina familiaren adierazpen altua ondesteko minbizia pairatzen duten pazienteetan gibeleranzko metastasiarekin erlazionatzen da (Chen et al., 2010). Gure

emaitzei dagokienez, SPANX-A/D familiak melanoman ere zelulen proliferazioa eta migrazioa susta ditzake funtzio nuklearrak erregulatuz. Izan ere, beste tumoreetan ikusi den bezala, melanoman nukleoaren azalean kokatutako SPANX-A proteina, Lamin A/C proteinarekin elkarrekiten du zeina nukleoaren barneko mintzean lokalizatzen den eta nukleoaren egonkortasunean parte hartzen duen (Urizar-Arenaza et al, 2018b errebisiopean; Dahl, 2004; Swift and Dischер, 2014; Berk et al., 2013). Hau ere bat dator giza espermatozoideetan deskribatu denarekin, izan ere, SPANX-A nukleoaren azalaren parte izanda melanoma zelulen kromatinaren iristagarritasunean parte hartu lezake histonak bezalako proteinekin elkarrekinez (Urizar-Arenaza et al., 2018b, errebisiopean). Horrela, aldamio bezala jardunez, SPANX-A/D familiak nukleoaren azalean proteina konplexuak eratzeko ahalmena eduki lezake zelularen funtzio nuklearrak erregulatuz. Azaldutakoa aintzat hartuta, badirudi giza espermatozoideen SPANX-A/D familiari buruzko informazio sakonagoa ezinbestekoa litzatekeela giza ugalkortasuna ulertzeko zein antzutasun eta tumoregenesian betetzen duen funtzio fisiopatologikoa deskribatzeko.

Laburbilduz, tesi honetan jasotako emaitza guztiak aztertuz, ondorioztatu dezakegu giza espermatozoideek GPCR hartzaileen azpitik mekanismo molekular espezifikoak dituztela, zelula somatikoengandik desberdinak direnak, eta zeintzuen bidez espermatozoideraren fisiologia erregulatzen dutenak. Proteina espermatozoide-espezifikoei dagokienez, SPANX-A/D proteina familia espermatozoideetan eta melanoman adierazten den CTA ezaguna da, zeinak funtzio fisiologiko eta fisiopatologiko anitz betetzen dituen arren, nukleoko antolakuntzan parte ere hartu lezake. Tesi honetan egindako aurkikuntzak beraz, erabilgarriak lirateke antzutasuna zein minbizia tratatzeko itu terapeutiko berrien identifikaziorako eta bigarren mailako eraginak murrizten dituen antisorgailuen zein tumoreetara bideraturiko farmakoen garapenerako.

6. Ondorioak
Conclusions

6. ONDORIOAK

1.- Beta-endorfina peptido opioide naturalak giza espermatozoideen erreakzio akrosomikoa aktibatzen du PKC seinaliztapen bidezidorraren bitartez.

2.- Hartzaile opioideek giza espermatozoideen erreakzio akrosomikoa erregulatzen dute. Bereziki, MOR, DOR eta KOR hartzaileek erreakzio akrosomikoa inhibitzen dute kaltzio kanaleak modulatuz.

3.- KOR hartzaile opioideak giza espermatozoideen mugikortasun hiperaktiboa eta erreakzio akrosomikoaren inhibizioan parte hartzen du espermatozoide-espezifikoak diren seinaliztapen bidezidorrak induzituz. Bereziki, espermatozoideetan soilik agertzen diren AKAP4, AKAP3, CABYR, ODF1, ODF2 edota CCDC familiako proteinetan fosforilazio aldaketak induzitzen ditu.

4.- Azterketa fosfoproteomikoen bitartez 3527 fosforilazio leku desberdin identifikatu dira giza espermatozoideen proteoman. Horietatik 2157 berriak dira. Hauek flagelo bidezko mugikortasunean, proteinen garraioan, axonemako dineina konplexuaren eraketan, espermatozoide-obozito ezagutzan, kapazitazioan, nukleoaren antolakuntzan eta txaperona bidezko proteinen tolesketan parte hartzen dute.

5.- SPANX-A/D proteina familiak giza espermatozoideetan funtzio anitz betetzen du. Izan ere, nukleoaren antolakuntzan, mitokondrioetako fosforilazio oxidatiboan eta mugikortasunean parte hartzen duten proteinekin elkarrekiten du: NUP, FH eta TEKT1, hurrenez hurren.

6.-SPANX-A/D proteina familiaren 7 fosforilazio leku aurkitu dira giza espermatozoideetan. Fosforilazio hauek proteina familiaren zelulan barreneko translokazioan eragiten ez badute ere, SPANX-A/D-ren proteina konplexuen egonkortasunean parte hartuko lukete.

7.- SPANX-A/D proteina familiak zelulen proliferazio eta migrazioan parte hartu lezake funtzio nuklearrak eraldatuz eta laminarekin elkarrekinez.

6. CONCLUSIONS

1.- Beta endorphin, the natural opioid peptide, stimulates the human sperm acrosome reaction through the PKC intracelular signaling pathway.

2.- Opioid receptors regulate the human sperm acrosome reaction. MOR, DOR and KOR opioid receptors inhibit the human sperm acrosome reaction through the modulation of calcium channels.

3.- KOR opioid receptor inhibits the human sperm hyperactive motility and acrosome reaction inducing sperm-specific molecular mechanisms. Specifically, KOR induces phosphorylation changes in sperm-specific proteins like AKAP3, AKAP4, CABYR, ODF1, ODF2 and CCDC protein family.

4.- Phosphoproteomic approaches present the most update phosphoproteome to date with 3527 phosphosites in human sperm proteins, considered 2157 novel. These proteins are involved in flagellum-dependent cell motility, protein transport, axonemal dynein complex assembly, sperm-egg recognition, sperm capacitation, nuclear organization and chaperone mediated protein assembly.

5.- SPANX-A/D protein family is involved in processes related to nuclear pore organization and envelope disassembly, oxidative phosphorylation in mitochondria and cilium movement, by the interaction with NUPs, FH and TEKT1.

6.-Phosphoproteomic approaches reveal 7 phosphosites of SPANX-A/D protein family in human spermatozoa. Although these phosphosites are not involved in the protein translocation within the cell, they participate in the stabilization of the SPANX-A/D protein complexes.

7.- SPANX-A/D protein family may be involved in cell proliferation and migration regulating nuclear functions by the interaction with Lamin A/C.

7.Bibliografia
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8. CURRICULUM VITAE

Publications

1.- Authors: **Itziar Urizar-Arenaza** and Nerea Osinalde, Vyacheslav Akimov, Michele Puglia, Luz Candenas, Francisco Pinto, Iraia Muñoa-Hoyos, Marta Gianzo, Roberto Matorras, Jon Irazusta, Blagoy Blagoev, Nerea Subiran and Irina Kartchmarova.
Título: Phosphoproteomics reveals changes in sperm-specific proteins underlying KOR in human spermatozoa.
Journal: Molecular and Cellular Proteomics,Under revision. Year: 2018

2.- Authors: **Itziar Urizar-Arenaza**, Aitor Benedicto, Arantza Perez-Valle, Nerea Osinalde, Iraia Muñoa-Hoyos, Vyacheslav Akimov, Michele Puglia, Aintzane Asumendi, Maria Dolores Boyano, Blagoy Blagoev, Nerea Subiran and Irina Kratchmarova I.
Title: Functional characterization of SPANX-A/D in human spermatozoa and melanoma.
Journal: Cellular and Molecular Life Sciences, Submitted. Year: 2018

3.- Authors: **Itziar Urizar-Arenaza**, Marta Gianzo, Iraia Muñoa-Hoyos and Nerea Subiran.
Title: Sperm-specific molecular mechanisms underlying G protein coupled receptors in human spermatozoa
Journal: Human Reproduction Update. Submitted. Year: 2018.

4.- Authors: Marta Gianzo, **Itziar Urizar-Arenaza**, Iraia Muñoa-Hoyos, Zaloa Larreategui, Nicolas Garrido, Luis Casis, Jon Irazusta and Nerea Subiran.
Title:Sperm Aminopeptidase N helps determine human embryo quality and blastocyst development during Assisted Reproduction Techniques.
Journal:Journal of Translational Meidicine. Submitted. Year: 2018

5.-Authors: Marta Gianzo, **Itziar Urizar-Arenaza**, Iraia Muñoa-Hoyos, Zaloa, Jon Irazusta and Nerea Subiran.
Title: Human Sperm Testicular Angiotensin-Converting Enzyme is related to human embryo quality.
Journal: Asian Journal of Andrology. Year: 2018

6.- Authors: Iraia Muñoa, **Itziar Urizar-Arenaza**, Jon Irazusta and Nerea Subiran
Title: The epigenetic regulation of the opioid system: New individualized prompt prevention and treatment estrategies.
Journal: Journal of Cellular Biochemistry. Year: 2016.

7.- Authors: **Itziar Urizar-Arenaza**, Haizea Estomba, Iraia Muñoa-Hoyos, Marta Gianzo, Roberto Matorras, Antonia Exposito, Luz Candenas, Francisco Maria Exposito, Asier Valdivia, Jon Irazusta and Nerea Subiran.
Title: The opioid beta-endorphin stimulates acrosome reaction in human spermatozoa.
Journal: Andrology. Year: 2016.

8.- Authors: Haizea Estomba, Iraia Muñoa-Hoyos, Marta Gianzo, **Itziar Urizar-Arenaza,** Luis Casis, Jon Irazusta and Nerea Subiran.
Title: Opioid receptors are present in male germ cells and regulate meiosis.
Journal: PLOS ONE. Year: 2015

9.- Authors: Marta Gianzo, Iraia Muñoa-Hoyos, **Itziar Urizar-Arenaza**, Zaloa Larreategui, Fernando Quintana, Nicolás Garrido, Nerea Subirán and Jon Irazusta.
Title: Angiotensin II type 2 receptor is expressed in human sperm cells and is involved in sperm motility. Journal: Fertility and Sterility. Year: 2015

Patents

Title: Method for obtaining a spermatozoid cell population with improved fitness.
Inventor: Nerea Subirán Ciudad.
Authors: Nerea Subiran, Yosu Franco, Maria Velasco, Jon Irazusta, Luis Casis, **Itziar Urizar-Arenaza**, Iraia Muñoa-Hoyos, Marta Gianzo.
Owner: UPV/EHU – IVF Donostia.
Application number: EP18382084.4. Approved patentability phase. ABG patents. Priority date: 14/02/18

Proyects

1.- Project Title: Creation of a startup, MEPRO (Medical Reproductive Solutions) Financing entity: Proyect Txekintek/Ekintzaile (Bic Gipuzkoa).
Grant: 55000€
Start date (dd/mm/yy): 01/01/2018. End date (dd/mm/yy): 31/12/2019.
Principal Investigator: Nerea Subirán.

2.- Project Title: Proyecto SPERM SELECT de transferencia del conocimineto. Programa La Caixa Impulse.
Financing Entity: Fundación La Caixa y La Caixa Capital Risk.
Grant: 70000€
Start Date (dd/mm/yy): 07/2017. End date (dd/mm/yy): 07/2019.
Principal Investigator: Nerea Subirán.

3.- Project Title: Papel de los receptores metabotrópicos en la capacidad fértil de los espermatozoides: Estudio de los receptores opioides: EHU14/17
Financing entity: UPV/EHU
Grant: 5100 €
Start Date (dd/mm/yy): 09/10/2014 End date (dd/mm/yy): 09/10/2015
Principal Investigator: Nerea Subirán Ciudad

4.- Project Title: Estudio de Sistemas Peptidícos en Reproducción y Cáncer. Grupos consolidados. GIC12/173
Financing entity: Gobierno Vasco
Grant: 90.000 €
Start Date (dd/mm/yy): 2013 End date (dd/mm/yy): 2014
Principal Investigator: Jon Irazusta Astiazaran

5.- Project Title: CD10 como biomarcador espermático para la obtención no invasiva de embriones de mejor calidad mediante las diferentes técnicas de reproducción asistida SAI13/103
Financing entity: UPV/EHU
Grant: 3.861.45€
Start date (dd/mm/yy): 2013 End date date (dd/mm/yy): 2014
Principal Investigator: Nerea Subiran Ciudad

6.- Project Title: Proyecto Estratégico de la UPV/EHU. Estudio de Sistemas Peptidicos en la Comunicación Celular
Financing entity:Universidad del Pais Vasco/Euskal Herriko Unibertsitatea
Grant: 10.056 €
Start date (dd/mm/yy): 2013 End date (dd/mm/yy): 2017
Principal Investigator: Javier Gil Goikouria

7.-Project Title: Proyecto de Grupo de Investigación del Gobierno Vasco. Grupo IT811-13

Financing entity: Gobierno Vasco
Grant: 90.000 €
Start date (dd/mm/yy):2013
End date (dd/mm/yy): 2015
Principal Investigator: Jon Irazusta Astiazaran

8.-Project Title: UFI, Unidad de Formación e Investigación: Reproducción, Desarrollo, Envejecimiento y Cáncer (El sistema opioide y su función en la Reprodución).
Financing Entity: Universidad del Pais Vasco /Euskal Herriko Unibertsitatea
Grant: 33.796,93 €
Start date (dd/mm/yy): 2012 End date (dd/mm/yy): 2014
Principal Investigator: Fernando Unda Rodriguez

9.- Project Title: Proyecto Saiotek Estudio del Sistema Opioide en la Función del Espermatozoide y su Aplicación para la Mejora de las Técnicas de Reproducción Asistida S-PE12UN092
Financing Entity: Gobierno Vasco
Grant: 26.628,67 €
Start date (dd/mm/yy): 01/01/2012 End date (dd/mm/yy):31/12/2013
Principal Investigator: Jon Irazusta Astiazaran

Congresses

1.- - Authors: **Itziar Urizar-Arenaza** and Nerea Osinalde, Iraia Muñoa-Hoyos, Marta Gianzo, Nerea Subiran and Irina Kratchmarova. Title:. Analisi fosfoproteomiko eta funtzionalak, giza espermatozoideen GPCR hartzaileek induzitutako seinaliztapen bidezidor intrazelularren azterketarako. Type of participation: ORAL. Congress: Osasun Zientzietako Ikertzaileen III. Topaketak. Place: Eibar. Year: 2018.

2.-Authors: Iraia Muñoa-Hoyos, Manu Araolaza, **Itziar Urizar-Arenaza**, Marta Gianzo and Nerea Subiran. Title: Morfinak saguein zelula ametan sortzen duen memoria epgigenetikoaren azterketa, RNA-seq teknikaren bidez. Type of participation: ORAL. Congress: Osasun Zientzietako Ikertzaileen III. Topaketak. Place: Eibar. Year: 2018

3.- Authors: Iraia Muñoa-Hoyos, **Itziar Urizar-Arenaza,** Marta Gianzo, John A Halsall, Carl Ward, Paloma Garcia, Bryan Turner and Nerea Subiran. Title: ChIP-sequencing revelas changes in the genomic distribution of the epigenetic repressive mark H3K27me3 at ICR/DMRs of imprinted genes induced by morphine in mouse Embryonic Stem Cells (mESCs). Type of participation: POSTER. Congress: 3rd Danube Conference on Epigenetics. Place: Budapest . Year: 2018.

4.- Authors: **Itziar Urizar-Arenaza** and Nerea Osinalde, Vyacheslav Akimov, Michele Puglia, Iraia Muñoa-Hoyos, Marta Gianzo, Roberto Matorras, Antonia Expósito, Jon Irazusta, Nerea Subiran and Irina Kratchmarova. Title: Quantitative phosphoproteomics reveals novel phosphosites downstream Kappa Opioid receptor in human spermatozoa. Type of Participation: ORAL. Congress: XII European Proteomics Congress (EuPA). **Best Oral comunication Award**. Place: Santiago de Compostela. Year: 2018.

5.- Authors: **Itziar Urizar-Arenaza** , N. Osinalde , V. Akimov , I. Muñoa-Hoyos , M. Gianzo , T. Ganzabal, J. Irazusta , N. Subirán and I. Kratchmarova. Title: SPANX A/D subfamily plays a key role in nuclear organisation, metabolism and flagellar-motility of human spermatozoa. Type of participation: ORAL. Congress: ESHRE 2018. Place: Barcelona. Year: 2018.

6.-Authors: M. Gianzo , **Itziar Urizar-Arenaza**, I. Muñoa-Hoyos , Z. Larreategui , J. Irazusta , N. Subirán. Title: Human Sperm Aminopeptidase N is related to embryo development and viability during ART.Type of participation: POSTER.Congress: ESHRE 2018.Place: Barcelona. Year: 2018.

7.- Authors: Muñoa-Hoyos, I, **Itziar Urizar-Arenaza** , Gianzo, M, Subirán N. Title: Different dynamic distribution of H3K27ac and H3K4me3 epigenetic active marks at imprinted genes during in vitro primordial germ cells differentiation. Type of participation: POSTER.Congress: ESHRE 2018. Place: Barcelona. Year: 2018.

8-Authors: **Itziar Urizar-Arenaza**, Nerea Osinalde, Marta Gianzo, Roberto Matorras, Antonia Expósito, Jon Irazusta, Nerea Subiran and Irina Kratchmarova. Title: Label-free proteomics reveals a novel signalling pathway modulated via kappa-opioid receptor.Type of participation: ORAL. Congress: ESHRE 2017. Place: Geneva. Year: 2017.

9.- Authors Marta Gianzo, **Itziar Urizar-Arenaza**, Iraia Muñoa-Hoyos, Zaloa Larreategui, Jon Irazusta and Nerea Subirán.Title: Sperm Aminopeptidase N is related to embryo development and blastocyst viability. Participation Type: POSTER Congress: International IVI Congress. Place: Bilbao. Year: 2017.

10.- Authors: Marta Gianzo, Iraia Muñoa-Hoyos, **Itziar Urizar-Arenaza**, Jon Irazusta and Nerea Subiran.Title: The (Pro) Renin receptor is expressed in human sperm cells and y related to sperm quality and embryo development.Type of participation: POSTER. Congress: ESHRE 2017 .Place: Geneva. Year: 2017.

11.- Authors: **Itziar Urizar-Arenaza**, Nerea Osinalde, Marta Gianzo, Roberto Matorras, Antonia Expósito, Jon Irazusta, Nerea Subiran and Irina Kratchmarova. Title: Label-free proteomics reveals a novel signalling pathway modulated via kappa-opioid receptor. Type of participation: POSTER. Congress: International IVI Congress. Place: Bilbao. Year: 2017.

12.- Authors: **Itziar Urizar-Arenaza**, Marta Gianzo, Teresa Ganzabal, Roberto Matorras, Antonia Exposito, Luis casis, Jon Irazusta and Nerea Subirán. Title: El receptor Kappa-opioide regula la

capacitación y reacción acrosómica en los espermatozoides humanos mediante la activación de diversas vías de señalización intracelulares.Type of participation: ORAL. Congress: Sociedad Española de Fertilidad. Place: Malaga. Year: 2016.

13.-Authors: **Itziar Urizar-Arenaza**, Iraia Muñoa, Haizea Estomba, Marta Gianzo, Jon Irazusta and Nerea Subirán. Title: Beta-endorfinaren eragina giza espermatozoideen erreakzio akrosomikoan.Type of participation: ORAL. Congress: Ikergazte, Nazioarteko Ikerketa Euskeraz. Place: Durango. Year: 2014.

14.-Authors: Haizea Estomba, Iraia Muñoa, **Itziar Urizar-Arenaza** , Marta Gianzo, Jon Irazusta and Nerea Subirán.Title: Opioide hartzaileen deskribapena eta kokapena saguen hozi-zelula maskulinoetan.Type of participation: ORAL. Congress: Ikergazte, Nazioarteko Ikerketa Euskeraz. Place: Durango. Year: 2015.

15.-Authors: **Itziar Urizar-Arenaza**, Iraia Muñoa , Haizea Estomba, Marta Gianzo, Roberto Matorras, Exposito A, Jon Irazusta and Nerea Subiran. Title: The kappa-opioid receptor modulates the human sperm capacitation and acrosome reaction.Type of participation: POSTER. Congress: European Society of Human Reproduction and Embriology (ESHRE 2015). Place: Lisbon. Year: 2015.

16.- Authors: Marta Gianzo, Larreategui Z, **Itziar Urizar-Arenaza**, Muñoa I, Estonba H, Irazusta J and Subiran N. Title: Angiotensin II AT2 receptor is expressed in human sperm cells and is involved in sperm motility.Type of participation: POSTER. Congress: ESHRE 2015. Place: Lisbon. Year: 2015.

17.-Authors: Estonba H, Muñoa I, Gianzo M, **Itziar Urizar-Arenaza**, Casis L, Irazusta J and Subirán N. Title: Opioid receptors are present in male germ cells and regulate meiosis.Type of participation: ORAL. Congress: ESHRE 2015. Place: Lisbon. Year: 2015.

18.-Authors: Marta Gianzo, Muñoa I , **Itziar Urizar-Arenaza**, Estonba H, Larreategui Z, Irazusta J and Subiran N. Title: Estudio de la expresión del receptor angiotensina II Tipo 2 en espermatozoides humanos y su implicación en la movilidad espermática. Type of participation: ORAL. Congress: Asociación para el Estudio de la Biologiía de la Reproducción (ASEBIR). Place: San Sebastián. Year: 2015.

19.-Authors: **Itziar Urizar-Arenaza**, Haizea Estomba, Iraia Muñoa, Marta Gianzo, Asier Valdivia, Luz Candenas, Roberto Matorras, Luis Casis, Jon Irazusta and Nerea Subiran. Title: La beta-endorfina potencia la reacción acrosómica inducida por progesterona por una vía PKC calcio independiete.Type of participation: POSTER. Congress: Sociedad Española de Fertilidad. Place: Barcelona. Year: 2014.

20.-Authors: Haizea Estomba, Iraia Muñoa**, Itziar Urizar-Arenaza**, Marta gianzo, Asier Valdivia, Luis Casis, Jon Irazusta and Nerea Subiran. Title: Localización y expresión de los receptores opioides mu, delta y kappa en células germinales masculinas de ratón. Type of participation: ORAL. Congress: Sociedad Española de Fertilidad (SEF). Place: Barcelona. Year: 2014.

21.-Authors: Iraia Muñoa, Haizea Estomba, **Itziar Urizar-Arenaza**, Marta Gianzo, Jon Irazusta, Luis Casis, Asier Valdivia and Nerea Subiran.Title: Sistema opioidearen bidezko gizonezkoen ugalketaren erregulazioa. Type of participation: ORAL. Congress: Osasun-zientziaetako ikertzaileen II.topaketak . Place: Eibar. Year: 2014.

22.-Authors: Carolina Romeu-Periz, M Lierta, A Chueca, C de Bonrostro, I Gimenez, M Sobreviela, A Urries, **Itziar Urizar-Arenaza**, H Estonba and L Casis. Title: Variación del

metabolismo peptídico en el líquido folicular de mujeres patológicas. Type of participation: POSTER. Congress: ASEBIR. Place: Sevilla. Year:2013

23.-Authors: Estonba H, Muñoa I, **Itziar Urizar-Arenaza**, Irazusta J and Subirán N. Title: Expression of opioid receptors (mu,delta and kappa) in mouse male germline cells. Type of participation: ORAL. Congress: 5th Florence-Utah Symposium on Genetics of Male Infertility. Place: Florence. Year: 2013

24.-Authors: **Itziar Urizar-Arenaza**, H Estomba, R. Matorras, A. Valdivia, A.Exposito, J. Irazusta y N.Subirán. Title :Capacitation and acrosome reaction inhibition by the opioid peptide beta-endorphin. Type of participation: POSTER. Congress: 5th International IVI Congress. Place: Sevilla. Year: 2013.

25.-Authors: N. Subirán, H. Estomba, **Itziar Urizar-Arenaza**, L. Crisol, R. Matorras, L. Casis and J. Irazusta. Title: La beta-endorfina inhibe los procesos de capacitación y reacción acrosómica. Type of participation: POSTER. Congress: 29º Congreso Nacional de la Sociedad Española de Fertilidad. Place: Granada. Year: 2012.

26.-Authors: Nerea Subirán, Haizea Estomba, **Itziar Urizar-Arenaza**, M Luz Candenas, Francisco M Pinto, Asier Valdivia and Jon Irazusta. Title: Efecto contrario del péptido opioide beta-endorfina sobre los procesos de capacitación y reacción acrosómica en espermatozoides humanos.Type of participation: POSTER. Congress: XXIX Congreso de la Sociedad Española de Bioquímica y Biología Molecular.Place: Barcelona. Year: 2011.

Stays in International Centers

1.- Center: Center for Experimental BioInformatics (CEBI), Department of Biochemistry and Molecular Biology, University of Southern Denmark.

Place: Odense M Country: Dinamarca
Start day (dd/mm/yy): 01/04/2016 End date (dd/mm/yy): 08/07/2016
Field: Phosphoproteomics. Supervisor: Professor Irina Kratchmarova

2.- Center: Center for Experimental BioInformatics (CEBI), Department of Biochemistry and Molecular Biology, University of Southern Denmark.

Place: Odense M Country:Dinamarca
Start date (dd/mm/yy):01/05/2017 End date (dd/mm/yy):30/06/2017
Field:Fosfoproteomics. Supervisor: Professor Irina Kratchmarova.

Courses

1.- 2018: Characterisation of post translational modifications in cellular signalling. EMBO Course. Odense. Beneficiaria de la EMBO TRavel Grant. Denmark

2.- 2017: Análisis del Semen: según criterios de la OMS. Clínica CEIFER

3.- 2017: Ensembl. UPV/EHU – EMBL-EBI.
4.- 2017: Ecole thématique Bioinformatics on protein-protein interactions for wet lab scientists. Instituto Pasteur.

5.-2016: SPPS paquete estadístico. UPV/EHU

6.-2013: Curso de Riesgos Biológicos. UPV/EHU

7.-2014: Fundamentos y Aplicaciones de la Microscopia confocal, la microscopia electronica y a la citometria de flujo en Biomedicina. UPV/EHU

8.-2014: Bioforo: "Pain:from individual perception to treatment, through molecular biology". UPV/EHU

9.-2014: Seminario BD Biosciences Discovery. UPV/EHU

10.-2014: Curso: Avances en Nutrición, Actividad física e Hidratación.UPV/EHU

12.-2015: Cursos de Coursera: "Drugs and the Brain", "Bioinformatics"

13.-2015: Seminario del Real Time y PCR D. UPV/EHU

14.-2015: Curso "Writing in Sciences". Stanford University.

15.-2015: Cursos de Verano: Potencial terapéutico de los cannabinoides: Evidencias actuales y perspectivas de futuro.

16.-2015: Doctoriales Transfronterizos (Cross-border doctorials) en Arantzazu, Octubre 2014.

17.-2015: 2 Encuentro Vasco-Chileno de Investigación Biomédica

Other Merits

- Assistant teacher in 'Cellular Physiology' subject, First year of Medicine. 2015-2018.

-Assistant teacher in ' Experimental Oncology'subject, Biomedical Research Master. Year 2012/2013.

- Assistant teacher in 'Reproductive Biology and Embrionary Development' subject. Year 2012-2013.

Awards

- Toribio Echeverria Award. SPERM SELECT Best Idea

- Best Oral communication: Quantitative phosphoproteomics reveals novel phosphosites downstream Kappa Opioid receptor in human spermatozoa, Congress EUPA. Santiago de Compostela, 2018.