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Universidad del País Vasco
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The role of protein NEDDylation in the pathogenesis of cholangiocarcinoma: new potential therapeutic target

Tesis presentada por

PAULA OLAIZOLA REBÉ

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Para la obtención del título de doctora en

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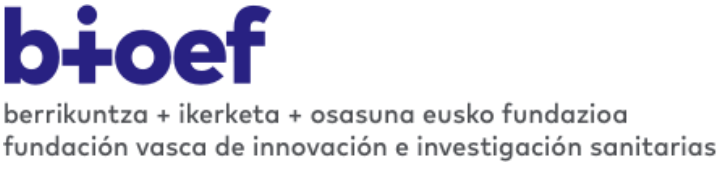

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Abbreviations

3D	Three dimensional
5-FU	5-fluoroacil
α-SMA	α-smooth muscle actin
ABC	ATP-binding cassette
ACTB	β-actin
AGRN	Agrin
AKR1C1	Aldo-keto reductase family 1 member C1
AKR1C3	Aldo-keto reductase family 1 member C3
AMP	Adenosine 5'-monophosphate
ANXA2	Annexin A2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and rad3-related protein
BAK	BCL2 agonist/killer
BAX	BCL2 associated X
BIM	BCL2 like 11
BS^3	Bis(sulfosuccinimidyl)suberate
BSA	Bovine serum albumin
CA19-9	Carbohydrate antigen 19-9
CAF	Cancer-associated fibroblasts
CCA	Cholangiocarcinoma
CCT3	T-complex protein 1 subunit gamma
CDK1	Cyclin-dependent kinase 1 (Cdc2)
CDC25A	Cell division cycle 25 homolog A
cDNA	Complementary DNA
CDT1	Chromatin licensing and DNA replication factor 1
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
Cis	Cisplastin
CK19	Cytokeratin 19
COL6A1	Collagen alpha-1(VI) chain
COPB1	Coatomer subunit beta
COX-2	Cyclooxygenase-2
CRISPR	Clustered regularly interspaced short palindromic repeats
CRL	Cullin-RING ligase
CSN	COP9 signalosome
CT	Computed tomography
DAB	3,3-diaminobenzidine
dCCA	Distal CCA
DDB2	DNA damage binding protein 2
DDR	DNA damage response
DLST	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
DMC1	Meiotic recombination protein DMC1/LIM15 homolog
Doxo	Doxorubicin
DSB	Double-strand break

eCCA	Extrahepatic CCA
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIF3E	Eukaryotic translation initiation factor 3 subunit E
EIF3F	Eukaryotic translation initiation factor 3 subunit F
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ERBB2	Erb-b2 receptor tyrosine kinase 2
ERCP	Endoscopic retrograde cholangiopancreatography
FAP1	Fibroblast activated protein 1
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gem	Gemcitabine
GemCis	Gemcitabine and cisplatin combination
GO	Gene ontology
gRNA	RNA guide
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hh	Hedgehog
HIFα	Hypoxia inducible factor α
HMGA	High mobility group A
HMGB1	High mobility group B protein 1
HRP	Horseradish peroxidase
HSC	Hepatic stellate cell
HSP90AA1	Heat shock protein HSP 90-alpha
HSPA1A	Heat shock 70 kDa protein 1A
IBDU	Intrahepatic bile duct unit
iCCA	Intrahepatic CCA
IDH	Isocitrate dehydrogenase
i.e.	Latin: *id est* (it is)
IF	Immunofluorescence
IG-iCCA	Intraductal-growing iCCA
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-12	Interleukin 12
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IRE1	Serine/threonine-protein kinase/endoribonuclease IRE1
KIF1C	Kinesin-like protein KIF1C
KPNA2	Importin subunit alpha-1

MCM1	DNA replication licensing factor MCM1
MDM2	Murine double minute 2
MDR1	Multidrug resistant protein 1 or P-glycoprotein
MF-iCCA	Mass-forming iCCA
MMP	Matrix metalloprotease
MOC	Mechanism of chemoresistance
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
NAE	NEDD8 activating enzyme E1
NAE1	NEDD8 activating enzyme E1, regulatory subunit
NAFLD	Non-alcoholic fatty liver disease
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NBD	Normal bile duct
ncRNA	Non-coding RNA
NDUFA8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NEDP1	NEDD8-specific protease
NEM	N-ethylmaleimide
NER	Nucleotide excision repair
NHC	Normal human cholangiocytes
NHEJ	Non-homologous end joining
NICD1	Notch intracellular domain 1
NK	Natural killer
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
OCT4	POU class 5 homeobox 1
ORC1	Origin recognition complex 1
P/S	Penicillin-streptomycin
p-H2AX	Phosphorylated histone H2A histone family member X
PAM	Protospacer adjacent motif
PBC	Primary biliary cholangitis
pCCA	Perihilar CCA
PCNA	Proliferating cell nuclear antigen
PD	Padua
PDGF	Platelet-derived growth factor
PEBP1	Phosphatidylethanolamine-binding protein 1
PHB	Prohibitin-1
PI-iCCA	Periductal-infiltrating iCCA
PLD	Polycystic liver disease
POLD3	DNA polymerase delta subunit 3
PRDX1	Peroxiredoxin-1
PRDX2	Peroxiredoxin-1
PRKDC	DNA-dependent protein kinase catalytic subunit
PSC	Primary sclerosing cholangitis
PTMs	Post-translational modifications
qPCR	Quantitative polymerase chain reaction

RAN	GTP-binding nuclear protein Ran
RBX	RING-box protein
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SEC22B	Vesicle-trafficking protein SEC22b
SOX2	(Sex-determining region Y)-box transcription factor 2
SOX17	(Sex-determining region Y)-box transcription factor 2
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC	Solute carrier
SMURF1	SMAD-specific E3 ubiquitin-protein ligase 1
SN	Surrounding normal
SS	San Sebastian
SUCLA2	Succinate-CoA ligase [ADP-forming] subunit beta, mitochondrial
T_0	Time zero
TACE	Transarterial chemoembolization
TAMs	Tumor-associated macrophages
TARE	Transarterial radioembolization
TBS-T	Tris-buffered saline with 0.1% Tween® 20
TCA	Tricarboxylic cycle
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor β
TGF-βR	Transforming growth factor β receptor
TIGER	The Thailand Initiative in Genomics and Expression Research
TILs	Tumor-infiltrating lymphocytes
TLDA	TaqMan Low-Density Array
TME	Tumor microenvironment
Tregs	Regulatory T cells
TXN	Thioredoxin
UBA3	Ubiquitin-activating enzyme or NEDD8 activating enzyme E1, catalytic subunit
UBE2F	Ubiquitin-conjugating enzyme E2F
UBE2M	Ubiquitin-conjugating enzyme E2M
UBL	Ubiquitin-like protein
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial
VAMP3	Vesicle-associated membrane protein 3
VCP	Transitional endoplasmic reticulum ATPase
VEGF	Vascular endothelial growth factor
WB	Western blot
WEE1	WEE1 G_2 checkpoint kinase
WNT	Wingless
WT	Wild type
XRCC5	X-ray repair cross complementing 5 (Ku80)
XRCC6	X-ray repair cross complementing 6 (Ku70)
ZO-1	Zona occludens 1

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Introduction

I.1. The liver

I.1.1. Physiology

The liver is the largest internal organ of the human body and one of the most important for the maintenance of physiological homeostasis.[1] The liver performs several and complex metabolic functions including carbohydrate, lipid and amino acid metabolism.[2] Additionally, this organ serves as nutrient storage for glucose, lipids, iron and vitamins.[2] The broad spectrum of functions accomplished by the liver also includes the synthesis and secretion of albumin, transferrin, fibrinogen, apolipoproteins, and other plasma proteins into blood.[2] Bile production and secretion is a major function of the liver and is crucial for nutrient absorption and biliary clearance of organic and inorganic solutes.[3] Furthermore, the liver receives a dual blood supply (i.e., the hepatic portal vein and the hepatic artery), becoming exposed to a variety of toxic compounds. In this regard, the liver has the ability to metabolize and secrete potentially harmful biochemical products that are produced by the body (i.e., bilirubin or ammonia), to detoxify and eliminate pathogenic and xenobiotic agents, as well as to regulate the immune response.[2,4] Of note, hepatic functions are maintained even after massive liver damage or partial resection, due to its unique regenerative capacity.[5]

I.1.2. Macroscopic and microscopic anatomy

Anatomically, the liver is divided into two large lobes (i.e., right and left) and two small central ones (i.e., quadrate and caudate), which are mostly covered by a fibrous layer, known as the Glisson's capsule.[2,6] The liver parenchyma is arranged in thousands of hexagonal units named hepatic lobules (**Figure I.1**).[2] Each hepatic lobule represents the functional and structural entity of the liver, consisting of a central vein from which hepatocytes radiate forming linear cords towards a portal triad, formed by connective tissue enclosing branches of the hepatic artery, portal vein and bile duct (**Figure I.1**).[6] Oxygen, nutrients, bile acids and hormones delivered by venous and arterial blood are drained from the terminal branches of the portal vein and hepatic artery to the lobule's central vein through the hepatic sinusoids (**Figure I.1**).[2] Similarly, hepatocyte-secreted bile reaches the bile duct branches at the portal triad through a network of canaliculi.[2] The sinusoidal capillaries lie in between the cords of hepatocytes separated by a narrow perisinusoidal space (also known as the space of Disse), which comprises reticular fibers and nutrient-rich blood plasma. The direct contact between sinusoidal capillaries and hepatocytes improves metabolic exchange.[2]

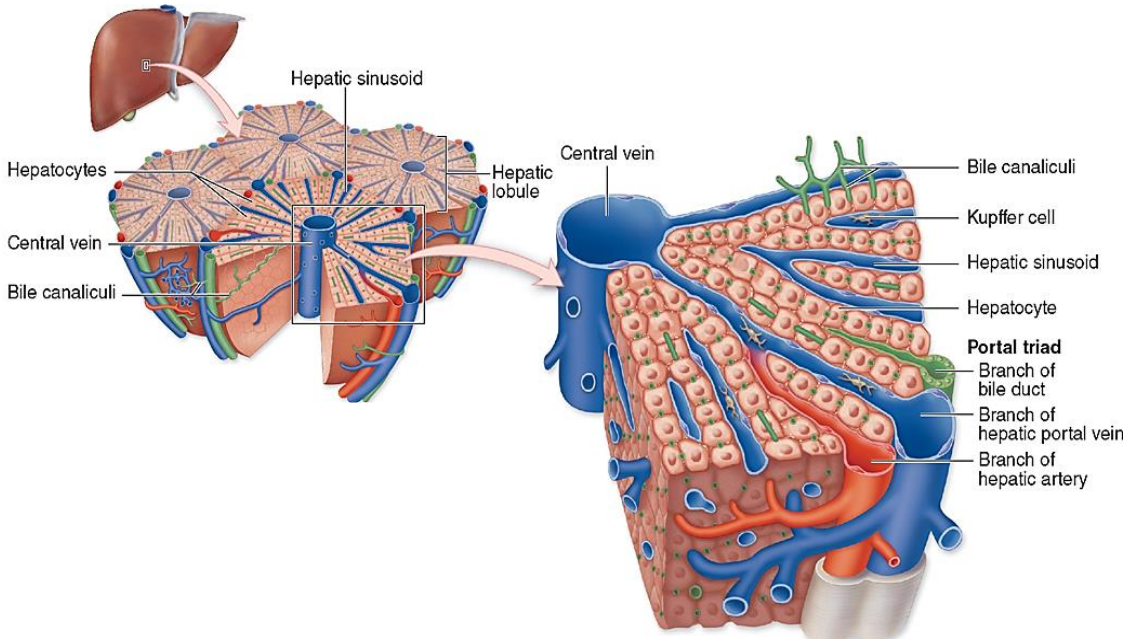

Figure I.1. Microscopic structure of the liver. The liver is structured in hexagonal hepatic lobules composed of cords of hepatocytes radiating from the central vein outwards to the portal triads. (Adapted from Mescher AL, 2013)[7]

Multiple cell populations (i.e., parenchymal and non-parenchymal) coexist within the liver and coordinately govern the hepatic function at multiple levels.[1,2] Hepatocytes and cholangiocytes are the two main epithelial cell types of this organ. Roughly, 70-80% of the liver volume consists of parenchymal hepatocytes, which are responsible for the majority of the metabolic functions in the liver, whereas cholangiocytes, the epithelial cells lining the bile ducts, only represent 3-5% of the total liver cells, even though they carry out crucial functions in the modification and transport of the bile.[1,2] Other non-parenchymal cells of the liver include the liver resident macrophages or Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells that are involved in immunological, fibrogenic and substance exchange processes, respectively.[1,2]

I.2. The biliary tract

I.2.1. Anatomy

The biliary tract is comprised of several ducts lined by cholangiocytes that regulate the production, composition and transport of the bile from the liver to the duodenum. As aforementioned, primary bile is secreted from the hepatocytes into the canaliculi (i.e. a narrow tubular space between the apical membranes of two adjacent hepatocytes) and is subsequently collected by the canals of Hering, leading to the ductule-canalicular junction.[8] These specialized channels serve as the anatomical and physiological transition from the hepatocyte-lined canaliculi to cholangiocyte-lined ductules (<15 µm), which ultimately form the biliary tree (**Figure I.2**).[8,9] These small structures serially converge at the portal space to form the interlobular ducts (15-100 µm), which progressively enlarge to form septal ducts (100-300 µm), area ducts (300-400 µm) and segmental ducts (400-800 µm) (**Figure I.2**).[8,9] The bile collected from the right and left lobes is then drained to the corresponding hepatic ducts (>800 µm), which are considered the limit of the intrahepatic biliary tree (**Figure I.2**).[8,9] Finally, the bile flows through the extrahepatic biliary tree (i.e., common hepatic duct, cystic duct, gallbladder, and common bile duct) ultimately reaching the duodenum (**Figure I.2**), where it enables lipid digestion and absorption.[8,9]

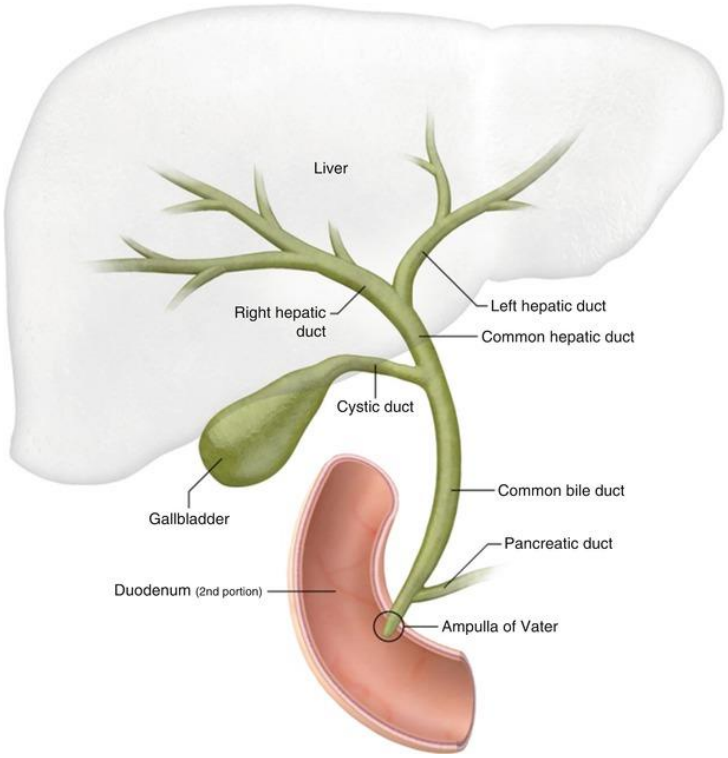

Figure I.2. Biliary tree architecture. The biliary tree consists of a network of intrahepatic and extrahepatic tubular ducts where the hepatocyte-secreted bile is modified and transported to the duodenum.[10]

I.2.2. Cholangiocytes

Cholangiocytes constitute a small proportion of all liver cells but are very important in health and disease. Biologically, these epithelial cells play essential roles for normal liver function and are key in the regulation of hepatocyte-derived bile composition, facilitating biliary salt reabsorption and contributing to its fluidization and alkalinization. Cholangiocytes express primary cilia that arise from their apical membrane.[11–13] This microtubule-based organelle possesses mechano-, chemo- and osmo-sensor properties that allow the detection of changes in bile flow and composition, and is able to transduce such stimuli into intracellular signaling ultimately modulating bile formation.[14,15] Furthermore, cholangiocytes display multiple transmembrane carriers (i.e., aquaporins, transporters and exchangers) at the apical and/or basolateral sides that are involved in bile composition regulation and biliary bicarbonate secretion,[3,16–18] protecting cholangiocytes from damaging or toxic agents.[19,20]

I.2.3. Cholangiopathies

Biliary diseases, also termed as cholangiopathies, refer to a large group of chronic liver diseases that share cholangiocytes as their central target.[16] Cholestasis, chronic inflammation, ductular reaction and fibrosis seem to be common events among biliary disorders. However, cholangiopathies are generally classified in different categories attending to their etiology in: a) immune-mediated [such as primary biliary cholangitis (PBC)[16] or primary sclerosing cholangitis (PSC)],[21] b) infectious (caused by opportunistic infections with *Cryptosporidium parvum*),[22] c) genetic [e.g., polycystic liver disease (PLD),[23] cystic fibrosis[24] or Alagille's syndrome],[25] d) vascular (post-ischemic cholangiopathies),[26] e) neoplastic [e.g., biliary tract cancer or cholangiocarcinoma (CCA)], f) drug-induced [e.g., amoxicillin/clavulanic acid, carbamazepine, 5- fluorouracil (5-FU), among others],[27,28] or g) idiopathic (e.g., biliary atresia, idiopathic childhood/adulthood ductopenia).[16] Although being considered rare diseases, cholangiopathies account for substantial morbidity and mortality, being a major indication for liver transplantation as curative therapy.[29–31] Therefore, elucidating the molecular mechanisms underlying the development and progression of these diseases is of utmost importance to find potential targets for therapy.

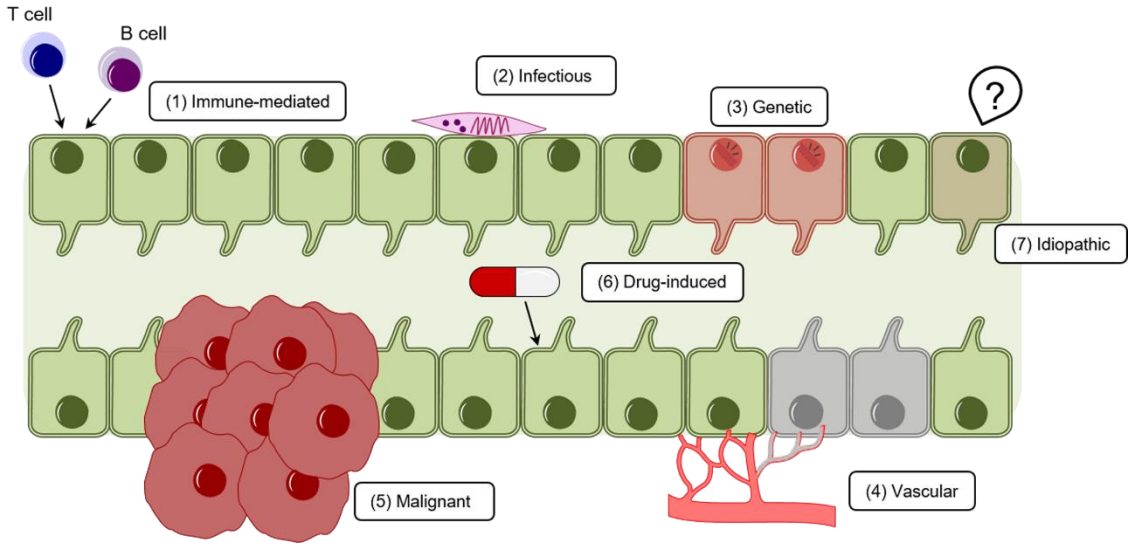

Figure I.3. Classification of cholangiopathies according to their etiology. Cholangiopathies are chronic liver diseases that affect cholangiocytes and are categorized as (1) Immune-mediated, (2) Infectious, (3) Genetic, (4) Ischemic, (5) Malignant, (6) Drug-induced and (7) Idiopathic.

I.3. Cholangiocarcinoma

I.3.1. General features

CCA comprises a heterogeneous group of malignancies arising along the biliary tree. These tumors emerge from the malignant transformation of the epithelial cells lining the bile ducts (i.e. cholangiocytes), although it can derive from peribiliary glands, hepatic stem cells or even hepatocytes under transdifferentiation.[32] CCA is the second most frequent primary liver tumor (~15%), after hepatocellular carcinoma (HCC), and represents ~3% of all gastrointestinal cancers. The global trend of CCA over the past decades indicates an increase in both incidence (0.3-6 per 100,000 inhabitants per year)[33–35] and mortality (1-6 per 100,000 inhabitants per year).[36–39] Despite being a rare tumor in most Western countries (<6 cases per 100,000 people), the global geographical distribution of CCA is asymmetrical and Southeast Asian countries, such as China, South Korea, Thailand and Japan, present significantly higher incidence.[37,39,40] Such discrepancy is likely due to differences in exposure to specific risk factors, particularly to endemic liver fluke parasites, and because of a high hepatitis B virus (HBV) and hepatitis C virus (HCV) prevalence in Asia.[35,41–43]

I.3.2. Classification

Considering the heterogeneity and diversity of CCAs, several classifications have been proposed.[38,44,45] The most widely used CCA classification is based on the anatomical location of the tumor. However, other parameters, such as tumor growth pattern or the cell of origin may be better predictors of CCA behavior.[32,46,47]

Anatomically, CCAs are classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA). iCCAs can emerge from any portion of the intrahepatic biliary tree, from segmental bile ducts to smaller branches (**Figure I.4**). pCCAs arise in the right and/or left hepatic duct and/or surrounding their junction, while dCCAs affect the common bile duct. iCCAs can be further divided attending to their growth pattern into mass-forming (MF-iCCA), periductal infiltrating (PI-iCCA) and intraductal growing (IG-iCCA), although mixed growth patterns have been described (**Figure I.4**).[48] MF-iCCA encompasses a mass of tumor cells affecting the biliary duct and the liver parenchyma.[49] In contrast, PI-iCCAs grow longitudinally along the wall of large bile ducts leading to progressive wall thickening and stricture development,[37,50,51] whereas IG-iCCAs present a papillary growth pattern towards the duct lumen.[51,52] On the other hand, pCCAs and dCCAs generally present as poorly defined sclerosing tumors and, less frequently, as papillary tumors, and exhibit similar growth patterns to PI- and IG-type of iCCAs.[53–55]

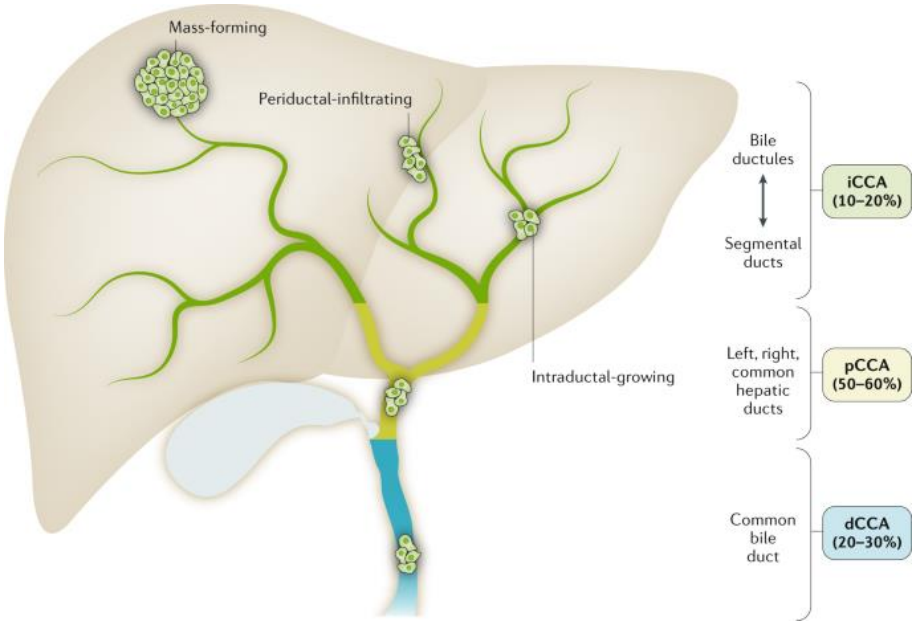

Figure I.4. CCA classification. Depending on their anatomical site of origin, CCAs are classified as intrahepatic (iCCA), perihilar (pCCA) or distal (dCCA). iCCAs are also classified into mass-forming, periductal infiltrating or intraductal growing according to their growth pattern.[37]

Histologically, pCCA and dCCA are predominantly mucinous adenocarcinomas or papillary tumors,[50,56] while iCCAs are more heterogeneous and show several histological variants. In this regard, two main histological subtypes of iCCA are usually distinguished according to the level or size of the affected bile duct. Thus, small bile duct (mixed) type iCCA arises as a small-sized tubular or acinar adenocarcinoma with nodular growth invading the liver parenchyma, and with minimal or no mucin production.[57–61] Alternatively, large bile duct (mucinous) type iCCA affects large intrahepatic bile ducts and is constituted by mucin-producing columnar tumor cells arranged in a large-duct or papillary architecture.[61–64] The distinction between small and large bile duct types does not only have histopathological implications but also distinguishes iCCA subtypes with different clinicopathological and molecular features.[58,61]

I.3.3. Risk factors

The etiologies of most CCAs are unknown; however, several risk factors with different degree of predisposition to CCA development have been established.[39,65] The presence of certain biliary pathologies such as choledochal cysts, stones within the bile ducts, cirrhosis, chronic biliary diseases (such as Caroli Disease or PSC) are strongly associated with CCA. In fact, among PSC patients (global incidence ~1/100,000) there is a 10-15% risk of developing CCA,[66–68] while in the case of Caroli Disease the risk reaches 6-30%.[69,70] Moreover, viral infections due to HBV and HCV, as well as liver fluke parasites, such as *Opisthorchis viverrini* and *Clonorchis sinensis*, have been reported to augment the risk of CCA development. Exposure to certain toxins (asbestos, dioxins or nitrosamines) has also been associated with CCA. On the other hand, alcoholic liver disease, cirrhosis, diabetes, tobacco and non-alcoholic fatty liver disease (NAFLD) is a less strong but highly prevalent risk factor.[37]

I.3.4. Molecular mechanisms of pathogenesis

The process of biliary tumorigenesis involves multiple complex mechanisms to drive the malignant transformation of cholangiocytes. Among them, sustained proliferation, death evasion, neo-angiogenesis as well as the development of invasive and colonizing capacities are some of the main hallmarks of CCA cells.[71] Underlying these hallmarks are genetic, epigenetic and molecular alterations affecting the target cells.[37]

I.3.4.1 Genetic and epigenetic alterations

Several studies, using whole and targeted DNA sequencing approaches, have emphasized the genomic complexity of CCA tumors, identifying the most prevalent gene mutations affecting crucial genes in cell growth promotion (*KRAS*, *BRAF*, *SMAD4*, *FGFR1-3*, *EGFR*, *NOTCH*, *WNT*), DNA rearrangements and genomic instability (*TP53*, *CDK1NA*, *CCND1*, *ATM*, *ROBO2*, *BRCA1* and *BRCA2*), de-ubiquitination (*BAP1*) and chromatin remodeling (*ARID1A*, *ARID1B*, *ARID2A*, *SMARCA4*, *PBRM1*, *MLL2*, *MLL3*, *KMT2C*).[37] Furthermore, mutations deregulating Wnt/β-catenin, Notch or PI3K signaling networks have been described. Of note, the discovery of hotspot *IDH1* and *IDH2* mutations, as well as the constitutive *FGFR2* fusions are driving mutational profile-based clinical trials testing specific compounds targeting these alterations.[37,39]

Despite displaying shared mutations, CCA subtypes present different genomic profiles. Thus, *FGFR*-fusions together with *TP53*, *KRAS*, *IDH1/2* and *BAP1* mutations are the most common events in iCCA, whereas *PRKACA* and *PRKACB* fusions, as well as mutations in *ELF3* preferentially occur in p/dCCA.[72,73] Integrative genomic

studies have aimed to stratify CCA patients based on prognosis.[74,75] In this regard, mutations in *TP53* or *KRAS* have been associated with higher tumor recurrence and lower overall survival in CCA patients after surgical resection,[72] compared to patients with *IDH* mutations or patients without mutations in any of those 3 genes. Although most CCA tumor mutations are somatic, a proportion of patients (5-10%) harbor germline mutations in *BRCA1/2*, *ATM* or *BAP1*, which may predispose to CCA development.[76,77]

Deregulated DNA methylation, histone modifications and aberrant non-coding RNA (ncRNA) expression can also trigger unbalanced transcription of a plethora of target genes that sustain malignant cell transformation without modifying the DNA sequence.[78,79] In this regard, CpG hypermethylation has been reported in CCA, supporting the relevance of epigenetic modifications in these tumors. However, the epigenetic modifications in CCAs are still poorly studied and a better understanding of these processes may hold promising translational potential, serving as diagnostic and prognostic tools, but also as targets for new therapeutic strategies.

I.3.4.2 Signaling and molecular networks

CCAs often arise in the context of prolonged biliary inflammation and cholestasis, which provide a rich milieu of pro-inflammatory cytokines, growth factors and toxic bile acids that might contribute to cholangiocarcinogenesis.[37,80,81] This setting presumably triggers aberrant signaling leading to uncontrolled cellular proliferation, survival, angiogenesis and invasion, overall promoting CCA development and sustaining tumor progression (**Figure I.5**). Transcriptomic profiling identified the presence of two subclasses of iCCA: the “inflammation” (38%) and “proliferative” (62%) subtypes, characterized by the activation of immune-mediated and oncogenic pathways, respectively.[75] Among the pro-inflammatory cytokines sustaining CCA growth and progression, interleukin 6 (IL-6) is a major player, being involved in the activation of the JAK/STAT3, ERK1/2 or the mitogenic p38 signaling pathways promoting tumor proliferation and growth.[82–85] On the other hand, multiple signals [e.g., inducible nitric oxide synthase (iNOS) activation, bile acids, oxysterol, among others) can induce the expression of the inflammatory mediator cyclooxygenase-2 (COX-2), triggering proliferation and preventing apoptosis through prostaglandin E2-mediated AKT or epidermal growth factor (EGF) pathway activation.[86,87]

Multiple signaling networks involved in biliary development, including Notch, Wnt/β-catenin, Hedgehog (Hh) or Hippo/YAP, are re-activated during liver repair or in an inflammatory setting.[88] Regarding CCA, a prominent activation of Notch, Wnt/β-catenin and transforming growth factor-β (TGF-β) was observed in comparison to

HCC.[89] The Notch pathway mediates biliary repair, growth and hepatocyte transdifferentiation into cholangiocytes during carcinogenesis.[90] Indeed, iCCA development in mouse models has been observed after experimental overexpression of Notch intracellular domain 1 (NICD1) in hepatocytes.[91,92] Moreover, the majority of CCAs present augmented Wnt/β-catenin signaling, in part as a consequence of the activated macrophage-mediated release of Wnt ligands[93,94] but also as a result of mutations[95] or DNA methylation alterations affecting components of this pathway,[96] altogether regulating cell growth and survival.[95] Likewise, most CCAs display activated Hh signaling,[97,98] which could be induced by myofibroblasts[99] or hepatic stellate cells (HSC)-secreted platelet-derived growth factor BB (PDGF-BB),[100] enhancing cell proliferation, migration and invasion. On the other hand, the Hippo/YAP signaling pathway is known to modulate organ size, cell proliferation and apoptosis.[101] In CCA, upregulation of YAP has been reported and correlates with worse prognosis.[102–104] Despite genetic alterations of the YAP pathway being infrequent,[105] up to 14% of CCAs present mutations in *ARID1A*, which encodes for a subunit of the chromatin remodeling complex SWI/SNF that reduces YAP transcriptional activity.[106]

Receptor tyrosine kinase (RTK) signaling activation is a common event in all CCA subtypes. Overactivation of *EGFR1*, *ERBB2* and *MET* RTK signaling has been reported in CCA and is associated with worse prognosis.[74,75] RAS-MAPK and PI3K-AKT-mTOR pathways are triggered by RTK signaling, resulting in augmented proliferation, apoptosis evasion and enhanced tumor growth.[74,75,107–109] In addition, chromosomal fusion rearrangements in *FGFR2* occur in CCA. Noteworthy, molecular alterations in RTK signaling pathways constitute amenable targets for therapy.

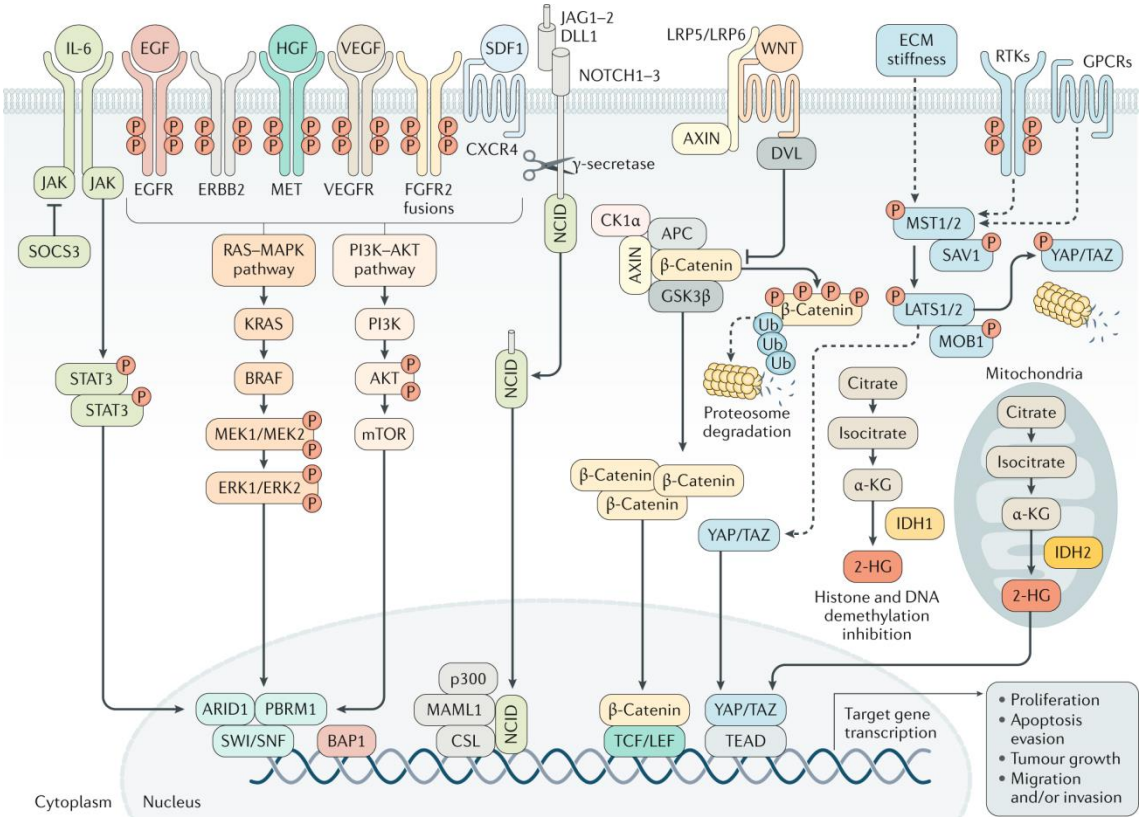

Figure I.5. Signaling pathways driving cholangiocarcinogenesis. CCA development, growth and progression involve complex molecular processes that include the interplay between extracellular ligands and the increased expression of aberrant activation of cell surface receptors that lead to deregulation of signaling pathways, ultimately enhancing cell proliferation, survival, migration or invasion. The most commonly mutated genes that might result in the overactivation of some of these pathways are *KRAS*, *BRAF*, *ARID1*, *PBRM1*, *BAP1*, *IDH1* and *IDH2*. Abbreviations: 2-HG, 2-hydroxyglutarate; ECM, extracellular matrix; RTK, receptor tyrosine kinase.[37]

I.3.5. Tumor microenvironment

CCAs present an extensive desmoplastic tumor microenvironment (TME) and, even though epithelial cells are generally considered as the coordinators of tumor growth, the crosstalk between the tumor and its stroma cannot be understated. In fact, TME can drive the neoplastic transformation of epithelial cells and regulate numerous cancer hallmarks.[110–115] The CCA stroma consists of a complex network of extracellular matrix proteins[116,117] and diverse cell types, including infiltrating immune cells (e.g., macrophages, neutrophils, natural killer or T cells), endothelial cells and cancer-associated fibroblasts (CAFs),[118] that interact with the tumor epithelium to support and sustain cancer progression (**Figure I.6**).

CAFs are a heterogeneous spindle-shaped cell population with mesenchymal origin that contributes to tumor progression.[119] In CCA, tumor growth and reduced survival positively correlate with the abundance of CAFs.[120] Although their origin remains uncertain, CAFs most likely derive from quiescent HSCs, tissue-resident portal fibroblasts, pericytes, bone marrow-derived mesenchymal stem cells and monocyte precursor-derived fibrocytes through transdifferentiation and activation.[121–123] CAFs can stimulate CCA growth through the release of short-ranged and direct morphogenetic signals such as Notch[124] or Hh[98]. Additionally, CAFs can express several matrix metalloproteases (MMPs) themselves[125,126] or communicate with other TME cells to release them, promoting a malignant CCA phenotype.[127] In turn, CCA cells can secrete PDGF-D and TGF-β that stimulate the recruitment and activation of fibroblasts.[128,129] Moreover, malignant cholangiocyte-derived PDGF-D induces CAFs secretion of vascular growth factors (e.g., VEGF-A, VEGF-C) which attract lymphatic endothelial cells, favoring CCA cell intravasation and metastasis.[130]

Among the immune cells residing within the TME, tumor-associated macrophages (TAMs) are the most relevant population.[113] These are mainly alternatively activated M2 macrophages, with anti-inflammatory and immunosuppressive characteristics that contribute to cancer progression.[131] As aforementioned, activated macrophages can secrete Wnt ligands activating the Wnt/β-catenin signaling in CCA cells, promoting their proliferation.[93,94] Tumor-infiltrating neutrophils (TINs) and lymphocytes (TILs) are also present in CCA TME. TINs seem to inversely correlate with $CD8^+$ T cells and positively correlate with regulatory T cells (Tregs).[132] In this regard, the abundance of TINs and Tregs together with reduced $CD8^+$ T cell infiltrates are associated with poor prognosis in patients with CCA.[132] In contrast, improved prognosis was described in CCA patients with enhanced $CD4^+$ and $CD8^+$ T cell infiltrates.[133–135] For this reason, a decrease in adaptive immune response

components and an increase of immunosuppressive Tregs has been suggested to permit immune scape of the tumor and has been related to CCA progression.[133,136]

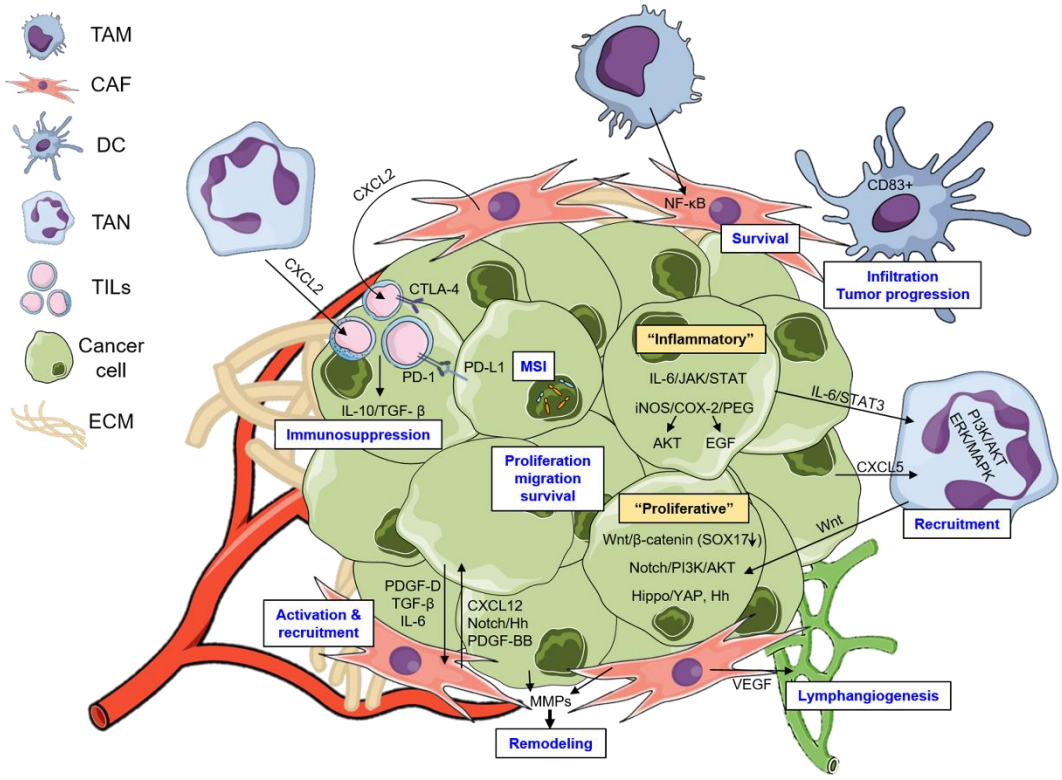

Figure I.6. Tumor microenvironment and the pathogenesis of cholangiocarcinoma. The crosstalk between cancer cells and their stroma triggers the activation of several signaling pathways in tumor tissue that results in cancer cell survival, proliferation and migration, immune cell recruitment and infiltration, immunosuppression, microsatellite instability, extracellular matrix remodeling and lymphangiogenesis, thus supporting tumor growth and progression. Abbreviations: CAF, cancer-associated fibroblast; COX-2, cyclooxygenase; CTLA-4, cytotoxic T lymphocyte antigen 4; DC, dendritic cell; ECM, extracellular matrix; iNOS, inducible nitrogen oxide synthase; MSI, microsatellite instability; PDGF, platelet-derived growth factor; PD-1, programmed death protein 1; PD-L1, programmed death ligand 1; PGE, prostaglandin E; TAM, tumor-associated macrophage, TAN, tumor-associated neutrophil; TILs, tumor-infiltrating lymphocytes. (Adapted from Rodrigues PM *et al*., 2020)[137]

I.3.6. Diagnosis

CCAs are generally asymptomatic in early stages thus, most patients are diagnosed at advanced phases (~70%) when the disease is already widespread. Late diagnosis, together with the highly chemoresistant nature of these tumors, compromise the possible therapeutic options and contribute to their dismal prognosis. Although there are no specific symptoms, abdominal pain, malaise, fatigue, pruritus, weight loss and/or jaundice, among others, might appear during tumor progression.

Diagnosis is usually conducted by combining imaging methods [i.e., computed tomography (CT), magnetic resonance imaging (MRI) or endoscopic retrograde cholangiopancreatography (ERCP)], analysis of non-specific serum tumor markers [i.e., carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9)] and histological analysis of tumor biopsies.[37,39,138,139] Nonetheless, the current non-invasive diagnostic tools (i.e., imaging methods and tumor markers in serum) display low sensibility and specificity, and always require histological confirmation. The lack of accurate non-invasive markers and prognosis predictors in CCA claims for an urgent need to combine efforts and search for precise and valid diagnostic biomarkers to improve patient welfare and outcome.

I.3.7. Therapeutic strategies

Currently, surgical resection of the tumor or liver transplantation are the only potentially curative options for CCA. The eligibility of CCA patients for surgical resection is conditioned to their clinical status, tumor extension as well as the presence or absence of metastasis or locally-advanced disease.[140] However, most CCA patients present with advanced unresectable tumors, and thus, less than one third undergo complete resection.[140] Besides, relapse after surgical resection is frequent and patients present a short 5-year survival (22-44% for iCCA, 11-41% for pCCA and 27-37% for dCCA),[141] prompting studies aiming to identify patients at risk of recurrence and focused on adjuvant therapy research. In this regard, the BILCAP study, a chemotherapy-based phase III clinical trial, reported benefits in terms of overall survival and relapse-free survival when employing capecitabine as adjuvant therapy in biliary tract cancers.[142] Based on the favorable results obtained, international guidelines recommend capecitabine as adjuvant therapy after curative resection of CCA.[142] Liver transplantation for CCA is controversial, and even though different multicenter studies have accomplished promising results in terms of disease-free or overall survival rates,[143–146] liver allograft supply and life-long immunosuppression are important limitations of this strategy.

In unresectable cases, palliative treatment remains the only possible option. Robust data derived from the phase III ABC-02 and the phase II BT22 trials support the use of first-line gemcitabine and cisplatin combination (GemCis) chemotherapy in patients with advanced CCA.[147,148] Once resistance to first-line therapy is developed, FOLFOX (folinic acid, 5-FU and oxaliplatin) has shown potential benefit as second-line therapy for CCA.[149] Additionally, more intensive approaches using triple chemotherapy are currently being assessed as first-line chemotherapeutic strategies.[150,151] Locoregional therapies such as transarterial chemoembolization (TACE), transarterial radioembolization (TARE) and liver chemosaturation constitute promising therapeutic options[152–154] but evidence supporting their efficacy is modest and further studies confirming their value are needed.[154]

Aiming to set the basis for precision medicine, the currently explored treatment options are based on the mutational signatures driving CCA. Several ongoing clinical trials are evaluating multiple molecules targeting specific genetic alterations such as *IDH1/2* mutations, *FGFR* alterations, RTK fusions or *EGFR*, *MET* and *ERBB2* mutations. Based on their promising achievements, molecular profiling in cancer, identifying mutations/amplifications/fusions amenable for targeted therapy, could represent a significant improvement in patient management.[137] Finally, in spite of emerging as an attractive anti-cancer therapeutic option, clinical data on immunotherapy for CCA is limited.

I.4. Posttranslational modifications

I.4.1. General concepts

Posttranslational modifications (PTMs) refer to the covalent attachment or proteolytic cleavage of functional groups or proteins to or from substrate proteins. These chemical changes alter the structure and properties of individual proteins, affecting their stability, activity, turnover, localization and/or interaction with other molecules. To date, more than 450 PTMs have been identified and such wide variety includes phosphorylation, methylation, acetylation, ubiquitination, SUMOylation, NEDDylation, glycosylation and lipidation, among others. These proteome modifications constitute a pivotal mechanism that regulates protein levels and function, allowing cells to rapidly respond to diverse stimuli.[155,156] Indeed PTMs can activate or inhibits multiple signaling networks, being determinant in numerous biological processes such as gene expression, signal transduction, proliferation, survival, protein-protein and cell-cell interactions, as well as in mediating communication between cells and their environment. [155,156] Given their relevance in physiological processes, perturbation of PTMs commonly lead to cell

disturbances.[156] Moreover, altered cellular states including differentiation or malignant transformation of cells could be accompanied by the acquisition of unique PTM hallmarks.[156]

I.4.2. The NEDDylation pathway

Protein NEDDylation results from the covalent and reversible binding of *neural precursor cell expressed developmentally down-regulated protein 8* (NEDD8) to a lysine residue in the substrate protein.[157] NEDD8 attachment to proteins is catalyzed by a three-step enzymatic cascade that involves the heterodimer NEDD8-activating enzyme E1 (NAE), NEDD8-conjugating E2 enzymes [ubiquitin-conjugating enzyme E2F (UBE2F) and ubiquitin-conjugating enzyme E2M (UBE2M)] and substrate-specific E3 ligases (**Figure I.7**). Briefly, NEDD8-specific protease (NEDP1) first processes the Gly76 residue at the C-terminal tail of the NEDD8 precursor form. The next step in NEDD8 activation requires the binding of Mg^{2+}, ATP and NEDD8 to NAE [constituted by the heterodimer of NEDD8 activating enzyme E1 regulatory subunit (NAE1) and the NEDD8 activating enzyme E1 catalytic subunit also known as ubiquitin-activating enzyme 3 (UBA3)] that leads to the formation of an acyl adenylate intermediate, NEDD8-AMP, and the release of inorganic pyrophosphate.[158,159] The NEDD8-AMP subsequently reacts with an active thiol site of the NAE E1 enzyme leading to the formation of NEDD8-NAE thioester and the release of AMP.[159–161] The binding of a second NEDD8-AMP, resulting from a second round of NEDD8, ATP and Mg^{2+} reaction, yields an open conformation of the NEDD8-charged NAE structure allowing the transfer of NEDD8 to one of the E2 NEDD8-conjugating enzymes (UBE2F and UBE2M) through a transthiolation reaction.[160–164] Finally, a substrate-specific E3 ligase transfers NEDD8 to a lysine residue in its target protein.[165–167] Most NEDD8 E3 ligases reported to date belong to the RING family of E3s [e.g., cullin-associated RING-box proteins 1 and 2 (RBX1/2), murine double minute 2 (MDM2), Von Hippel–Lindau (VHL), among others]. Other NEDD8 E3 ligases include Parkin or SMAD-specific E3 ubiquitin-protein ligase 1 (SMURF1).[161] Protein NEDDylation is a reversible process in which deNEDDylases [e.g., NEDP1 or COP9 signalosome (CSN)] are able to cleave the peptide bond between the substrate and NEDD8, freeing NEDD8 and facilitating the restart of the NEDDylation conjugation cycle.[168] Curiously, while NEDP1 is able to process the precursor form of NEDD8, CSN complexes do not present a high affinity for free NEDD8 and are very inefficient in processing its precursor form.[161] In contrast, NEDP1 exhibits an insignificant activity when it comes to removing a single NEDD8 from cullins. Nevertheless, NEDP1 mediates deNEDDylation of hyperNEDDylated cullins, resulting in mono-NEDDylated substrates.[161] Moreover, NEDP1 can deconjugate NEDD8 from multiple non-cullin substrates.[161]

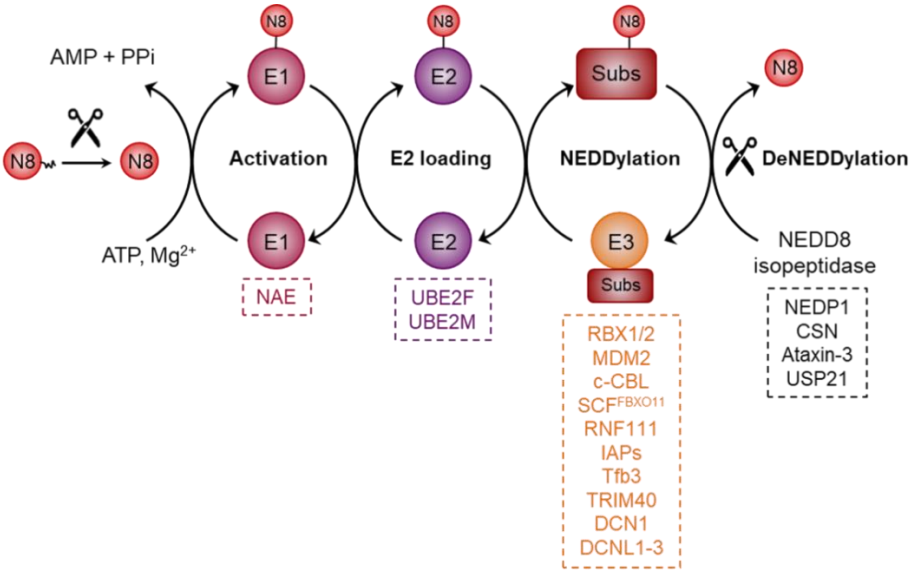

Figure I.7. The NEDDylation pathway. Schematic representation of each step of the NEDD8 conjugation pathway, including NEDD8 precursor processing, NEDD8 activation by NAE, E2 loading, conjugation to a substrate by an E3 and recycling of NEDD8 by a deNEDDylating isopeptidase. The involving enzymes in each step are listed. Abbreviations: c-CBL, casitas B-lineage lymphoma; CSN, COP9 signalosome; DCN1, defective in cullin neddylation protein 1; DCNL 1-3, DCN1-like protein 1-3; IAP, inhibitor of apoptosis; MDM2, murine double minute 2; N8, NEDD8; NAE, NEDD8-activating enzyme; NEDP1, NEDD specific protease 1; RBX1/2, RING-box protein 1/2; RNF111, ring finger protein 111; SCF^{FBXO11}, Skp1-Cul1-F-box; subs, substrate; Tfb3; RNA polymerase II transcription factor B subunit 3; TRIM40, tripartite motif-containing protein 40; UBE2F, ubiquitin-conjugating enzyme 2F; UBE2M, ubiquitin-conjugating enzyme 2M; USP21, ubiquitin carboxyl-terminal hydrolase 21. (Adapted from Zhao Y *et al*., 2014)[169]

NEDD8 is a ubiquitin-like protein (UBL), and these, including ubiquitin and SUMO1, are able to form chains of consecutive SUMO or ubiquitin residues on their substrates. Nevertheless, NEDD8 substrates are thought to be mainly mono-NEDDylated on a single or several conserved lysine residues, and NEDD8 chains have only been reported *in vitro*.[170]

Even though NEDD8 is a UBL that shares 59% amino acid identity and 80% homology with ubiquitin;[171] protein NEDDylation is specific. In this regard, NEDP1 is specific for the NEDD8 precursor form and does not process other UBL precursors.[161] Additionally, a single amino acid difference in the C-terminal of the two UBLs, Ala72 in NEDD8 and Arg72 in ubiquitin, which is recognized by their respective E1 enzymes, represents an important specificity mark.[162] Furthermore, the binding of NEDD8 to the E2 enzymes occurs in a UBA3-specific site that is not present in other E1s, preventing cross-reactivity with other UBL pathways such as ubiquitination or SUMOylation.[162] Finally, NAE can recognize and distinguish both NEDD8 E2 conjugating enzymes, incorporating additional specificity when it comes to cullin modification since UBE2M and UBE2F specifically NEDDylate different cullins (cullin 1-4 and cullin 5, respectively).[161]

I.4.3. NEDDylation substrates

The best characterized substrate of NEDD8 is the cullin family of proteins.[172] In humans, 8 cullin family members have been identified and these include cullins 1-3, 4A, 4B, 5, 7 and 9.[172] Cullins act as a molecular scaffold together with an adaptor protein, a substrate receptor and a RING protein to form the cullin-RING ligases (CRLs), well-known E3 ubiquitin ligases. NEDDylation of cullins activates CRLs, and therefore, promotes ubiquitination and proteasomal degradation of multiple CRL substrates modulating important biological processes such as cell cycle progression, survival, DNA repair and signal transduction, among others (**Figure I.8**).[159,173] The plethora of proteins that can be targeted by CRLs include DNA licensing proteins (e.g., CDT1, ORC1), cell cycle mediators (e.g., p21, p27) or kinases (e.g., WEE1, RhoA).

In addition to CRLs, several non-cullin proteins have been identified to become NEDDylated (**Figure I.8**). These include transcription factors (e.g., p53, p73, E2F, IκBα, HIF1α), receptors (e.g., EGFR, TGF-βR2), kinases (e.g., PINK1, CK1α), E3 ligases (e.g., MDM2, Parkin) and others such as Histone 4 or ribosomal proteins.[161,174–180] NEDDylation of transcription factors, generally suppresses their activity by altering their stability, subcellular localization or interaction with DNA. For instance, MDM2-mediated p53 NEDDylation, unlike MDM2-mediated ubiquitination, does not lead to proteasomal degradation but inhibits its transcriptional activity.[174] The p53 family member, p73 can also become NEDDylated by MDM2, impeding its nuclear translocation and therefore, downregulating its transcriptional activity.[181] Similarly, the transcriptional activity of E2F transcription factors is reduced upon E2F NEDDylation.[182] Protein NEDDylation can potentially regulate RTK signaling. EGFR is a RTK that is activated by binding to extracellular growth factors, which in turn trigger several signaling networks. However, hyper-activation of the downstream signaling cascades can be detrimental, thus EGFR is rapidly phosphorylated or ubiquitinated to mediate its internalization through endocytosis and degradation.[161] Moreover, the E3 ligase c-CBL has been reported to NEDDylate EGFR, resulting in increased ubiquitination and degradation.[161]

On the other hand, protein NEDDylation can result in protein stabilization. In this regard, MDM2 mediates its auto-NEDDylation to enhance its stability and promotes NEDDylation of ribosomal proteins (i.e., L11 and S4) modulating their stability and subcellular location.[161] Likewise, NEDDylation of the oncoprotein HuR leads to its stabilization and nuclear localization, protecting this protein from degradation and hence stimulating cell proliferation and survival.[175] Taken together, these data highlight the relevance of protein NEDDylation, and its fine-tuning, in numerous physiological processes (**Figure I.8**).

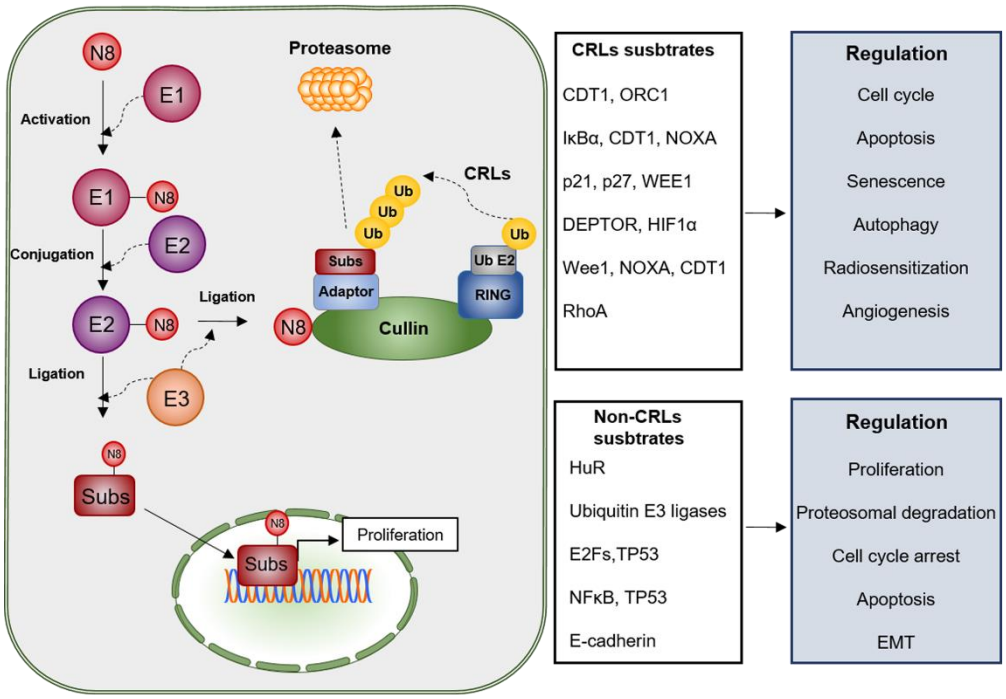

Figure I.8. Molecular mechanisms modulated by the NEDDylation pathway. The NEDD8 conjugation pathway is involved in several physiological processes including cell cycle progression, proliferation, survival, migration, invasion, angiogenesis, among others. Abbreviations: CRL, cullin RING ligase; EMT, epithelial-mesenchymal transition; N8, NEDD8; subs, substrate; Ub, ubiquitin.

I.4.4. NEDDylation and disease

Deregulation in NEDDylation conjugation has been reported in several human diseases such as different types of cancers,[183–187] inflammatory and autoimmune disorders[188,189] as well as neurodegenerative[190,191] and cardiac conditions.[192] Regarding cancer, aberrant protein NEDDylation has been found in distinct types of tumors and multiple NEDD8 target proteins have been identified. As aforementioned, these include cell cycle regulators, tumor suppressors and oncoproteins. Therefore, disruption of normal NEDDylation adversely affects normal cell cycle progression, cell proliferation and survival, ultimately promoting tumor growth.

Considering hepatic disorders, liver fibrosis, early stages of NAFLD (i.e., hepatic steatosis), HCC and iCCA have been shown to exhibit aberrant protein NEDDylation.[193–197] In fact, upregulated protein NEDDylation was observed in patients with liver fibrosis as well as in two animal models mimicking liver fibrosis progression.[193] Similarly, *NEDD8* mRNA levels were increased in patients with hepatic steatosis compared to healthy controls,[195] and both *NEDD8* and *NAE1* mRNA levels were found augmented in a large cohort of HCC patients.[198] Furthermore, the expression of the NEDDylation pathway components (i.e., *NAE1*, *UBA3*, *UBE2M*), as well as NEDD8-conjugation, were determined by immunohistochemistry (IHC) in a cohort of iCCA patients, of which two-thirds displayed upregulation of the NEDDylation pathway.[197] Besides, global levels of NEDDylation and NAE1 protein expression significantly correlated with poor disease outcome in HCC[194] and *NAE1* expression was shown to be an independent prognostic factor for postoperative recurrence in iCCA.[197] Furthermore, knockdown of *UBE2M* reduced cell proliferation and survival in iCCA cells.[199] Overall, these findings indicate that upregulated NEDDylation pathway is involved in liver disease and interference in this pathway could be a promising therapeutic target.

I.4.5. Pevonedistat – A first-in-class NEDDylation inhibitor

Pevonedistat (Takeda Oncology) was developed as a result of perseverant medicinal chemical efforts on N6-benzyl adenosine, which had been previously identified as an inhibitor of NAE through high throughput screening methods.[173] Pevonedistat is an adenosine sulfamate analog and a small highly selective first-in-class inhibitor of NAE and, therefore, of the NEDDylation pathway (**Figure I.9**).[173] Since Pevonedistat is structurally related to AMP, a tight binding product of the first step of the NEDDylation cascade, Pevonedistat is able to form a covalent adduct with NEDD8, impeding further steps in the NEDDylation cascade by a novel mechanism termed substrate-assisted inhibition. This Pevonedistat-NEDD8 adduct resembles the NEDD8-AMP intermediate, but cannot be further transferred to E2s, stopping the subsequent reactions and blocking NEDD8 conjugation.[200] By doing so, Pevonedistat effectively inhibits cullin NEDDylation, inactivating CRLs, which leads to the accumulation of CRL substrates and, thus, triggers cell cycle arrest, apoptosis, senescence and multiple other cellular responses. Likewise, inhibition of NEDD8 conjugation to certain oncoproteins also halts disturbed cell growth. Preclinical studies have proven its potent antitumor activity and well-tolerated toxicity.[159,173] In addition, phase I trials have ensured the safety of Pevonedistat and demonstrated promising clinical effects in terms of disease stabilization and partial or complete responses to treatment.[200,201] Thus, Pevonedistat is currently being investigated in several clinical trials for the treatment of patients suffering from solid and hematological tumors, alone or in combination with other chemotherapeutic compounds.

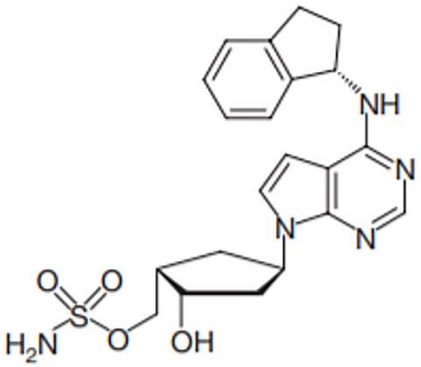

Figure I.9. Chemical structure of Pevonedistat. Pevonedistat (((1S,2S,4R)-4-{4-[(S)-2,3-Dihydro-1H-inden-1-ylamino]-7H-pyrrolo[2,3-d]pyrimidin-7-yl}-2-hydroxycyclopentyl)methyl sulfamate hydrochloride) is an adenosine sulfamate analog that forms with NEDD8 and adduct, which impedes NEDD8 conjugation and blocks the NEDDylation pathway.[173]

Furthermore, increasing evidence is highlighting the role of NEDDylation in the regulation of TME.[202] Importantly, CAFs derived from Pevonedistat-treated HCC tissues presented downregulation of genes involved in cell cycle and DNA replication pathways, suggesting that Pevonedistat could inhibit CAFs proliferation.[203] Moreover, Pevonedistat was found to reduce endothelial cell migration and capillary tube formation, overall suppressing angiogenesis.[202,204] By contrast, T cell and dendritic cell activation, which contributes to antitumor immune response, seems to be impaired upon NEDDylation inhibition.[205,206] It is, therefore, important to determine the relevance of protein NEDDylation in tumor-promoting TME and to assess the effect of NEDDylation inhibition on the different populations of TME *in vivo*, providing further foundation for the use of Pevonedistat as an anticancer therapeutic strategy.

Hypothesis and Objectives

PTMs are essential mechanisms to modulate cellular responses to diverse stimuli. The relevance of protein NEDDylation, in particular, has been demonstrated in different diseases including cancer. Upregulation of the NEDDylation pathway in CCA pointed out the relevance of this PTM in cholangiocarcinogenesis. Therefore, this dissertation aims to further depict the potential role of NEDDylation in the pathogenesis of CCA as well as its regulatory value using Pevonedistat.

Hence, the following objectives were proposed to be assessed:

I. Analysis of the expression levels of the NEDDylation activation components in human CCA tissue compared to controls.

II. Analysis of the expression levels of the NEDDylation activation components in CCA cell lines compared to normal controls.

III. Evaluation of the impact of pharmacological or genetic NEDDylation inhibition in the pathogenesis of CCA *in vitro*.

IV. Evaluation of the impact of pharmacological or genetic NEDDylation inhibition in the pathogenesis of CCA *in vivo*.

V. Identification of the NEDDylation targets involved in cholangiocarcinogenesis.

VI. Ascertain of the role of NEDDylation in the crosstalk between CCA cells and the TME.

References

1. Juza RM, Pauli EM. Clinical and surgical anatomy of the liver: A review for clinicians. Clin Anat 2014;27:764–769.

2. Trefts E, Gannon M, Wasserman DH. The liver. Curr Biol 2017;27:R1147–R1151.

3. Boyer JL. Bile formation and secretion. Compr Physiol 2013;3:1035–1078.

4. Kubes P, Jenne C. Immune Responses in the Liver. Annu Rev Immunol 2018;36:247–277.

5. Bhatia SN, Underhill GH, Zaret KS, et al. Cell and tissue engineering for liver disease. Sci Transl Med 2014;6.

6. Cotoi CG, Quaglia A. Normal Liver Anatomy and Introduction to Liver Histology. In: *Textbook of Pediatric Gastroenterology, Hepatology and Nutrition*. Springer International Publishing; 2016:609–612.

7. Mescher A. *Junqueira's basic histology: text and atlas*. McGraw-Hill Medical (New York, NY); 2013.

8. Han Y, Glaser S, Meng F, et al. Recent advances in the morphological and functional heterogeneity of the biliary epithelium. Exp Biol Med 2013;238:549–565.

9. Tabibian JH, Masyuk AI, Masyuk T V., et al. Physiology of cholangiocytes. Compr Physiol 2013;3:541–565.

10. Anon. The Biliary Tree | Radiology Key. Available at: https://radiologykey.com/the-biliary-tree/ [Accessed November 28, 2020].

11. Goetz SC, Anderson K V. The primary cilium: A signalling centre during vertebrate development. Nat Rev Genet 2010;11:331–344.

12. Wheway G, Nazlamova L, Hancock JT. Signaling through the primary cilium. Front Cell Dev Biol 2018;6.

13. Wheatley DN, Wang AM, Strugnell GE. Expression of primary cilia in mammalian cells. Cell Biol Int 1996;20:73–81.

14. Masyuk AI, Masyuk T V., Splinter PL, et al. Cholangiocyte Cilia Detect Changes in Luminal Fluid Flow and Transmit Them Into Intracellular Ca2+ and cAMP Signaling. Gastroenterology 2006;131:911–920.

15. Gradilone SA, Masyuk AI, Splinter PL, et al. Cholangiocyte cilia express TRPV4 and detect changes in luminal tonicity inducing bicarbonate secretion. Proc Natl Acad Sci U S A 2007;104:19138–19143.

16. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: Disorders of biliary epithelia. Gastroenterology 2004;127:1565–1577.

17. Jensen K, Marzioni M, Munshi K, et al. Autocrine regulation of biliary pathology by activated cholangiocytes. Am J Physiol - Gastrointest Liver Physiol 2012;302.

18. Bogert PT, LaRusso NF. Cholangiocyte biology. Curr Opin Gastroenterol 2007;23:299–305.

19. Maroni L, Haibo B, Ray D, et al. Functional and Structural Features of Cholangiocytes in Health and Disease. CMGH 2015;1:368–380.

20. Beuers U, Hohenester S, Buy Wenniger LJM de, et al. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010;52:1489–1496.

21. Eaton JE, Talwalkar JA, Lazaridis KN, et al. Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management. Gastroenterology 2013;145:521–36.

22. O'Hara SP, Small AJ, Gajdos GB, et al. HIV-1 tat protein suppresses cholangiocyte toll- like receptor 4 expression and defense against cryptosporidium parvum. J Infect Dis 2009;199:1195–1204.

23. Gevers TJG, Drenth JPH. Diagnosis and management of polycystic liver disease. Nat Rev Gastroenterol Hepatol 2013;10:101–108.

24. Elborn JS. Cystic fibrosis. Lancet 2016;388:2519–2531.

25. Saleh M, Kamath BM, Chitayat D. Alagille syndrome: Clinical perspectives. Appl Clin Genet

2016;9:75–82.

26. Deltenre P, Valla DC. Ischemic cholangiopathy. Semin Liver Dis 2008;28:235.

27. Razumilava N, Gores GJ. Cholangiocarcinoma. Lancet 2014;383:2168–2179.

28. Visentin M, Lenggenhager D, Gai Z, et al. Drug-induced bile duct injury. Biochim Biophys Acta - Mol Basis Dis 2018;1864:1498–1506.

29. Rajapaksha IG, Angus PW, Herath CB. Current therapies and novel approaches for biliary diseases. World J Gastrointest Pathophysiol 2019;10:1–10.

30. Lazaridis KN, Larusso NF. The cholangiopathies. Mayo Clin Proc 2015;90:791–800.

31. Tam PKH, Yiu RS, Lendahl U, et al. Cholangiopathies – Towards a molecular understanding. EBioMedicine 2018;35:381–393.

32. Cardinale V. Multiple cells of origin in cholangiocarcinoma underlie biological, epidemiological and clinical heterogeneity. World J Gastrointest Oncol 2012;4:94.

33. Shaib YH, Davila JA, McGlynn K, et al. Rising incidence of intrahepatic cholangiocarcinoma in the United States: A true increase? J Hepatol 2004;40:472–477.

34. Khan SA, Toledano MB, Taylor-Robinson SD. Epidemiology, risk factor, and pathogenesis of cholangiocarcinoma. HPB 2008;10:77–82.

35. Khan SA, Tavolari S, Brandi G. Cholangiocarcinoma: Epidemiology and risk factors. Liver Int 2019;39:19–31.

36. Bertuccio P, Malvezzi M, Carioli G, et al. Global trends in mortality from intrahepatic and extrahepatic cholangiocarcinoma. J Hepatol 2019;71:104–114.

37. Banales JM, Marin JJG, Lamarca A, et al. Cholangiocarcinoma 2020: the next horizon in mechanisms and management. Nat Rev Gastroenterol Hepatol 2020;17:557–588.

38. Nakeeb A, Pitt HA, Sohn TA, et al. Cholangiocarcinoma: A spectrum of intrahepatic, perihilar, and distal tumors. Ann Surg 1996;224:463–475.

39. Banales JM, Cardinale V, Carpino G, et al. Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). Nat Rev Gastroenterol Hepatol 2016;13:261–280.

40. Bridgewater J, Galle PR, Khan SA, et al. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. J Hepatol 2014;60:1268–1289.

41. Hughes T, O'Connor T, Techasen A, et al. Opisthorchiasis and cholangiocarcinoma in Southeast Asia: An unresolved problem. Int J Gen Med 2017;10:227–237.

42. Lim MK, Ju Y-H, Franceschi S, et al. *Clonorchis Sinensis infection and increasing risk of cholangiocarcinoma in the Republic of Korea*. 2006.

43. Lee TY, Lee SS, Jung SW, et al. Hepatitis B virus infection and intrahepatic cholangiocarcinoma in Korea: A case-control study. Am J Gastroenterol 2008;103:1716–1720.

44. Ebata T, Kosuge T, Hirano S, et al. Proposal to modify the International Union Against Cancer staging system for perihilar cholangiocarcinomas. Br J Surg 2014;101:79–88.

45. Cai Y, Cheng N, Ye H, et al. The current management of cholangiocarcinoma: A comparison of current guidelines. Biosci Trends 2016;10:92–102.

46. Bragazzi MC, Ridola L, Safarikia S, et al. New insights into cholangiocarcinoma: Multiple stems and related cell lineages of origin. Ann Gastroenterol 2018;31:42–55.

47. Patel T. New insights into the molecular pathogenesis of intrahepatic cholangiocarcinoma. J Gastroenterol 2014;49:165–172.

48. Yamasaki S. Intrahepatic cholangiocarcinoma: Macroscopic type and stage classification. J Hepatobiliary Pancreat Surg 2003;10:288–291.

49. Vijgen S, Terris B, Rubbia-Brandt L. Pathology of intrahepatic cholangiocarcinoma. HepatoBiliary Surg Nutr 2017;6:22–34.

50. Nakanuma Y, Kakuda Y. Pathologic classification of cholangiocarcinoma: New concepts. Best

Pract Res Clin Gastroenterol 2015;29:277–293.

51. Nakanuma Y, Sato Y, Harada K, et al. Pathological classification of intrahepatic cholangiocarcinoma based on a new concept. World J Hepatol 2010;2:419–427.

52. Aishima S, Oda Y. Pathogenesis and classification of intrahepatic cholangiocarcinoma: Different characters of perihilar large duct type versus peripheral small duct type. J Hepatobiliary Pancreat Sci 2015;22:94–100.

53. Blechacz B, Komuta M, Roskams T, et al. Clinical diagnosis and staging of cholangiocarcinoma. Nat Rev Gastroenterol Hepatol 2011;8:512–522.

54. Alvaro D, Bragazzi MC, Benedetti A, et al. Cholangiocarcinoma in Italy: A national survey on clinical characteristics, diagnostic modalities and treatment. Results from the " Cholangiocarcinoma" committee of the Italian Association for the Study of Liver disease. Dig Liver Dis 2011;43:60–65.

55. Rose AM de, Cucchetti A, Clemente G, et al. Prognostic Significance of Tumor Doubling Time in Mass-Forming Type Cholangiocarcinoma. J Gastrointest Surg 2013;17:739–747.

56. Krasinskas AM. Cholangiocarcinoma. Surg Pathol Clin 2018;11:403–429.

57. Aishima S, Kuroda Y, Nishihara Y, et al. Proposal of progression model for intrahepatic cholangiocarcinoma: Clinicopathologic differences between hilar type and peripheral type. Am J Surg Pathol 2007;31:1059–1067.

58. Akita M, Fujikura K, Ajiki T, et al. Dichotomy in intrahepatic cholangiocarcinomas based on histologic similarities to hilar cholangiocarcinomas. Mod Pathol 2017;30:986–997.

59. Liau JY, Tsai JH, Yuan RH, et al. Morphological subclassification of intrahepatic cholangiocarcinoma: Etiological, clinicopathological, and molecular features. Mod Pathol 2014;27:1163–1173.

60. Hayashi A, Misumi K, Shibahara J, et al. Distinct Clinicopathologic and Genetic Features of 2 Histologic Subtypes of Intrahepatic Cholangiocarcinoma. Am J Surg Pathol 2016;40:1021–1030.

61. Komuta M, Govaere O, Vandecaveye V, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. Hepatology 2012;55:1876–1888.

62. Cardinale V, Wang Y, Carpino G, et al. Mucin-producing cholangiocarcinoma might derive from biliary tree stem/progenitor cells located in peribiliary glands. Hepatology 2012;55:2041–2042.

63. Igarashi S, Sato Y, Ren XS, et al. Participation of peribiliary glands in biliary tract pathophysiologies. World J Hepatol 2013;5:425–32.

64. Carpino G, Cardinale V, Folseraas T, et al. Neoplastic Transformation of the Peribiliary Stem Cell Niche in Cholangiocarcinoma Arisen in Primary Sclerosing Cholangitis. Hepatology 2019;69:622–638.

65. Clements O, Eliahoo J, Kim JU, et al. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma: A systematic review and meta-analysis. J Hepatol 2019;72.

66. Hirschfield GM, Karlsen TH, Lindor KD, et al. Primary sclerosing cholangitis. In: *The Lancet*.Vol 382. Lancet Publishing Group; 2013:1587–1599.

67. Weismüller TJ, Wedemeyer J, Kubicka S, et al. The challenges in primary sclerosing cholangitis - Aetiopathogenesis, autoimmunity, management and malignancy. J Hepatol 2008;48.

68. Razumilava N, Gores GJ. Surveillance for cholangiocarcinoma in patients with primary sclerosing cholangitis: Effective and justified? Clin Liver Dis 2016;8:43–47.

69. Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. Hepatology 2011;54:173–184.

70. Chapman RW. Risk factors for biliary tract carcinogenesis. In: *Annals of Oncology*.Vol 10. Springer Netherlands; 1999.

71. Sacks D, Baxter B, Campbell BCV, et al. Multisociety Consensus Quality Improvement Revised Consensus Statement for Endovascular Therapy of Acute Ischemic Stroke. Int J Stroke 2018;13:612–632.

72. Nepal C, O'Rourke CJ, Oliveira DVNP, et al. Genomic perturbations reveal distinct regulatory networks in intrahepatic cholangiocarcinoma. Hepatology 2018;68:949–963.

73. Nakamura H, Arai Y, Totoki Y, et al. Genomic spectra of biliary tract cancer. Nat Genet 2015;47:1003–1010.

74. Andersen JB, Spee B, Blechacz BR, et al. Genomic and genetic characterization of cholangiocarcinoma identifies therapeutic targets for tyrosine kinase inhibitors. Gastroenterology 2012;142.

75. Sia D, Hoshida Y, Villanueva A, et al. Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes that have different outcomes. Gastroenterology 2013;144:829–840.

76. Lin J, Shi J, Guo H, et al. Alterations in DNA damage repair genes in primary liver cancer. Clin Cancer Res 2019;25:4701–4711.

77. Maynard H, Stadler ZK, Berger MF, et al. Germline alterations in patients with biliary tract cancers: A spectrum of significant and previously underappreciated findings. Cancer 2020;126:1995–2002.

78. Łuczak MW, Jagodzinski PP. *The role of DNA methylation in cancer development*. 2006.

79. O'Rourke CJ, Munoz-Garrido P, Aguayo EL, et al. Epigenome dysregulation in cholangiocarcinoma. Biochim Biophys Acta - Mol Basis Dis 2018;1864:1423–1434.

80. Jaiswal M, LaRusso NF, Burgart LJ, et al. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 2000;60:184–190.

81. Zabron A, Edwards RJ, Khan SA. The challenge of cholangiocarcinoma: Dissecting the molecular mechanisms of an insidious cancer. DMM Dis Model Mech 2013;6:281–292.

82. Andersen JB. Molecular pathogenesis of intrahepatic cholangiocarcinoma. J Hepatobiliary Pancreat Sci 2015;22:101–113.

83. Sia D, Tovar V, Moeini A, et al. Intrahepatic cholangiocarcinoma: Pathogenesis and rationale for molecular therapies. Oncogene 2013;32:4861–4870.

84. Frampton G, Invernizzi P, Bernuzzi F, et al. Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 2012;61:268–277.

85. Tadlock L, Patel T. Involvement of p38 mitogen-activated protein kinase signaling in transformed growth of a cholangiocarcinoma cell line. Hepatology 2001;33:43–51.

86. Han C, Wu T. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. J Biol Chem 2005;280:24053–24063.

87. Yoon JH, Canbay AE, Werneburg NW, et al. Oxysterols Induce Cyclooxygenase-2 Expression in Cholangiocytes: Implications for Biliary Tract Carcinogenesis. Hepatology 2004;39:732–738.

88. Banales JM, Huebert RC, Karlsen T, et al. Cholangiocyte pathobiology. Nat Rev Gastroenterol Hepatol 2019;16:269–281.

89. Xue TC, Zhang BH, Ye SL, et al. Differentially expressed gene profiles of intrahepatic cholangiocarcinoma, hepatocellular carcinoma, and combined hepatocellular-cholangiocarcinoma by integrated microarray analysis. Tumor Biol 2015;36:5891–5899.

90. Gil-García B, Baladrón V. The complex role of NOTCH receptors and their ligands in the development of hepatoblastoma, cholangiocarcinoma and hepatocellular carcinoma. Biol Cell 2016;108:29–40.

91. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. J Clin Invest 2012;122:3914–3918.

92. Fan B, Malato Y, Calvisi DF, et al. Cholangiocarcinomas can originate from hepatocytes in mice. J Clin Invest 2012;122:2911–2915.

93. Loilome W, Bungkanjana P, Techasen A, et al. Activated macrophages promote Wnt/β-catenin signaling in cholangiocarcinoma cells. Tumor Biol 2014;35:5357–5367.

94. Boulter L, Guest R V., Kendall TJ, et al. WNT signaling drives cholangiocarcinoma growth and can be pharmacologically inhibited. J Clin Invest 2015;125:1269–1285.

95. Perugorria MJ, Olaizola P, Labiano I, et al. Wnt–β-catenin signalling in liver development, health

and disease. Nat Rev Gastroenterol Hepatol 2019;16:121–136.

96. Goeppert B, Konermann C, Schmidt CR, et al. Global alterations of DNA methylation in cholangiocarcinoma target the Wnt signaling pathway. Hepatology 2014;59:544–554.

97. Riedlinger D, Bahra M, Boas-Knoop S, et al. Hedgehog pathway as a potential treatment target in human cholangiocarcinoma. J Hepatobiliary Pancreat Sci 2014;21:607–615.

98. Khatib M El, Kalnytska A, Palagani V, et al. Inhibition of hedgehog signaling attenuates carcinogenesis in vitro and increases necrosis of cholangiocellular carcinoma. Hepatology 2013;57:1035–1045.

99. Fingas CD, Bronk SF, Werneburg NW, et al. Myofibroblast-derived PDGF-BB promotes hedgehog survival signaling in cholangiocarcinoma cells. Hepatology 2011;54:2076–2088.

100. Kim Y, Kim MO, Shin JS, et al. Hedgehog signaling between cancer cells and hepatic stellate cells in promoting cholangiocarcinoma. Ann Surg Oncol 2014;21:2684–2698.

101. Pan D. Hippo signaling in organ size control. Genes Dev 2007;21:886–897.

102. Li H, Wolfe A, Septer S, et al. Deregulation of Hippo kinase signalling in Human hepatic malignancies. Liver Int 2012;32:38–47.

103. Tao J, Calvisi DF, Ranganathan S, et al. Activation of β-catenin and Yap1 in human hepatoblastoma and induction of hepatocarcinogenesis in mice. Gastroenterology 2014;147:690–701.

104. Pei T, Li Y, Wang J, et al. YAP is a critical oncogene in human cholangiocarcinoma. Oncotarget 2015;6:17206–17220.

105. Farshidfar F, Zheng S, Gingras MC, et al. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. Cell Rep 2017;18:2780–2794.

106. Moeini A, Sia D, Bardeesy N, et al. Molecular Pathogenesis and Targeted Therapies for Intrahepatic Cholangiocarcinoma. Clin Cancer Res 2016;22:291–300.

107. Endo K, Yoon B Il, Pairojkul C, et al. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. Hepatology 2002;36:439–450.

108. Han C, Leng J, Demetris AJ, et al. Cyclooxygenase-2 Promotes Human Cholangiocarcinoma Growth: Evidence for Cyclooxygenase-2-Independent Mechanism in Celecoxib-Mediated Induction of p21waf1/cip1 and p27kip1 and Cell Cycle Arrest. Cancer Res 2004;64:1369–1376.

109. Leone F, Cavalloni G, Pignochino Y, et al. Somatic mutations of epidermal growth factor receptor in bile duct and gallbladder carcinoma. Clin Cancer Res 2006;12:1680–1685.

110. Fabris L, Perugorria MJ, Mertens J, et al. The tumour microenvironment and immune milieu of cholangiocarcinoma. Liver Int 2019;39:63–78.

111. Høgdall D, Lewinska M, Andersen JB. Desmoplastic Tumor Microenvironment and Immunotherapy in Cholangiocarcinoma. Trends in Cancer 2018;4:239–255.

112. Senthebane DA, Rowe A, Thomford NE, et al. The role of tumor microenvironment in chemoresistance: To survive, keep your enemies closer. Int J Mol Sci 2017;18.

113. Brivio S, Cadamuro M, Strazzabosco M, et al. Tumor reactive stroma in cholangiocarcinoma: The fuel behind cancer aggressiveness. World J Hepatol 2017;9:455–468.

114. Erdogan B, Ao M, White LM, et al. Cancer-associated fibroblasts promote directional cancer cell migration by aligning fibronectin. J Cell Biol 2017;216:3799–3816.

115. Karagiannis GS, Poutahidis T, Erdman SE, et al. Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. Mol Cancer Res 2012;10:1403–1418.

116. Govaere O, Wouters J, Petz M, et al. Laminin-332 sustains chemoresistance and quiescence as part of the human hepatic cancer stem cell niche. J Hepatol 2016;64:609–617.

117. Szendröi M, Lapis K. Distribution of fibronectin and laminin in human liver tumors. J Cancer Res Clin Oncol 1985;109:60–64.

118. Tamma R, Annese T, Ruggieri S, et al. Inflammatory cells infiltrate and angiogenesis in locally

advanced and metastatic cholangiocarcinoma. Eur J Clin Invest 2019;49.

119. Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer 2016;16:582–598.

120. Chuaysri C, Thuwajit P, Paupairoj A, et al. Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. Oncol Rep 2009;21:957–969.

121. Vaquero J, Guedj N, Clapéron A, et al. Epithelial-mesenchymal transition in cholangiocarcinoma: From clinical evidence to regulatory networks. J Hepatol 2017;66:424–441.

122. LeBleu VS, Kalluri R. A peek into cancer-associated fibroblasts: Origins, functions and translational impact. DMM Dis Model Mech 2018;11.

123. Itou RA, Uyama N, Hirota S, et al. Immunohistochemical characterization of cancer-associated fibroblasts at the primary sites and in the metastatic lymph nodes of human intrahepatic cholangiocarcinoma. Hum Pathol 2019;83:77–89.

124. Guest R V., Boulter L, Dwyer BJ, et al. Notch3 drives development and progression of cholangiocarcinoma. Proc Natl Acad Sci U S A 2016;113:12250–12255.

125. Terada T, Okada Y, Nakanuma Y. Expression of immunoreactive matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in human normal livers and primary liver tumors. Hepatology 1996;23:1341–1344.

126. Prakobwong S, Yongvanit P, Hiraku Y, et al. Involvement of MMP-9 in peribiliary fibrosis and cholangiocarcinogenesis via Rac1-dependent DNA damage in a hamster model. Int J Cancer 2010;127:2576–2587.

127. Sirica AE. The role of cancer-associated myofibroblasts in intrahepatic cholangiocarcinoma. Nat Rev Gastroenterol Hepatol 2012;9:44–54.

128. Cadamuro M, Nardo G, Indraccolo S, et al. Platelet-derived growth factor-D and Rho GTPases regulate recruitment of cancer-associated fibroblasts in cholangiocarcinoma. Hepatology 2013;58:1042–1053.

129. Clapéron A, Mergey M, Aoudjehane L, et al. Hepatic myofibroblasts promote the progression of human cholangiocarcinoma through activation of epidermal growth factor receptor. Hepatology 2013;58:2001–2011.

130. Cadamuro M, Brivio S, Mertens J, et al. Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor lymphangiogenesis in cholangiocarcinoma. J Hepatol 2019;70:700–709.

131. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. Immunity 2014;41:49–61.

132. Kitano Y, Okabe H, Yamashita YI, et al. Tumour-infiltrating inflammatory and immune cells in patients with extrahepatic cholangiocarcinoma. Br J Cancer 2018;118:171–180.

133. Goeppert B, Frauenschuh L, Zucknick M, et al. Prognostic impact of tumour-infiltrating immune cells on biliary tract cancer. Br J Cancer 2013;109:2665–2674.

134. Oshikiri T, Miyamoto M, Shichinohe T, et al. Prognostic Value of Intratumoral CD8+ T Lymphocyte in Extrahepatic Bile Duct Carcinoma as Essential Immune Response. J Surg Oncol 2003;84:224–228.

135. Lim YJ, Koh J, Kim K, et al. High ratio of programmed cell death protein 1 (PD-1)+/CD8+ tumor-infiltrating lymphocytes identifies a poor prognostic subset of extrahepatic bile duct cancer undergoing surgery plus adjuvant chemoradiotherapy. Radiother Oncol 2015;117:165–170.

136. Ghidini M, Cascione L, Carotenuto P, et al. Characterisation of the immune-related transcriptome in resected biliary tract cancers. Eur J Cancer 2017;86:158–165.

137. Rodrigues PM, Olaizola P, Paiva NA, et al. Pathogenesis of Cholangiocarcinoma. Annu Rev Pathol 2020;Accepted.

138. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 2013;145:1215–1229.

139. Rizvi S, Khan SA, Hallemeier CL, et al. Cholangiocarcinoma-evolving concepts and therapeutic strategies. Nat Rev Clin Oncol 2018;15:95–111.

140. Radtke A, Königsrainer A. Surgical Therapy of Cholangiocarcinoma. Visc Med 2016;32:422–426.

141. Khan SA, Davidson BR, Goldin RD, et al. Guidelines for the diagnosis and treatment of cholangiocarcinoma: An update. Gut 2012;61:1657–1669.

142. Primrose JN, Fox RP, Palmer DH, et al. Capecitabine compared with observation in resected biliary tract cancer (BILCAP): a randomised, controlled, multicentre, phase 3 study. Lancet Oncol 2019;20:663–673.

143. Darwish Murad S, Kim WR, Harnois DM, et al. Efficacy of neoadjuvant chemoradiation, followed by liver transplantation, for perihilar cholangiocarcinoma at 12 US centers. Gastroenterology 2012;143.

144. Ethun CG, Lopez-Aguiar AG, Anderson DJ, et al. Transplantation versus resection for hilar cholangiocarcinoma: An argument for shifting treatment paradigms for resectable disease. Ann Surg 2018;267:797–805.

145. Sapisochin G, Facciuto M, Rubbia-Brandt L, et al. Liver transplantation for “very early” intrahepatic cholangiocarcinoma: International retrospective study supporting a prospective assessment. Hepatology 2016;64:1178–1188.

146. Sapisochin G, Lope CR De, Gastaca M, et al. Intrahepatic cholangiocarcinoma or mixed hepatocellular-cholangiocarcinoma in patients undergoing liver transplantation: A spanish matched cohort multicenter study. Ann Surg 2014;259:944–952.

147. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus Gemcitabine versus Gemcitabine for Biliary Tract Cancer. N Engl J Med 2010;362:1273–1281.

148. Okusaka T, Nakachi K, Fukutomi A, et al. Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: A comparative multicentre study in Japan. Br J Cancer 2010;103:469–474.

149. Lamarca A, Palmer DH, Wasan HS, et al. ABC-06 | A randomised phase III, multi-centre, open-label study of active symptom control (ASC) alone or ASC with oxaliplatin / 5-FU chemotherapy (ASC+mFOLFOX) for patients (pts) with locally advanced / metastatic biliary tract cancers (ABC) previously-treated with cisplatin/gemcitabine (CisGem) chemotherapy. J Clin Oncol 2019;37:4003–4003.

150. Shroff RT, Javle MM, Xiao L, et al. Gemcitabine, Cisplatin, and nab-Paclitaxel for the Treatment of Advanced Biliary Tract Cancers: A Phase 2 Clinical Trial. JAMA Oncol 2019;5:824–830.

151. Park JO, Feng Y-H, Chen Y-Y, et al. Updated results of a phase IIa study to evaluate the clinical efficacy and safety of erdafitinib in Asian advanced cholangiocarcinoma (CCA) patients with FGFR alterations. J Clin Oncol 2019;37:4117–4117.

152. Park SY, Kim JH, Yoon HJ, et al. Transarterial chemoembolization versus supportive therapy in the palliative treatment of unresectable intrahepatic cholangiocarcinoma. Clin Radiol 2011;66:322–328.

153. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science (80-) 2017;357:409–413.

154. Al-Adra DP, Gill RS, Axford SJ, et al. Treatment of unresectable intrahepatic cholangiocarcinoma with yttrium-90 radioembolization: A systematic review and pooled analysis. Eur J Surg Oncol 2015;41:120–127.

155. Deribe YL, Pawson T, Dikic I. Post-translational modifications in signal integration. Nat Struct Mol Biol 2010;17:666–672.

156. Wang YC, Peterson SE, Loring JF. Protein post-translational modifications and regulation of pluripotency in human stem cells. Cell Res 2014;24:143–160.

157. Yavuz AS, Sözer NB, Sezerman OU. Prediction of neddylation sites from protein sequences and sequence-derived properties. BMC Bioinformatics 2015;16:S9.

158. Gong L, Yeh ETH. Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. J Biol Chem 1999;274:12036–12042.

159. Soucy TA, Dick LR, Smith PG, et al. The NEDD8 conjugation pathway and its relevance in cancer biology and therapy. Genes and Cancer 2010;1:708–716.

160. Bohnsack RN, Haas AL. Conservation in the mechanism of Nedd8 activation by the human

AppBp1-Uba3 heterodimer. J Biol Chem 2003;278:26823–26830.

161. Enchev RI, Schulman BA, Peter M. Protein neddylation: Beyond cullin-RING ligases. Nat Rev Mol Cell Biol 2015;16:30–44.

162. Walden H, Podgorski MS, Huang DT, et al. The Structure of the APPBP1-UBA3-NEDD8-ATP Complex Reveals the Basis for Selective Ubiquitin-like Protein Activation by an E1. Mol Cell 2003;12:1427–1437.

163. Walden H, Podgorski MS, Schulman BA. Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. Nature 2003;422:330–334.

164. Huang DT, Ayrault O, Hunt HW, et al. E2-RING Expansion of the NEDD8 Cascade Confers Specificity to Cullin Modification. Mol Cell 2009;33:483–495.

165. Kamura T, Koepp DM, Conrad MN, et al. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science (80-) 1999;284:657–661.

166. Skowyra D, Koepp DM, Kamura T, et al. Reconstitution of G1 cyclin ubiquitination with complexes containing SCF(Grr1) and Rbx1. Science (80-) 1999;284:662–665.

167. Xirodimas DP, Saville MK, Bourdon JC, et al. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell 2004;118:83–97.

168. Delgado TC, Barbier-Torres L, Zubiete-Franco I, et al. Neddylation, a novel paradigm in liver cancer. Transl Gastroenterol Hepatol 2018;3.

169. Zhao Y, Morgan MA, Sun Y. Targeting neddylation pathways to inactivate cullin-RING ligases for anticancer therapy. Antioxidants Redox Signal 2014;21:2383–2400.

170. Xirodimas DP, Sundqvist A, Nakamura A, et al. Ribosomal proteins are targets for the NEDD8 pathway. EMBO Rep 2008;9:280–286.

171. Hjerpe R, Thomas Y, Chen J, et al. Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. Biochem J 2012;441:927–936.

172. Pan ZQ, Kentsis A, Dias DC, et al. Nedd8 on cullin: Building an expressway to protein destruction. Oncogene 2004;23:1985–1997.

173. Soucy TA, Smith PG, Milhollen MA, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009;458:732–736.

174. Zhou L, Zhang W, Sun Y, et al. Protein neddylation and its alterations in human cancers for targeted therapy. Cell Signal 2018;44:92–102.

175. Embade N, Fernández-Ramos D, Varela-Rey M, et al. Murine double minute 2 regulates Hu antigen R stability in human liver and colon cancer through NEDDylation. Hepatology 2012;55:1237–1248.

176. Zuo W, Huang F, Chiang YJ, et al. C-Cbl-Mediated Neddylation Antagonizes Ubiquitination and Degradation of the TGF-β Type II Receptor. Mol Cell 2013;49:499–510.

177. Loftus SJ, Liu G, Carr SM, et al. NEDDylation regulates E2F-1-dependent transcription. EMBO Rep 2012;13:811–818.

178. Ryu JH, Li SH, Park HS, et al. Hypoxia-inducible factor α subunit stabilization by NEDD8 conjugation is reactive oxygen species-dependent. J Biol Chem 2011;286:6963–6970.

179. Xie P, Zhang M, He S, et al. The covalent modifier Nedd8 is critical for the activation of Smurf1 ubiquitin ligase in tumorigenesis. Nat Commun 2014;5.

180. Mergner J, Schwechheimer C. The NEDD8 modification pathway in plants. Front Plant Sci 2014;5.

181. Watson IR, Blanch A, Lin DCC, et al. Mdm2-mediated NEDD8 modification of TAp73 regulates its transactivation function. J Biol Chem 2006;281:34096–34103.

182. Aoki I, Higuchi M, Gotoh Y. NEDDylation controls the target specificity of E2F1 and apoptosis induction. Oncogene 2013;32:3954–3964.

183. Salon C, Brambilla E, Brambilla C, et al. Altered pattern of Cul-1 protein expression and neddylation in human lung tumours: Relationships with CANDI and cyclin e protein levels. J Pathol 2007;213:303–310.

184. Hori T, Osaka F, Chiba T, et al. Covalent modification of all members of human cullin family proteins by NEDD8. Oncogene 1999;18:6829–6834.

185. Chairatvit K, Ngamkitidechakul C. Control of cell proliferation via elevated NEDD8 conjugation in oral squamous cell carcinoma. Mol Cell Biochem 2007;306:163–169.

186. Nakayama KI, Nakayama K. Ubiquitin ligases: Cell-cycle control and cancer. Nat Rev Cancer 2006;6:369–381.

187. Melchor L, Saucedo-Cuevas LP, Muñoz-Repeto I, et al. Comprehensive characterization of the DNA amplification at 13q34 in human breast cancer reveals TFDP1 and CUL4A as likely candidate target genes. Breast Cancer Res 2009;11.

188. Chang FM, Reyna SM, Granados JC, et al. Inhibition of neddylation represses lipopolysaccharide-induced proinflammatory cytokine production in macrophage cells. J Biol Chem 2012;287:35756–35767.

189. Mathewson N, Toubai T, Kapeles S, et al. Neddylation plays an important role in the regulation of murine and human dendritic cell function. Blood 2013;122:2062–2073.

190. Mori F, Nishie M, Piao YS, et al. Accumulation of NEDD8 in neuronal and glial inclusions of neurodegenerative disorders. Neuropathol Appl Neurobiol 2005;31:53–61.

191. Lin Y, Xue J, Deng J, et al. Neddylation activity modulates the neurodegeneration associated with fragile X associated tremor/ataxia syndrome (FXTAS) through regulating Sima. Neurobiol Dis 2020;143.

192. Kandala S, Kim IM, Su H. Neddylation and deneddylation in cardiac biology. Am J Cardiovasc Dis 2014;4:140–158.

193. Zubiete-Franco I, Fernández-Tussy P, Barbier-Torres L, et al. Deregulated neddylation in liver fibrosis. Hepatology 2017;65:694–709.

194. Barbier-Torres L, Delgado TC, García-Rodríguez JL, et al. Stabilization of LKB1 and Akt by neddylation regulates energy metabolism in liver cancer. Oncotarget 2015;6:2509–2523.

195. Ju U Il, Jeong DW, Seo J, et al. Neddylation of sterol regulatory element-binding protein 1c is a potential therapeutic target for nonalcoholic fatty liver treatment. Cell Death Dis 2020;11.

196. Yu J, Huang W long, Xu Q guo, et al. Overactivated neddylation pathway in human hepatocellular carcinoma. Cancer Med 2018;7:3363–3372.

197. Gao Q, Yu GY, Shi JY, et al. Neddylation pathway is up-regulated in human intrahepatic cholangiocarcinoma and serves as a potential therapeutic target. Oncotarget 2014;5:7820–7832.

198. Roessler S, Jia HL, Budhu A, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res 2010;70:10202–10212.

199. Zhao B, Gao C, Shi D, et al. Knockdown of Nedd8-conjugating enzyme UBE2M suppresses the proliferation and induces the apoptosis of intrahepatic cholangiocarcinoma cells. Oncol Rep 2019;42:2670–2679.

200. Brownell JE, Sintchak MD, Gavin JM, et al. Substrate-Assisted Inhibition of Ubiquitin-like Protein-Activating Enzymes: The NEDD8 E1 Inhibitor MLN4924 Forms a NEDD8-AMP Mimetic In Situ. Mol Cell 2010;37:102–111.

201. Swords RT, Coutre S, Maris MB, et al. Pevonedistat, a first-in-class NEDD8-activating enzyme inhibitor, combined with azacitidine in patients with AML. Blood 2018;131:1415–1424.

202. Zhou L, Jiang Y, Luo Q, et al. Neddylation: A novel modulator of the tumor microenvironment. Mol Cancer 2019;18.

203. Luo Q, Wang CQ, Yang LY, et al. FOXQ1/NDRG1 axis exacerbates hepatocellular carcinoma initiation via enhancing crosstalk between fibroblasts and tumor cells. Cancer Lett 2018;417:21–34.

204. Yao WT, Wu JF, Yu GY, et al. Suppression of tumor angiogenesis by targeting the protein neddylation pathway. Cell Death Dis 2014;5.

205. Jin HS, Liao L, Park Y, et al. Neddylation pathway regulates T-cell function by targeting an adaptor protein Shc and a protein kinase Erk signaling. Proc Natl Acad Sci U S A 2013;110:624–629.

206. Cheng M, Hu S, Wang Z, et al. Inhibition of neddylation regulates dendritic cell functions via Deptor accumulation driven mTOR inactivation. Oncotarget 2016;7:35643–35654.

207. O'Rourke CJ, Matter MS, Nepal C, et al. Identification of a Pan-Gamma-Secretase Inhibitor Response Signature for Notch-Driven Cholangiocarcinoma. Hepatology 2020;71:196–213.

208. Chaisaingmongkol J, Budhu A, Dang H, et al. Common Molecular Subtypes Among Asian Hepatocellular Carcinoma and Cholangiocarcinoma. Cancer Cell 2017;32:57-70.e3.

209. Banales JM, Sáez E, Úriz M, et al. Up-regulation of microRNA 506 leads to decreased Cl -/HCO 3-anion exchanger 2 expression in biliary epithelium of patients with primary biliary cirrhosis. Hepatology 2012;56:687–697.

210. Holt AP, Haughton EL, Lalor PF, et al. Liver Myofibroblasts Regulate Infiltration and Positioning of Lymphocytes in Human Liver. Gastroenterology 2009;136:705–714.

211. Perugorria MJ, Wilson CL, Zeybel M, et al. Histone methyltransferase ASH1 orchestrates fibrogenic gene transcription during myofibroblast transdifferentiation. Hepatology 2012;56:1129–1139.

212. Chen Q, Kang J, Fu C. The independence of and associations among apoptosis, autophagy, and necrosis. Signal Transduct Target Ther 2018;3.

213. Adan A, Alizada G, Kiraz Y, et al. Flow cytometry: basic principles and applications. Crit Rev Biotechnol 2017;37:163–176.

214. Poon IKH, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. Cell Death Differ 2010;17:381–397.

215. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 2013;31:397–405.

216. Shalem O, Sanjana NE, Hartenian E, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science (80-) 2014;343:84–87.

217. Bhaya D, Davison M, Barrangou R. CRISPR-cas systems in bacteria and archaea: Versatile small RNAs for adaptive defense and regulation. Annu Rev Genet 2011;45:273–297.

218. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science (80-) 2013;339:819–823.

219. Wiśniewski JR, Zougman A, Nagaraj N, et al. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359–362.

220. Meier F, Beck S, Grassl N, et al. Parallel accumulation-serial fragmentation (PASEF): Multiplying sequencing speed and sensitivity by synchronized scans in a trapped ion mobility device. J Proteome Res 2015;14:5378–5387.

221. Meier F, Brunner AD, Koch S, et al. Online parallel accumulation–serial fragmentation (PASEF) with a novel trapped ion mobility mass spectrometer. Mol Cell Proteomics 2018;17:2534–2545.

222. Arbelaiz A, Azkargorta M, Krawczyk M, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology 2017;66:1125–1143.

223. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 2016;13:731–740.

224. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 2019;47:D607–D613.

225. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

226. Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res 2016;44:W147–W153.

227. Perez-Riverol Y, Csordas A, Bai J, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 2019;47:D442–D450.

228. Mita Y, Ajiki T, Kamigaki T, et al. Antitumor effect of gemcitabine on orthotopically inoculated

human gallbladder cancer cells in nude mice. Ann Surg Oncol 2007;14:1374–1380.

229. Jo HJ, Shim HE, Han ME, et al. WTAP regulates migration and invasion of cholangiocarcinoma cells. J Gastroenterol 2013;48:1271–1282.

230. Lin S, Shang Z, Li S, et al. Neddylation inhibitor MLN4924 induces G2 cell cycle arrest, DNA damage and sensitizes esophageal squamous cell carcinoma cells to cisplatin. Oncol Lett 2018;15:2583–2589.

231. Podhorecka M, Skladanowski A, Bozko P. H2AX phosphorylation: Its role in DNA damage response and cancer therapy. J Nucleic Acids 2010;2010.

232. Ward IM, Chen J. Histone H2AX Is Phosphorylated in an ATR-dependent Manner in Response to Replicational Stress. J Biol Chem 2001;276:47759–47762.

233. Chung D, Salsman J, Dellaire G. Inhibition of neddylation induces mitotic defects and alters MKLP1 accumulation at the midbody during cytokinesis. Cell Cycle 2019;18:1135–1153.

234. Chiba T, Tanaka K. Cullin-based Ubiquitin Ligase and its Control by NEDD8-conjugating System. Curr Protein Pept Sci 2005;5:177–184.

235. Petroski MD, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 2005;6:9–20.

236. Lin JJ, Milhollen MA, Smith PG, et al. NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells. Cancer Res 2010;70:10310–10320.

237. Lan H, Tang Z, Jin H, et al. Neddylation inhibitor MLN4924 suppresses growth and migration of human gastric cancer cells. Sci Rep 2016;6:1–12.

238. Horibata S, Vo T V., Subramanian V, et al. Utilization of the soft agar colony formation assay to identify inhibitors of tumorigenicity in breast cancer cells. J Vis Exp 2015;2015:52727.

239. Paiva C, Godbersen JC, Berger A, et al. Targeting neddylation induces DNA damage and checkpoint activation and sensitizes chronic lymphocytic leukemia b cells to Alkylating agents. Cell Death Dis 2015;6.

240. Garcia K, Blank JL, Bouck DC, et al. Nedd8-activating enzyme inhibitor mln4924 provides synergy with mitomycin C through interactions with ATR, BRCA1/BRCA2, and chromatin dynamics pathways. Mol Cancer Ther 2014;13:1625–1635.

241. Blank JL, Liu XJ, Cosmopoulos K, et al. Novel DNA damage checkpoints mediating cell death induced by the NEDD8-activating enzyme inhibitor MLN4924. Cancer Res 2013;73:225–234.

242. Zhao Y, Sun Y. Cullin-RING Ligases as Attractive Anti-cancer Targets. Curr Pharm Des 2013;19:3215–3225.

243. Rhind N, Russell P. Tyrosine Phosphorylation of Cdc2 Is Required for the Replication Checkpoint in Schizosaccharomyces pombe. Mol Cell Biol 1998;18:3782–3787.

244. Schmidt M, Rohe A, Platzer C, et al. Regulation of G2/M transition by inhibition of WEE1 and PKMYT1 Kinases. Molecules 2017;22.

245. Meek DW, Anderson CW. Posttranslational modification of p53: cooperative integrators of function. Cold Spring Harb Perspect Biol 2009;1.

246. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: The demons of the guardian of the genome. Cancer Res 2000;60:6788–6793.

247. Haupt S, Agostino S Di, Mizrahi I, et al. Promyelocytic leukemia protein is required for gain of function by mutant p53. Cancer Res 2009;69:4818–4826.

248. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene 1999;18:7644–7655.

249. Brown JS, Lukashchuk N, Sczaniecka-Clift M, et al. Neddylation Promotes Ubiquitylation and Release of Ku from DNA-Damage Sites. Cell Rep 2015;11:704–714.

250. Cadamuro M, Brivio S, Spirli C, et al. Autocrine and paracrine mechanisms promoting chemoresistance in cholangiocarcinoma. Int J Mol Sci 2017;18.

251. Zhou Y, Ling XL. Establishment of a cisplatininduced multidrug resistance cell line SK-Hep1/ DDP. Chin J Cancer 2010;29:167–171.

252. Young LC CBCSDRGJ. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels - PubMed. Clin Cancer Res 2001;6:1798–1804.

253. Herlevsen M, Oxford G, Owens CR, et al. Depletion of major vault protein increases doxorubicin sensitivity and nuclear accumulation and disrupts its sequestration in lysosomes. Mol Cancer Ther 2007;6:1804–1813.

254. Zhang G, Wang Z, Qian F, et al. Silencing of the ABCC4 gene by RNA interference reverses multidrug resistance in human gastric cancer. Oncol Rep 2015;33:1147–1154.

255. Hagmann W, Jesnowski R, Löhr JM. Interdependence of gemcitabine treatment, transporter expression, and resistance in human pancreatic carcinoma cells. Neoplasia 2010;12:740–747.

256. Wigle TJ, Tsvetkova E V., Welch SA, et al. DPYD and fluorouracil-based chemotherapy: Mini review and case report. Pharmaceutics 2019;11.

257. Li J, Sun P, Huang T, et al. Individualized chemotherapy guided by the expression of ERCC1, RRM1, TUBB3, TYMS and TOP2A genes versus classic chemotherapy in the treatment of breast cancer: A comparative effectiveness study. Oncol Lett 2020;21:1–1.

258. Hwang IG, Jang JS, Do JH, et al. Different relation between ERCC1 overexpression and treatment outcomes of two platinum agents in advanced biliary tract adenocarcinoma patients. Cancer Chemother Pharmacol 2011;68:935–944.

259. Aleksakhina SN, Kashyap A, Imyanitov EN. Mechanisms of acquired tumor drug resistance. Biochim Biophys Acta - Rev Cancer 2019;1872.

260. Khawar IA, Park JK, Jung ES, et al. Three Dimensional Mixed-Cell Spheroids Mimic Stroma-Mediated Chemoresistance and Invasive Migration in hepatocellular carcinoma. Neoplasia (United States) 2018;20:800–812.

261. Jung HR, Kang HM, Ryu JW, et al. Cell Spheroids with Enhanced Aggressiveness to Mimic Human Liver Cancer in Vitro and in Vivo. Sci Rep 2017;7.

262. Wever O De, Demetter P, Mareel M, et al. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer 2008;123:2229–2238.

263. Schor SL, Schor AM, Rushton G, et al. Adult, foetal and transformed fibroblasts display different migratory phenotypes on collagen gels: Evidence for an isoformic transition during foetal development. J Cell Sci 1985;VOL. 73:221–234.

264. Schor SL, Schor AM, Rushton G. Fibroblasts from cancer patients display a mixture of both foetal and adult-like phenotypic characteristics. J Cell Sci 1988;90:401–407.

265. Wever O De, Westbroek W, Verloes A, et al. Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-β or wounding. J Cell Sci 2004;117:4691–4703.

266. Shuai S, Xian L, Huso T, et al. Abstract 4494: HMGA1 drives tumor progression and recruits cancer-associated fibroblasts in pancreatic ductal adenocarcinoma. In: *Cancer Research*.Vol 78. American Association for Cancer Research (AACR); 2018:4494–4494.

267. Strell C, Norberg KJ, Mezheyeuski A, et al. Stroma-regulated HMGA2 is an independent prognostic marker in PDAC and AAC. Br J Cancer 2017;117:65–77.

268. Zhang Y, Liu Z, Yang X, et al. H3K27 acetylation activated-COL6A1 promotes osteosarcoma lung metastasis by repressing STAT1 and activating pulmonary cancer-associated fibroblasts. 2021;11:1473–1492.

269. Lokman NA, Ween MP, Oehler MK, et al. The role of annexin A2 in tumorigenesis and cancer progression. Cancer Microenviron 2011;4:199–208.

270. Chakraborty S, Njah K, Hong W. Agrin Mediates Angiogenesis in the Tumor Microenvironment. Trends in Cancer 2020;6:81–85.

271. Jezierska-Drutel A, Attaran S, Hopkins BL, et al. The peroxidase PRDX1 inhibits the activated phenotype in mammary fibroblasts through regulating c-Jun N-terminal kinases. BMC Cancer 2019;19:812.

272. Zeng CM, Chang LL, Ying MD, et al. Aldo-keto reductase AKR1C1-AKR1C4: Functions, regulation, and intervention for anti-cancer therapy. Front Pharmacol 2017;8.

273. Lee-Law PY, Olaizola P, Caballero-Camino FJ, et al. Targeting UBC9-mediated protein hyper-SUMOylation in cystic cholangiocytes halts polycystic liver disease in experimental models. J Hepatol 2020;0.

274. Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol 2006;24:1241–1252.

275. Jia L, Li H, Sun Y. Induction of p21-dependent senescence by an NAE inhibitor, MLN4924, as a mechanism of growth suppression. Neoplasia 2011;13:561–569.

276. Milhollen MA, Narayanan U, Soucy TA, et al. Inhibition of NEDD8-activating enzyme induces rereplication and apoptosis in human tumor cells consistent with deregulating CDT1 turnover. Cancer Res 2011;71:3042–3051.

277. Groisman R, Polanowska J, Kuraoka I, et al. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell 2003;113:357–367.

278. Lippard SJ. New chemistry of an old molecule: cis-[Pt(NH3) 2Cl2]. Science (80-) 1982;218:1075–1082.

279. Moore JK, Haber JE. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol Cell Biol 1996;16:2164–2173.

280. Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. Transl Cancer Res 2013;2:130–143.

281. Misra S, Zhang X, Wani NA, et al. Both BRCA1-wild type and -mutant triple-negative breast cancers show sensitivity to the NAE inhibitor MLN4924 which is enhanced upon MLN4924 and cisplatin combination treatment. Oncotarget 2020;11:784–800.

282. Nawrocki ST, Kelly KR, Smith PG, et al. Disrupting protein NEDDylation with MLN4924 is a novel strategy to target cisplatin resistance in ovarian cancer. Clin Cancer Res 2013;19:3577–3590.

283. Swords RT, Watts J, Erba HP, et al. Expanded safety analysis of pevonedistat, a first-in-class NEDD8-activating enzyme inhibitor, in patients with acute myeloid leukemia and myelodysplastic syndromes. Blood Cancer J 2017;7.

284. Milhollen MA, Thomas MP, Narayanan U, et al. Treatment-Emergent Mutations in NAEβ Confer Resistance to the NEDD8-Activating Enzyme Inhibitor MLN4924. Cancer Cell 2012;21:388–401.

285. Li L, Kang J, Zhang W, et al. Validation of NEDD8-conjugating enzyme UBC12 as a new therapeutic target in lung cancer. EBioMedicine 2019;45:81–91.

286. Zou J, Su H. Targeting neddylation E2 for anticancer therapy, putting new wine into new bottles? EBioMedicine 2019;45:3–4.

287. Jia CC, Wang TT, Liu W, et al. Cancer-Associated Fibroblasts from Hepatocellular Carcinoma Promote Malignant Cell Proliferation by HGF Secretion. PLoS One 2013;8.

288. Sun DY, Wu JQ, He ZH, et al. Cancer-associated fibroblast regulate proliferation and migration of prostate cancer cells through TGF-β signaling pathway. Life Sci 2019;235.

289. Vaquero J, Aoudjehane L, Fouassier L. Cancer-associated fibroblasts in cholangiocarcinoma. Curr Opin Gastroenterol 2020;36:63–69.

290. Heits N, Heinze T, Bernsmeier A, et al. Influence of mTOR-inhibitors and mycophenolic acid on human cholangiocellular carcinoma and cancer associated fibroblasts. BMC Cancer 2016;16.

291. Zou J, Li J, Ma W, et al. Abstract 14914: Inactivation of Neddylation Activating Enzyme 1 (NAE1) in Mice and Rats Leads to Cardiac Developmental and Functional Defects. Circulation 2016;134.

292. Zou J, Ma W, Li J, et al. Neddylation mediates ventricular chamber maturation through repression of Hippo signaling. Proc Natl Acad Sci U S A 2018;115:E4101–E4110.

293. Vogl AM, Brockmann MM, Giusti SA, et al. Neddylation inhibition impairs spine development, destabilizes synapses and deteriorates cognition. Nat Neurosci 2015;18:239–251.

294. Pastore M, Lori G, Gentilini A, et al. Multifaceted Aspects of Metabolic Plasticity in Human Cholangiocarcinoma: An Overview of Current Perspectives. Cells 2020;9:596.

295. Pant K, Richard S, Peixoto E, et al. Role of Glucose Metabolism Reprogramming in the Pathogenesis of Cholangiocarcinoma. Front Med 2020;7:113.

296. Chen P, Hu T, Liang Y, et al. Neddylation inhibition activates the extrinsic apoptosis pathway through ATF4-CHOP-DR5 axis in human esophageal cancer cells. Clin Cancer Res 2016;22:4145–4157.

297. Pavlides S, Whitaker-Menezes D, Castello-Cros R, et al. The reverse Warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 2009;8:3984–4001.

298. Lee M. Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication. World J Biol Chem 2015;6:148.

299. Webber J, Yeung V, Clayton A. Extracellular vesicles as modulators of the cancer microenvironment. Semin Cell Dev Biol 2015;40:27–34.

300. Becker A, Thakur BK, Weiss JM, et al. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell 2016;30:836–848.

301. Erice O, Labiano I, Arbelaiz A, et al. Differential effects of FXR or TGR5 activation in cholangiocarcinoma progression. Biochim Biophys Acta - Mol Basis Dis 2018;1864:1335–1344.

302. Santos-Laso A, Izquierdo-Sanchez L, Rodrigues PM, et al. Proteostasis disturbances and endoplasmic reticulum stress contribute to polycystic liver disease: New therapeutic targets. Liver Int 2020;40:1670–1685.

303. Mackintosh C, García-Domínguez DJ, Ordóñez JL, et al. WEE1 accumulation and deregulation of S-phase proteins mediate MLN4924 potent inhibitory effect on Ewing sarcoma cells. Oncogene 2013;32:1441–1451.

Appendix

Publications during the PhD

1. Lee-Law, P.Y., **Olaizola, P.**, Caballero-Camino, F.J., Izquierdo-Sánchez, L., Rodrigues, P.M., Santos-Laso, A., Azkargorta, M., Elortza, F., Martinez-Chantar, M.L., Perugorria, M.J., Aspichueta, P., Marzioni, M., LaRusso, N.F., Bujanda, L., Drenth, J.P.H., Banales, J.M., 2020. Targeting UBC9-mediated protein hyper-SUMOylation in cystic cholangiocytes halts polycystic liver disease in experimental models. J. Hepatol. https://doi.org/10.1016/j.jhep.2020.09.010

2. Rodrigues, P.M., **Olaizola, P.**, Paiva, N.A., Olaizola, I., Agirre-Lizaso, A., Landa, A., Bujanda, L., Perugorria, M.J., Banales., J.M., 2020. Pathogenesis of Cholangiocarcinoma. Annu. Rev. Pathol. Mech. Dis. 2021;16:annurev-pathol-030220-020455.

3. Gordobil, O., **Olaizola, P.**, Banales, J.M., Labidi, J., 2020. Lignins from agroindustrial by-products as natural ingredients for cosmetics: Chemical structure and in vitro sunscreen and cytotoxic activities. Molecules 25.

4. Raggi, C., Fiaccadori, K., Pastore, M., Correnti, M., Piombanti, B., Forti, E., Navari, N., Abbadessa, G., Hall, T., Destro, A., Di Tommaso, L., Roncalli, M., Meng, F., Glaser, S., Rovida, E., Peraldo-Neia, C., **Olaizola, P.**, Banales, J.M., Gerussi, A., Elvevi, A., Droz dit Busset, M., Bhoori, S., Mazzaferro, V., Alpini, G., Marra, F., Invernizzi, P., 2019. Antitumor Activity of a Novel Fibroblast Growth Factor Receptor Inhibitor for Intrahepatic Cholangiocarcinoma. Am. J. Pathol. 189, 2090–2101.

5. Perugorria, M.J., **Olaizola, P.**, Labiano, I., Esparza-Baquer, A., Marzioni, M., Marin, J.J.G., Bujanda, L., Banales, J.M., 2019. Wnt–β-catenin signalling in liver development, health and disease. Nat. Rev. Gastroenterol. Hepatol.

6. Perugorria, M.J., **Olaizola, P.**, Banales, J.M., 2019. Cholangiocyte-to-Hepatocyte Differentiation: A Context-Dependent Process and an Opportunity for Regenerative Medicine. Hepatology.

7. Lapitz, A., Arbelaiz, A., **Olaizola, P.**, Aranburu, A., Bujanda, L., Perugorria, M.J., Banales, J.M., 2018. Extracellular vesicles in hepatobiliary malignancies. Front. Immunol.

8. **Olaizola, P**., Perugorria, M.J., Banales, J.M., 2018. Toward personalized medicine for intrahepatic cholangiocarcinoma: Pharmacogenomic stratification of patients. Hepatology.

9. Erice, O., Labiano, I., Arbelaiz, A., Santos-Laso, A., Munoz-Garrido, P., Jimenez-Agüero, R., **Olaizola, P.**, Caro-Maldonado, A., Martín-Martín, N., Carracedo, A., Lozano, E., Marin, J.J., O'Rourke, C.J., Andersen, J.B., Llop, J., Gómez-Vallejo, V., Padro, D., Martin, A., Marzioni, M., Adorini, L., Trauner, M., Bujanda, L., Perugorria, M.J., Banales, J.M., 2018. Differential effects of FXR or TGR5 activation in cholangiocarcinoma progression. Biochim. Biophys. Acta - Mol. Basis Dis. 1864, 1335–1344.

10. **Olaizola, P.**, Lee-Law, P.Y., Arbelaiz, A., Lapitz, A., Perugorria, M.J., Bujanda, L., Banales, J.M., 2018. MicroRNAs and extracellular vesicles in cholangiopathies. Biochim. Biophys. Acta - Mol. Basis Dis.