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SHORT AND LONG-TERM REGULATION OF AUTOPHAGY

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The cover represents the importance of time on the regulation of autophagy. This thesis shows both aspects, the short-term regulation based on histone modifications represents the minutes, as the response it is in a quick manner. Long-term effects on the regulation of autophagy occur by DNA methylation and these represents the hours.
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Short and long-term transcriptional regulation of autophagy

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To my Parents

“For their endless love and encouragement. You teach me to be brave and consistent with my ideas, to be strong enough to make it all the way until here and, to be grateful in life. You are my inspiration, and this is for YOU”

Para mis Padres

“Por vuestro amor infinito y apoyo. Me habéis convertido en la persona que soy hoy en día. Me enseñasteis a ser valiente y constante con mis ideas, a ser fuerte para conseguir lo que me proponga y a ser agradecida en la vida. Vosotros sois mi inspiración y esto, es por y para VOSOTROS”

*“The adventure of life is to **learn**. The purpose of life is to **grow**.
The essence of life is to **care**. The secret of life is to **dare**. The beauty of life is to **give**.”*
William Arthur Ward

ABSTRACT

Autophagy is a conserved catabolic pathway triggered by stress conditions in which portions of the cytoplasm, damage organelles, misfolded proteins and intracellular bacteria are delivered and degraded in the lysosome/vacuoles. Thus, an efficient induction and completion of the process is required to ensure a proper homeostasis of the cell. Autophagy has been considered a cytoplasmic event where the role of the nucleus on the regulation of this pathway was underestimated. However, recent findings elicited the role of histone modifying enzymes on the transcriptional regulation of autophagy-related (ATG) genes.

In line with those results, we focused on the role of the two histone modifying enzymes regulating the histone 3 lysine 36 (H3K36) trimethylation, Rph1/KDM4A and Set2/SETD2, on the regulation of autophagy. In **paper I**, we investigated the function of the histone demethylase, Rph1/KDM4 as a negative regulator of autophagy, whereas in **paper II** we uncovered the role of the histone methyltransferase, Set2/SETD2, as a positive transcriptional regulator of ATG genes, as the impact on the differential expression of ATG14 splice isoforms that results on the inhibition of the autophagosome-lysosome fusion. Moreover, in **paper III**, we identify that SETD2 inactivating mutations on clear cell renal cell carcinomas (ccRCC) lead to an aberrant ATG12-containing complexes and accumulation of free ATG12, which is associated with a differential expression of ATG12 isoforms and reduced autophagic flux.

Whereas the previous studies report the involvement of histone modifying enzymes and on the short-term regulation of autophagy, we also aimed to decipher the epigenetic mechanism responsible for the long-lasting effects of autophagy. In **paper IV**, we found that short autophagy stimulus is associated with an upregulation of *de novo* DNA methyltransferase 3A (DNMT3A) responsible of an increase of DNA methylation on selected ATG genes. Eventually, this epigenetic memory involves a persistent decrease of baseline autophagy. Moreover, in **paper V**, we uncovered the mechanism upstream on the regulation of DNMT3A expression by ULK3-mediated phosphorylation and activation of GLI1.

Overall, these insights bring light on novel mechanisms and signaling pathways controlling short and long-term transcriptional regulation of autophagy by histone modifying enzymes, alternative splicing and DNA methylation.

LIST OF SCIENTIFIC PAPERS

- I. Amélie Bernard, Meiyan Jin, **Patricia González-Rodríguez**, Jens Füllgrabe, Elizabeth Delorme-Axford, Steven K. Backues, Bertrand Joseph and Daniel J. Klionsky. Rph1/KDM4 Mediates Nutrient-Limitation signaling that leads to the transcriptional Induction of autophagy. *Current Biology*, 25, 546-555, March 2, 2015.
- II. **Patricia González-Rodríguez**, Elizabeth Delorme-Axford, Amélie Bernard, Kathleen Grabert, Vassilis Stratoulis, Jens Füllgrabe, Daniel J. Klionsky and Bertrand Joseph. Set2/SETD2 regulates expression of ATG14 isoforms and autophagosome-lysosome fusion. *Manuscript*.
- III. **Patricia González-Rodríguez**, Pinelopi Engskog-Vlachos, Hanzhao Zhang, Adriana Natalia Murgoci, Ioannis Zerdas and Bertrand Joseph. SETD2 mutation in renal clear cell carcinoma suppress autophagy via regulation of ATG12. *Cell Death and Diseases* (2020)11:69.
- IV. **Patricia González-Rodríguez**, Mathilde Cheray, Jens Füllgrabe, Maria Salli, Virginia Cunha, Agata Lupa, Wenbo Li, Qi Ma, Kristian Dreij, Michael G. Rosenfeld and Bertrand Joseph. The DNA methyltransferase DNMT3A contributes to autophagy long-term memory. *Autophagy* (Accepted)
- V. **Patricia González-Rodríguez**, Mathilde Cheray and Bertrand Joseph. ULK3-dependent activation of GLI1 promotes DNMT3A expression upon autophagy induction. *Manuscript*.

RELATED PAPERS, NOT INCLUDED IN THE THESIS

- SI. Carles Solà-Riera, Shawon Gupta, Kimia T. Maleki, **Patricia González-Rodríguez**, Dalel Saidi, Christine L. Zimmer, Sindhu Vaneti, Laura Rivino, Yee-Sin Leo, David Chien Lye, Paul A. MacAry, Clas Ahlm, Anna Sned-Sörensen, Bertrand Joseph, Niklas K. Björkström, Hans-Gustaf Ljunggren and Jonas Klingström. Hantavirus inhibits TRAIL-mediated killing infected cells by downregulating death receptor 5. *Cell Rep.* 2019. 28(8):2124-2139.

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LIST OF ABBREVIATIONS

A β	Amyloid- β
Ac	Acetylation
AD	Alzheimer Disease
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
APP	Amyloid precursor protein
ATG	Autophagy-related
ATG14L	ATG14-like
ATG16L	ATG16-like
AV	Autophagic vesicles
BCL-2	B-cell lymphoma 2
BECN1	Beclin-1
ccRCC	Clear cell renal cell carcinoma
CBZ	Carbamazepine
CK1	Casein kinase 1
CMA	Chaperone-mediated Autophagy
CL	Cardiolipin
CLEAR	Coordinated Lysosomal Expression and Regulation
CpG	Cytosine-phosphatase-guanine
Ctv	Cytoplasmic-to-Vacuole
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E2F1	E2-transcription factor 2
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
EZH2	Enhancer of Zeste 2 polycomb repressive complex 2 subunit
FBXO22	Ubiquitin ligase complex SCF
FOXO	Class O of forkhead box
FPKM	Fragments per Kilobase per million
FUND1	FUN14 domain-containing protein 1
GABARAP	γ -aminobutyric acid receptor-associated protein
GABARAPL	γ -aminobutyric acid receptor-associated protein-like

GFP	Green fluorescent protein
GIS	Glg1-2 suppressor
GLI	Glioma-associated oncogene homolog zinc finger protein
GRO-seq	Global run-on sequencing
GSK3b	Glycogen synthase kinase 3b
HAT	Histone acetyltransferase
HCQ	Hydroxychloroquine
HDAC	Histone deacetylase
HDM	Histone demethylase
HD	Huntington Disease
HEK293	Human embryonic kidney 293 cells
HIF1A	Hypoxia-inducible factor 1 α
JMJD	Jumonji-C domain containing
KAT8	Lysine acetyltransferase 8
hMOF	Human orthologue of males absent on the First
HMTs	Histone methyltransferases
HSC70	Heat shock cognate 71 kDa protein
HTT	Huntingtin
IL1 β	Interleukin-1 β
KDM4	Lysine (K)-specific demethylase 4
LAMP2A	Lysosome-associated membrane protein 2
MAP1LC3	Microtubule-associated protein 1 A/B light chain 3
MEF	Mouse embryonic fibroblasts
MHC class II	Major histocompatibility complex class II
LIR	LC3-interacting domain/region
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MTOR	Mechanistic target of rapamycin kinase
NBR1	Neighbor of BRCA1 gene 1
NF- κ B	nuclear factor κ -light-chain-enhancer of activated B cells
NIX	NIP-like protein X
PARP	Poly (ADP-ribose) polymerase
PAS	Phagophore assembly site
PBRM1	SWI/SNP chromatin remodeling complex 1
PD	Parkinson Disease
PE	Phosphatidylethanolamine

PI3KC3/VPS34	Serine/threonine-protein kinase VPS34
PI3KR4/VPS15	Serine/threonine-protein kinase VPS15
PINK1	PTEN-induced kinase 1
PKA	Protein Kinase A
PLA	Proximity ligation assay
PMN	Piecemeal microautophagy of the nucleus
PTMs	Postranslational modifications
PolyQ	Polyglutamine
PtdIns3K	Phosphatidylinositol 3-kinases
RAB	Ras-associated binding
RB1CC1/FIP200	RB1 inducible coiled-coil 1
RCC	Renal cell carcinoma
RFP	Red fluorescence protein
RIM15	Regulator of IME2
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
ROS	Reactive Oxygen Species
RT-qPCR	Real time quantitative polymerase chain reaction
S6K1	Ribosomal protein S6 kinase beta-1
SETD2	SET domain-containing 2
shRNA	Small hairpin RNA
shRNP	Small heterogeneous ribonucleoproteins
SNCA	Alpha-synuclein
SNX17	Syntaxin-17
SQTSM-1/p62	Sequestosome-1
SNAP29	Synaptosomal-associated protein 29
STAT	Signal transducer and activator transcription
STK36	Serine/threonine kinase 36
STS	Staurosporine
SUFU	Suppressor of fused protein
TET	Ten-eleven translocation
TFEB	Transcription factor EB
TIP60	TAT-interacting protein 60 kDa
TLR	Toll-like receptor
TP53	Tumor protein 53
ULK	Unc-51-like autophagy activating kinase

VAMP8	Vesicle-associated membrane protein 8
VHL	Von Hippel-Lindau
VPA	Valproic acid/Valproate
WIPI	WD repeat domain phosphoinositide-interacting protein 2
ZKSCAN3	Zinc-finger protein with KRAB and SCAN domain 3

1 INTRODUCTION

1.1 AUTOPHAGY

In 1963, Christian De Duve, who discovered the organelle responsible for degradation of macromolecules in the cell, the lysosome, later coined the concept of “Autophagy”. This term corresponds to the two Greek words “*auto*” and “*phagein*” (self-eating) and relates to the self-digestion of cytoplasmic components by a lysosomal degradation pathway (Ashford and Porter, 1962; Sutherland and De Duve, 1948). Autophagy, corresponds to an evolutionary conserved catabolic pathway where cytosolic components are degraded within the lysosome (in mammals) or vacuole (in yeast and plants). Subsequently, the degraded cargo is recycled depending on the requirement of the cell and is used for different anabolic pathways (Levine and Klionsky, 2004; Xie and Klionsky, 2007).

Currently, two types of cargo selection by autophagy are known, nonselective or selective autophagy. However, those two concepts were not clearly defined until the 1990s. Oshumi and colleagues demonstrated that nonselective autophagy is associated with a bulk of random cytoplasmic components that are engulfed by an “autophagic body”, also known as autophagosomes, upon nutrient deprivation in *Saccharomyces Cerevisiae* (Yeast) (Baba et al., 1994; Takeshige et al., 1992) (**Figure 1**). Interestingly, they found that within those cytoplasmic bodies, the cargo corresponded to lipid and glycogen granules, ribosomes, endoplasmic reticulum (ER) and mitochondria engulfed and were indistinguishable from the components in the cytoplasm. These findings suggested that the sequestering of cargo occurred in an unspecific manner, which are in turn degraded within the vacuole resulting in a global turnover of new macromolecules that are then released back to the cytoplasm (Takeshige et al., 1992).

At the same time, Klionsky et al., characterized for the first time the concept of selective autophagy, by the demonstration of the import of the aminopeptidase I within the vacuole in yeast (Klionsky et al., 1992). In selective autophagy, specific substrates such as protein aggregates, damaged mitochondria or bacteria are selectively targeted by autophagy receptors and therefore degraded through this pathway (**Figure 1**). Interestingly, each process involves specific cellular components and accordingly to the substrate/cargo recruited by the autophagosome it is identified as a unique name. For instance, mitophagy for selective degradation of mitochondria, ribophagy for ribosomes, etc.

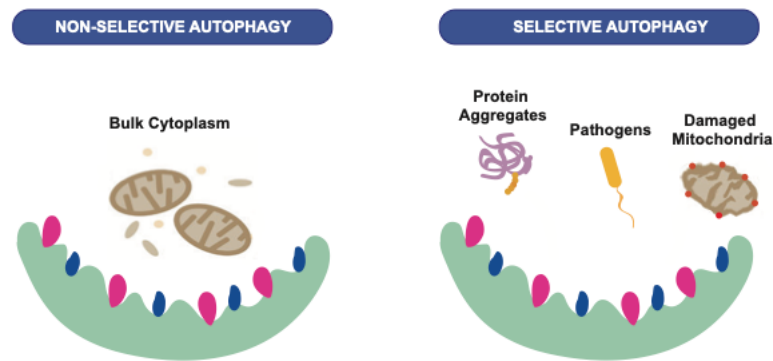


Figure 1. Non-selective and selective autophagy. Non-selective autophagy is a cellular response to the scarcity of available nutrients. A bulk of random cytoplasmic components are taken up into the precursor of autophagosomes, the phagophore (shown in green) and subsequently degraded. Selective autophagy degrades specific cytoplasmic components such as protein aggregates, pathogens and damaged organelles that are targeted by specific proteins (shown in red and yellow) or a specific sequence of amino acids that acts as a signal of recognition by selective autophagy receptors (shown as purple and pink). In both conditions, the cargo is engulfed by the “autophagic body” or phagophore.

To date, different types of non-selective and selective autophagy pathway have been described, which rely on the cargo delivered into the vacuole or lysosome. In yeast, selective autophagy includes the cytoplasm-to-vacuole (Ctv) pathway also known as macroautophagy, mitophagy, pexophagy, piecemeal microautophagy of the nucleus (PMN), reticulophagy and ribophagy. In eukaryotes, selective autophagy includes macroautophagy, mitophagy, pexophagy, PMN, reticulophagy, ribophagy as in yeast. Additionally, aggrephagy and xenophagy are exclusively described in higher eukaryotes (Kim et al., 2007; Nakagawa et al., 2004; Roberts et al., 2003; Sakai et al., 2006; Scott et al., 1996; Shintani et al., 2002; Shintani and Klionsky, 2004; Wickner and Schekman, 2005). Whereas there are several types of autophagy, the three types primary described are: Macroautophagy, microautophagy and chaperone-mediated autophagy.

Macroautophagy

Macroautophagy, generally referred to as autophagy, is the most studied type of autophagy. The induction of this process is triggered by several types of cellular stress such as nutrient depletion, hypoxia, pathogens or drugs. The hallmark of autophagy is the exclusive formation of a double membrane vesicle known as an autophagosome, whose arrangements take place by subsequent steps right after autophagy is induced. The first event after induction is the formation of the phagophore, the precursors of the autophagosome, the key organelle of this

process. The phagophore consists of a single sheet of a lipidic membrane that elongates and expands due to the addition of lipids from several sources until it closes. When the phagophore fuses, it generates the autophagosome. Upon the maturation process, microtubules support the movement of autophagosomes towards the lysosome. Eventually, the autophagosome fuses with the lysosome, generating an autolysosome. In this step, the release of the acid hydrolytic enzymes of the lysosome is essential for the degradation and breakdown of the autophagic-cargo (**Figure 2**). Thereafter, the obtained macromolecules such as amino acids, lipids, metabolites, etc. are re-used for different cellular purposes with the goal to keep cellular homeostasis (Feng et al., 2014; Mizushima et al., 2008; Xie and Klionsky, 2007; Yin et al., 2016). Of note, this is the main type of autophagy studied and focus of this thesis.

MACROAUTOPHAGY

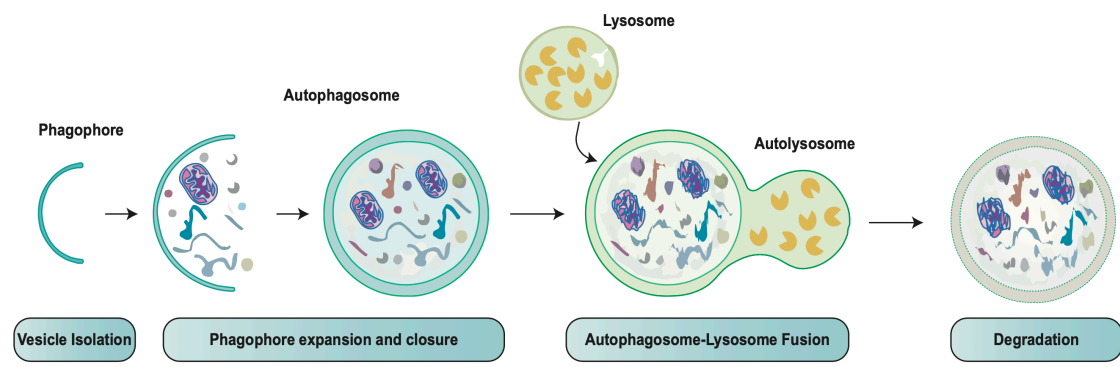


Figure 2. Macroautophagy. This pathway is divided in different steps: vesicle isolation, phagophore expansion and closure, Autophagosome lysosome-fusion and degradation. During the isolation of the membrane the phagophore starts to form and to recruit the cargo that potentially will be degraded. Next, the formation of the autophagosome takes place when the phagophore expands, recruit the cytosolic cargo and fuses, creating a double membrane vesicle. Later, the lysosome fuses with the autophagosome (generating an autolysosome) and promotes the degradation of the components inside the organelle.

Mitophagy

Mitophagy is a specific type of degradation of damaged or non-functional mitochondria by autophagy. Mitochondria is considered the one of the core organelles and as act as the “power source” of the cell for being able to generate energy from several metabolic pathways, but also cellular respiration and control of cell death (Ashrafi and Schwarz, 2013; Palikaras and Tavernarakis, 2014). Then a meticulous regulation and turn-over of mitochondria known as mitophagy is important to act as a quality control process within the cell. Mitochondria degradation through autophagy mostly occurs upon stress-triggered conditions such as generation of Reactive Oxygen Species (ROS), a process that decreases the efficiency of the

respiratory chain and therefore alteration of its function which is critical for cell homeostasis (Palikaras and Tavernarakis, 2014; Quinsay et al., 2010). Mitochondria can be degraded through autophagy in two ways: PINK/PARKIN-dependent autophagy or ubiquitin-independent autophagy. But there is also an autophagy-independent mitochondria degradation through the endo-lysosomal pathway (**Figure 3**) (Cadete et al., 2016; Hanna et al., 2012; Liu et al., 2012; McLelland et al., 2016).

MITOCHONDRIAL CLEARANCE

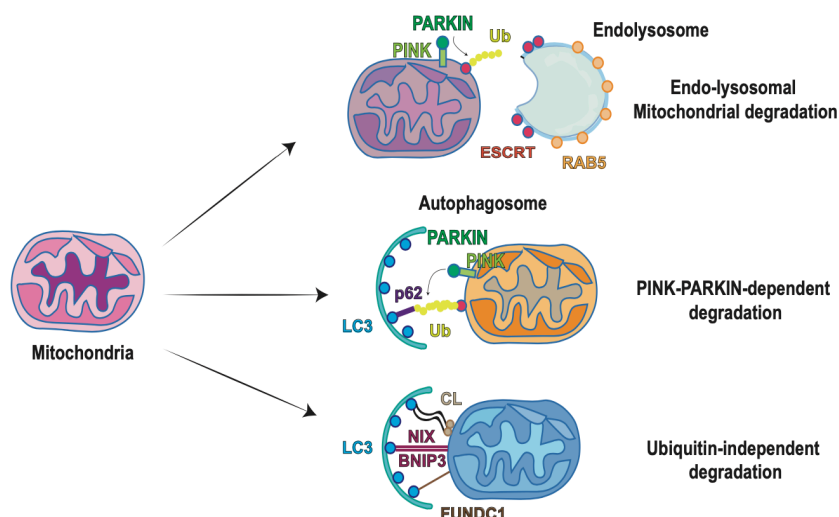


Figure 3. Mitochondrial degradation. Endo-lysosomal mitochondrial degradation corresponds to the internalization of the mitochondria directly within endosomes by the recruitment of Endosomal Sorting Complex Required for transport (ESCRT) on the endosomes. ESCRT recruitment is mediated by ubiquitination of proteins on the mitochondrial surface mediated by PARKIN (PARK). Moreover, mitochondria can be cleared in two different types of autophagy. On the PTEN-induced kinase 1 (PINK1) and PARKIN-dependent degradation, upon stress conditions PINK1 is translocated to the surface of the mitochondria, which phosphorylates PARK. Then, PARK interacts with its substrates by ubiquitination. The ubiquitin residues, are recognized by a ubiquitin-adaptor protein such as sequestosome-1 (SQSTM-1/p62), which contains a LIR (LC3-interacting domain) that allows its binding to MAP1LC3. Moreover, mitochondria can be also cleared in a ubiquitin-independent manner without the implication of PINK1 or PARK, but relying on LC3 contribution. In this case, NIX and BNIP3, CL or FUNDC1 contain LIR domain that allows the binding to LC3 and mitochondrial degradation through autophagy.

Microautophagy

Microautophagy, has been widely considered a non-selective degradative process. During this process, the cytoplasmic cargo “*in bulk*” is taken up by the vacuole (plants and yeast) or lysosome for degradation. The lysosomal membrane protrudes and invaginates in order to promote the degradation of the cytosolic components. Once the cargo is inside, its degradation occurs in a short and efficient manner by the hydrolytic proteases that are present in the lysosome (Nakamura et al., 1997)(**Figure 4**). Whereas microautophagy has been always considered a non-selective pathway, in yeast it has been found to degrade selective cargo such as peroxisomes, a mechanism known as micropexophagy, micromitophagy

(degradation of portions of the mitochondria), microlipophagy or piecemeal microautophagy (Kvam and Goldfarb, 2004) Vevea et al., 2016). All these types of microautophagy provide further evidence of a microautophagy selective degradation process in yeast. Instead, in higher eukaryotes some genes involved in this process are not conserved from yeast. However, in mammals recent studies reveal a similar degradative process with common features as in yeast, named endosomal-microautophagy, where the cargo is internalized within the lysosome or late endosomes (multivesicular bodies). The mechanism of internalization of the cargo can occur in two different manners: ESCRT-independent (microautophagy) or ESCRT-dependent (endosome-microautophagy) (Galluzzi et al., 2017; Mukherjee et al., 2016; Uytterhoeven et al., 2015).

MICROAUTOPHAGY

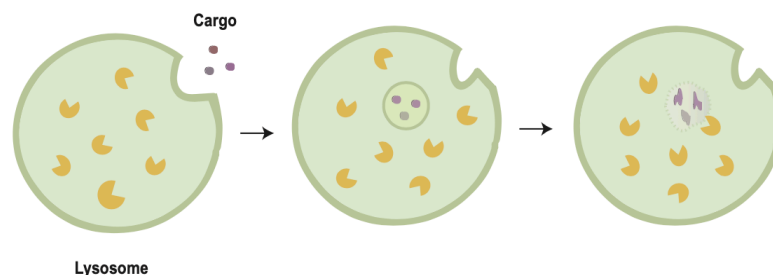


Figure 4. Microautophagy. Simplified representation of the mechanism for microautophagy. Firstly, the membrane of the lysosome is evaginated and then wrap and sequesters the cytoplasmic cargo. The material is engulfed in a small vesicle inside the lysosome that eventually is degraded by the action of the hydrolytic enzymes of the lysosome.

Chaperone-mediated autophagy (CMA)

Chaperone-mediated autophagy (CMA), is known as a type of autophagy that degrades selectively proteins that contains the KFRQ-domain in their amino acid sequence (Dice, 2007). This domain is recognized by the cytosolic chaperone protein, Heat shock cognate protein of 70 kDa (HSC70) that brings the protein to the surface of the lysosome. Lysosome-associated membrane protein 2A (LAMP2A) sits on the lysosome and acts as a receptor specific for this pathway by direct interaction with HSC70 and the protein to be degraded. The protein-chaperone recognition with the LAMP2A monomer drives the formation of a LAMP2A multimeric complex of 700 kDa (Bandyopadhyay et al., 2008; Cuervo, 2010). Ultimately, the protein to be degraded is unfolded and introduced into the lumen of the lysosome with the help of the luminal form of HSC70 (Lys-HSC70) within the lysosome (**Figure 5**). Once inside, the hydrolytic enzymes lead to the cargo degradation (Cuervo and Dice, 1996; Kaushik et al., 2006; Tekirdag and Cuervo, 2018).

CHAPERONE-MEDIATED AUTOPHAGY

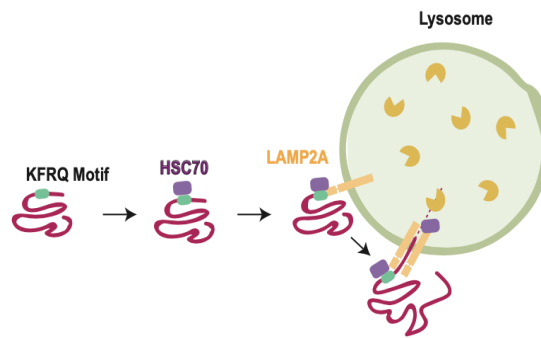


Figure 5. Chaperone-mediated autophagy (CMA). Degradation of proteins that contain the KFRQ motif by binding to the chaperone HSC70 allows an encounter with LAMP2A, which is a lysosomal transmembrane protein. HSC70-substrate and LAMP2A interaction promotes the formation of a multimeric complex of LAMP2A. The protein is unfolded and translocated within the lysosome mediated by the luminal HSC70. Finally, proteases inside the lysosome break-down the protein in simple molecules that can be reused in anabolic pathways.

1.2 CYTOPLASMIC REGULATION OF AUTOPHAGY

Autophagy is a dynamic process that requires a wide-range of components to ensure its completion. Studies in the yeast system have contributed to the identification of more than 30 ATG genes that are involved in this process (Klionsky et al., 2003). As previously mentioned, autophagy is a process that is an evolutionarily conserved mechanism from yeast to mammals. Noteworthy, although there are a large number of genes related to this pathway that differs through evolution, however, the core molecular machinery of autophagy is considered to be regulated by ATG genes that are also conserved (Yang and Klionsky, 2010). These core ATG proteins have distinct roles and functions, are classified by their contribution to the different steps of the autophagy pathway: autophagy induction and phagophore formation, autophagosome expansion, autophagosome maturation, cargo recruitment and degradation (Mizushima, 2007)(**Figure 7**).

Autophagy induction and phagophore formation

Induction of autophagy most commonly occurs upon stress conditions, which essentially requires, but is not limited to, the repression of the mechanistic target of rapamycin kinase (mTOR), a core component of several molecular pathways involved in protein synthesis, cell proliferation and cell cycle progression ((Saxton and Sabatini, 2017). Therefore, inhibition of mTORC1 is enough to induce autophagy upon limited nutrient availability (Kanazawa et al., 2004). Instead, autophagy can also be induced in an mTOR-independent manner, for

instance by AMP-activated protein kinase (AMPK) sensing (Sarkar, 2013). Downstream, Autophagy related 1 (Atg1) protein in yeast or Unc-51-like autophagy activating Kinase 1/2 (ULK1/2) in mammals is auto-phosphorylated and promotes the formation of a large complex with Autophagy related 13 (ATG13) and Autophagy related 17/ RB1 inducible coiled-coil 1/Focal adhesion kinase family interacting protein of 200 kDa (Atg17/RB1CC1/FIP200) (Hara et al., 2008; Kabeya et al., 2005; Kamada et al., 2000).

ULK proteins are a serine/threonine kinase that belongs to a family containing five different homologs: ULK1, ULK2, ULK3, ULK4 and the Serine/threonine kinase 36 (STK36)(Jung et al., 2009). Among these, so far only ULK1 and ULK2 play a role in the initiation of autophagy, while the function of the other homologs such as ULK3 or ULK4 still remains to be elucidated. However, new reports support the fact that ULK3 is upregulated upon nitrogen starvation or amino acid deprivation conditions (Jung et al., 2009; Young et al., 2006). Moreover, few studies report the involvement of ULK3 expression on the initiation of autophagy, as well as the contribution on the regulation of GLI zinc finger family proteins (GLI, also known as Glioma-associated oncogene homolog zinc finger protein) that are part of the Hedgehog signaling pathway (Goruppi et al., 2017; Kasak et al., 2018; Maloverjan et al., 2010b). The authors show that ULK3 is a kinase with an intrinsic auto-phosphorylation activity, is then able to phosphorylate GLI1 and GLI2 proteins in the cytoplasm. ULK3-mediated phosphorylation of GLI1 proteins promotes its translocation into the nucleus and enhances transcriptional activity of GLI-targeted genes (Maloverjan et al., 2010a; Maloverjan et al., 2010b)(**Figure 6**).

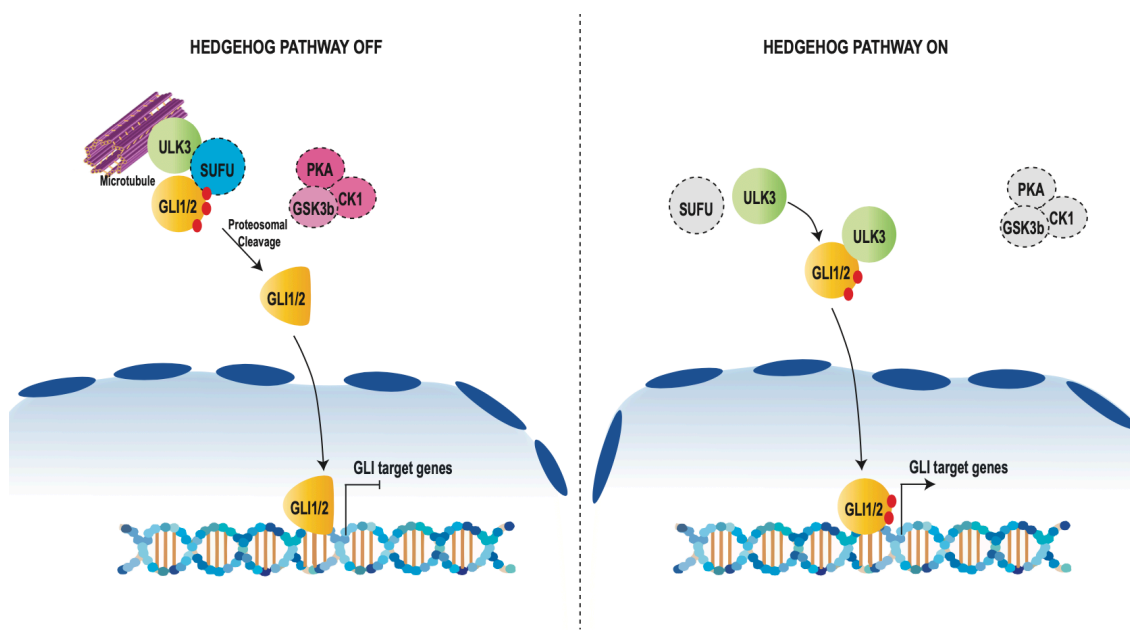


Figure 6. A model of ULK3-mediated GLI proteins regulation upon activation of the Hedgehog pathway. In the absence of a signal that triggers the Hedgehog pathway, ULK3 is recruited at the microtubules, which possibly interacts with the suppressor of fused protein (SUFU), which promotes the inactivation of ULK3 activity. SUFU binds to GLI1/2 and phosphorylates it. ULK3-SUFU promotes the C-terminal processing of GLI1/2 that it is exposed to the cytoplasm. Therefore, the protein kinase A (PKA), Casein kinase 1 (CK1) and Glycogen synthase kinase 3 b (GSK3b) are kinase proteins recruited to GLI1/2 sites and generates a truncated form of GLI1/2 that it is able to translocate into the nucleus acting as a repressor of GLI target genes. On the other hand, activation of the pathway leads to activation of ULK3, which mediates phosphorylation and activation of GLI1/2 proteins. Eventually, GLI1/2 translocate into the nucleus and enhance transcription of target genes.

Once ULK1 is activated, it promotes the recruitment of ATG13 and FIP200, which contributes to the initiation of the phagophore in a specific location known as the phagophore assembly sites (PAS). The proper recruitment of ULK1 to the PAS is essential for the binding and recognition of other effectors during the phagophore formation, the precursor of the autophagosome (Itakura and Mizushima, 2010).

In order to guarantee a proper vesicle nucleation, the activity of the ULK1-ATG13-FIP200-ATG101 complex and its recruitment to the PAS lead to association of an additional kinase complex, known as phosphatidylinositol 3-kinases (PtdIns3K) complex (He and Klionsky, 2009). This complex consists of the several components: class-III phosphatidylinositol 3-kinase Serine/threonine-protein kinase VPS34 (PI3KC3/VPS34), Serine/threonine-protein kinase VPS15 (PI3KR4/VPS15), BECLIN-1 (BECN1) and ATG14L (ATG14-like). The major role of this complex is the recruitment to the PAS and phosphorylation of Phosphatidylinositol 3-phosphate at the site of formation of the autophagosome and recruitment of other proteins such as WD-repeat protein Interacting with Phosphoinositide 2 (WIPI2) or ATG9. The latter will contribute to the transport and sealing of the autophagosome (Itakura and Mizushima, 2010; Matsunaga et al., 2010; Zhou et al., 2017).

Autophagosome expansion and closure by ubiquitin-like conjugation complexes

In this step, the action of two ubiquitin conjugation complexes work simultaneously with the purpose of promoting the lipidation and incorporation of the main autophagic marker, Microtubule associated protein 1A/1B-light chain 3 (MAP1LC3/LC3) or ATG8 in yeast, during the expansion of the autophagosome (Jatana et al., 2020; Nguyen et al., 2016; Xie et al., 2008).

The first ubiquitin-like complex corresponds to the ATG12-ATG5-ATG16L complex. Initially, ATG12 is activated by the E1-like enzyme ATG7. Next, the E2-like enzyme ATG10

promotes the covalent binding of ATG5 to ATG12, which forms the ATG12-ATG5 conjugate. Furthermore, ATG12-ATG5 forms a complex with a coiled-coil protein ATG16L (ATG16-like 1). The subsequent ATG12-ATG5-ATG16L starts to homo-oligomerize and form larger complexes (Hanada et al., 2007).

The second ubiquitin-like conjugation complex, is responsible for the conjugation of the group phosphatidylethanolamine (PE) to the MAP1LC3. During this process, the ATG4 protein cleaves the C-terminal of LC3, exposing a glycine residue. Following, ATG7 binds to LC3 and transfer this group to an E2-like ligase ATG3. This interaction promotes the conjugation of PE to the exposed glycine residue of LC3 (Ichimura et al., 2000; Satoo et al., 2009). The addition of PE allows ATG8/LC3 to remain attached to the phagophore membrane, this lipidated form is known as ATG8-PE or LC3-II (Geng and Klionsky, 2008). Under full nutrient conditions, LC3 is in the cytosol (known as LC3-I) while upon autophagy induction it is recruited to both sides of the phagophore, which then corresponds to ATG8-PE/LC3-II (Ichimura et al., 2000).

During this process, the ATG12-ATG5-ATG16 complex is necessary for a proper recruitment of LC3 to the PAS, however further studies are needed to fully understand this process. During the vesicle expansion LC3 locates on the surface controlling the size of the autophagosome and recognition of potential molecules to be degraded through this pathway (Xie and Klionsky, 2007).

Autophagosome maturation, cargo recruitment and degradation

One of the major differences between yeast and other organisms, is the presence of different homologs of the yeast Atg8 protein in higher eukaryotes. These, belong to the ATG family members and can be divided in two distinct subfamilies: MAP1LC3 (MAP1LC3A, MAP1LC3B, MAP1LC3B2 and MAP1LC3C), γ -aminobutyric acid receptor-associated protein (GABARAP) and γ -aminobutyric acid receptor-associated protein-like (GABARAPL) (containing GABARAPL1, GABARAPL2 and GABARAPL3) (Grunwald et al., 2020; Jatana et al., 2020; Weidberg et al., 2010). The major functions of LC3A/B/B2 isoforms is to promote phagophore elongation and identification of potential substrates that will then be degraded by this pathway, while LC3C has been reported to have a role in autophagosome formation and secretory pathways (Koukourakis et al., 2015). On the other hand, GABARAPL family members play a major role in the process autophagosome

maturation (Weidberg et al., 2010). Therefore, this suggest that LC3 and GABARAPL isoforms could play distinct roles in the different stages of autophagy.

It is extensively known that ATG8 family members, are able to recognize the cargo that potentially will be degraded through this pathway. As previously mentioned, the recruitment of the cargo is thought to occurs in a non-selective manner, however on the last decades studies support the idea that cargo recognition is carried out in a selective way. This selection is through direct interaction with autophagy receptor/adaptor proteins (Wild et al., 2014). For instance, the most known autophagy adaptor proteins are p62 and NBR1 autophagy cargo (NBR1) that are recruited to the autophagosome through their LC3-interacting region (LIR) to LC3. Later, p62 recognizes ubiquitinated substrates, such as protein aggregates or damaged mitochondria for their degradation. In fact, proteins involved in different cellular pathways that contain a LIR-motif are selectively degraded by autophagy (Birgisdottir Å et al., 2013; Komatsu et al., 2007; Liu et al., 2016) .

Later, the autophagosome fuses with the lysosome, creating the autolysosome by the action of Ras-associated binding (RAB) GTPases and several SNARE proteins such as Syntaxin-17 (STX17), Vesicle-associated membrane protein 8 (VAMP8) and the involvement of ATG14. (Kimura et al., 2007; Mizushima, 2007; Mizushima et al., 2011). Indeed, it has been reported that ATG14 contains two distinct isoforms: the long and the short. The long isoform, contains a cysteine domain that allows both the formation of the phagophore in the PtdIns3K complex but also is the unique isoform able to homo-oligomerize and promote autophagosome-lysosome fusion. Whereas, the short isoform lacks of the cysteine domain and therefore can only promote phagophore formation (Diao et al., 2015).

Eventually, the mature autophagosome fused with the lysosome, acquiring the name of autolysosome is acidified by the lysosomal hydrolytic enzymes. These enzymes degrade the cargo inside such as lipids, protein aggregates, pathogens and even organelles with the goal to provide nutrients in form of macromolecules. These are secreted back by lysosomal permeases towards the cytoplasm and can be re-used for a wide number of cellular pathways (Mizushima, 2007).

MACROAUTOPHAGY MACHINERY

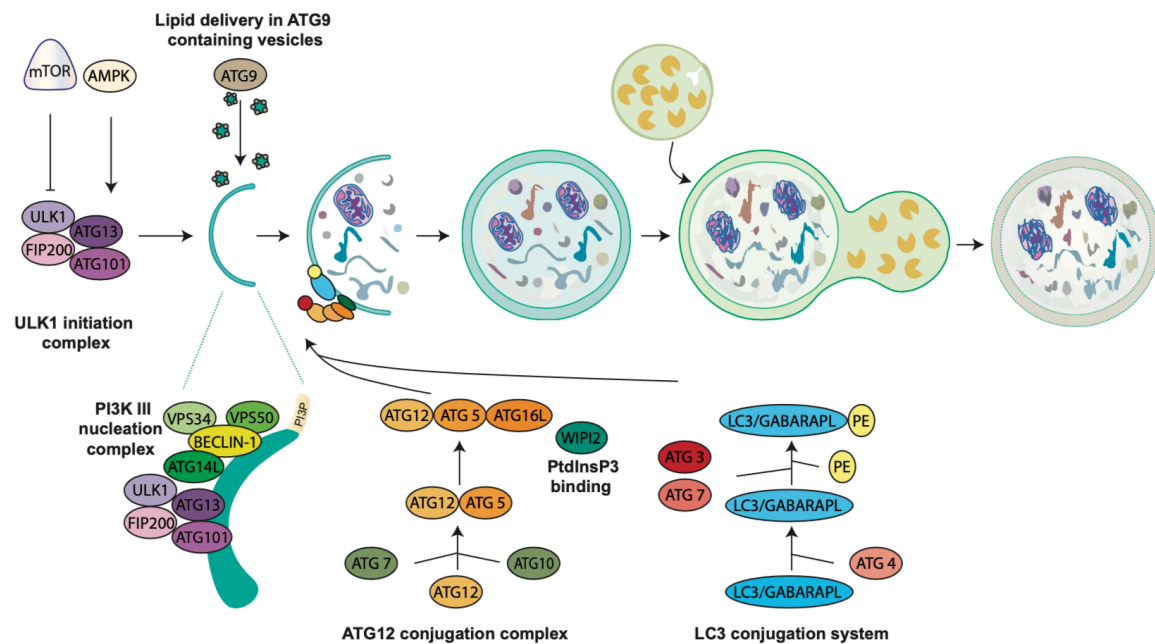


Figure 7. Autophagy machinery. A schematic process of the autophagy machinery and the proteins and complexes involved in the different steps of this process.

1.3 THE ROLE OF AUTOPHAGY IN PHYSIOLOGY AND DISEASES

As previously described, autophagy is a tightly regulated process that works in a coordinated and meticulously manner, essential to ensure a proper induction and completion of the pathway. Its induction is normally triggered upon different types of cellular stress such as starvation conditions, hypoxia, ER stress, protein aggregates or misfolded proteins but also, pathogens, damaged organelles or even drugs (Bartholomew et al., 2012; Feng et al., 2014; Levine and Kroemer, 2008). Thus, a basal and physiological level of autophagy is required for the maintenance of cell homeostasis and cellular functions.

Autophagy is considered to play an essential role in physiological events such as embryogenesis, cell differentiation, adaptation to environmental changes, aging, cell death and cell survival (Levine and Klionsky, 2004; Mizushima et al., 2008). However, impairment of autophagy may be involved in the pathogenesis of a wide range of human diseases including neurodegenerative diseases, diabetes, cardiovascular diseases, cancer, chronic inflammation and immune diseases (Levine and Kroemer, 2008).

AUTOPHAGY IN HEALTH AND DISEASES

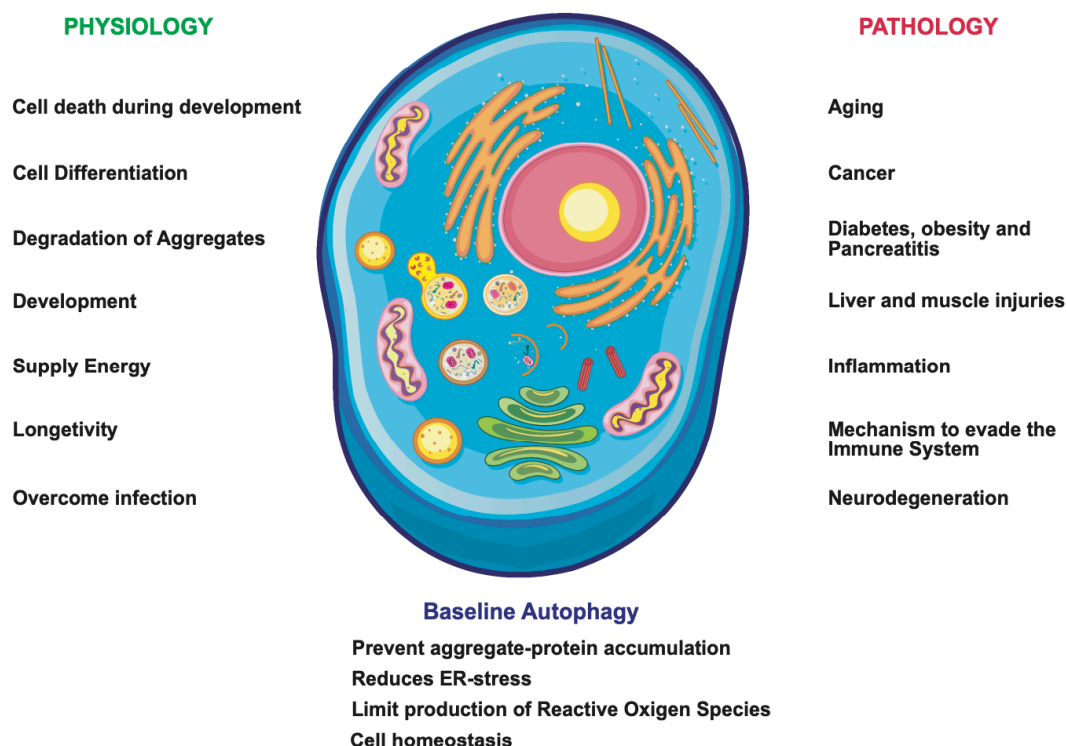


Figure 8. The physiological and pathological role of autophagy. Baseline autophagy is found to prevent aggregate-protein accumulation, reduce ER-stress and ROS that in this way maintains cellular homeostasis. Moreover, autophagy plays an essential role in different physiological events including cell death during developmental embryo stages, cell differentiation, degradation of misfolded proteins as a quality control mechanism, energy supplier under stress conditions, longevity and a mechanism to overcome infections. On the other hand, autophagy deficiency enhances aging, promotes cancer among others such as inflammation and neurodegenerative diseases and acts as a mechanism that pathogens use to evade the immune system.

Thus, autophagy is considered as a versatile pathway, which can be considered as a double-edged sword since its activation/inhibition could be either beneficial by promoting cell survival or deleterious by leading to cell damage (Levine and Klionsky, 2004; Shintani and Klionsky, 2004).

1.3.1 PHYSIOLOGICAL ROLE OF AUTOPHAGY

In normal conditions, autophagy is in a low basal level, being essential for cellular and physiological functions and cell homeostasis.

Autophagy - A quality control checkpoint under physiological conditions

Recent studies confirm that neuron specific *Atg5* and *Atg7* knockout mice exhibit a delay in neuronal development that leads to a motor and behavioral retardation compared to wild-type animals. Moreover, histological approaches showed the loss of Purkinje cells in the cerebellum in autophagy deficient neuron specific mice, as well as distinct morphology that

might be responsible for the developmental defect shown. It has been suggested that these results are an accumulation of cytosolic protein aggregates within the neurons. Overall, this suggests that the impact of basal autophagy in neurons, is an important mechanism for proper neuronal development, but also to promote the clearance of damaged organelles and misfolded proteins that otherwise could promote progression of neurodegenerative diseases (Hara et al., 2006).

Interestingly, in the liver during fasting, proteins are degraded in a quicker manner in order to generate the fuel required to compensate the punctual lack of nutrients. However, in *Atg7*-deficient hepatocytes, and then autophagy deficient, a decrease on protein turn-over due to the impossibility to generate autophagosomes and therefore protein degradation is observed. These cells also exhibit an accumulation of protein aggregates as well as damaged mitochondria, suggesting that autophagy is important in normal physiological conditions in order to remove damage components upon fasting (Komatsu et al., 2005).

Role in organism development and cell fate decision

One of the first indications that autophagy is important for embryo development is the role it plays during fertilization and during pre-implantation. During this event, autophagy is highly upregulated, due to a dramatic decrease on the phosphorylation of the ribosomal protein S6 kinase 1 (S6K1) activity by an inhibition of mTOR. In fact, oocytes that lack *Atg5*^{-/-} in mice causes a developmental delay upon fertilization, and blastocyst divisions are retained at a stage of 4-8 cells due to an impairment of protein recycling. In fact, it is well known that during fertilization there is a need of increased protein turn-over as well as removal of maternal factors that are no longer required. On the other hand, production of other factors that are essential during embryo development must be robustly produced during these early stages (Merz et al., 1981; Tsukamoto et al., 2008).

Autophagy also plays an important role at birth and after placenta withdrawal. During this process, the termination of the placental nutrient source to the fetus provokes a temporary but severe starvation, which triggers an acute autophagy response (Kuma et al., 2004). Indeed, the lack of nutrients during the embryo-to-neonatal transition is one of the main causes of death of *Atg*-deficient mice, which corroborate that autophagy is essential during this process. Here, autophagy acts as a survival mechanism, which acts as a source of amino

acids and energy, required before nutrient supply that latter restore through the milk (Schiaffino et al., 2008).

Recent studies also report the physiological requirement of autophagy during cell differentiation and cell fate. For instance, autophagy is necessary for the generation of neurons from neuronal stem cells during neuronal development. In fact, lack of *Atg5*, *Atg7* or *Vps34* in neurons impaired their differentiation and neurogenesis, affecting neuronal depolarization and axon growth (Casares-Crespo et al., 2018; Inaguma et al., 2016; Lv et al., 2014). Whereas *Ambra1* and *Beclin-1* mutants show severe effects on the neuronal tube formation and an increase of proliferation rate of neuronal tissues during embryonic development (Fimia et al., 2007; Yazdankhah et al., 2014). These studies bring evidence that autophagy is essential of cell differentiation and proliferation.

Modulating inflammation and the immune response

A specific type of autophagy named xenophagy, is triggered upon pathogens infection. During xenophagy, autophagosomes target, encase and eradicate pathogens such bacteria and viruses, suggesting that this process is crucial for the maintenance of an efficient immune system (Yang and Klionsky, 2020; Zhou and Zhang, 2012). Beyond the role of autophagy on pathogens clearance, it also mediates cellular events required to for innate and adaptive cell immunity, such autophagy-mediated antigen presentation to the major histocompatibility complex (MHC class II), or delivery of the antigen viral nucleic acids in the form of an antigens to the Toll-like receptor 7 (TLR7), with the consequent activation of interferon signaling (Schmid and Münz, 2007). In addition, basal autophagy prevents the auto-activation of the immune system. The importance to maintain a low basal level of autophagy it fundamental for inhibiting inflammation to keep down leukocyte activation upon infection. In fact, baseline autophagy inhibits interleukin 1 β (IL1- β) secretion whereas autophagy induction increases its secretion (Dupont et al., 2011).

Physiological cell death and aging

During the last decades, whether autophagy is considered a survival or deleterious process remains a subject of debate. Nevertheless, recently in the field autophagy has been considered as a survival mechanism, as produce nutrients upon stress conditions. Thus, this pro-survival acquisition is considered as an adaptive response and cytoprotective mechanism upon cell

damage. However, if the stimulus is prolonged over the time, excessive and uncontrolled autophagy eventually can be deleterious leading to cell death (Levine and Klionsky, 2004). Thus, the importance of cellular adaptation is essential for survival to acute environmental cues. Physiological autophagy-mediated cell death occurs mainly upon development. For instance, in *drosophila* autophagic degradation of the anti-apoptotic protein BRUCE, activates caspases leading to cell death (Nezis et al., 2010).

One unstoppable physiological event during life is aging. Aging is a multifactorial and physiological process defined as a decline and deterioration that occurs in most of the organisms (Harman, 1991; López-Otín et al., 2013). One of the cellular signatures that occurs during aging is a decline in baseline autophagy. Indeed, calorie restriction or fasting (one of the most robust inducers of autophagy) that it is started during adulthood is known to have beneficial effects on several physiological mechanistic processes by decreasing oxidative stress or inflammatory responses but also increasing longevity in mice (Barbosa et al., 2018). In fact, rapamycin was the first pharmacological drug to extend lifespan in mice (Harrison et al., 2009). However, while this study suggests that activation of autophagy may play a role in lifespan extension and slow down the ageing process, the responsible molecular mechanism still need to be elucidated.

1.3.2 AUTOPHAGY AND DISEASES

Autophagy dysregulation it has been reported to be involved in the pathogenesis of a wide range of human diseases including neurodegenerative diseases, diabetes, cardiovascular diseases, cancer, chronic inflammation and immune diseases (Levine and Kroemer, 2008). It follows a description of the most relevant for this thesis.

Neurodegenerative diseases

The dependence of neurons on autophagy in many instances acts as a beneficial mechanism that allows the clearance of protein aggregates. The inefficient degradation of those aggregates eventually leads to diseases and death (Komatsu et al.; Komatsu et al., 2005). In fact, alterations on the degradation of protein aggregates have been confirmed in Alzheimer disease (AD), which is the most common cause of early dementia (Harman, 2001). AD is characterized by the development of senile plaques that are constituted mainly of Amyloid β (A β) and neurofibrillary tangles composed of tau proteins (Armstrong et al., 1998). Patients with AD develop a gradual loss of their cognitive abilities and one of the main features of this patients is the increased of accumulation of autophagic vesicles (AV) containing

Amyloid precursor proteins (APP) and A β peptides, which can then lead to neurotoxicity (Zhang et al., 2012).

Not limited to AD, autophagy is also a key pathway for other types of neurodegenerative diseases such as Parkinson disease (PD) and Huntington diseases (HD). PD patients are characterized by an accumulation of α -synuclein (SNCA) into cytoplasmic inclusions named Lewy bodies where macroautophagy, and specifically mitophagy, appears to be the main pathway involved in the clearance of protein aggregates. For example, mutations in genes that regulates mitophagy, such PARKIN and PINK1, are common in PD patients (Ishihara-Paul et al., 2008). These mutations abolish the ability to remove damage mitochondria and brings evidence that this pathway is involved on SNCA clearance. Eventually, accumulation of mitochondria in PD leads to an increase in ROS and oxidative stress, leading to death of dopaminergic neurons. But also, SNCA protein contains a KFERQ-like motif making it susceptible as a substrate for CMA degradation (Alcalay et al., 2010; Issa et al., 2018). However, SNCA mutants and dopamine-modified SNCA can bind to both Hsc70 and LAMP2A but cannot translocate within the lysosome, which eventually blocks CMA and accumulation of other cytosolic proteins that are degraded through this pathway (Martinez-Vicente et al., 2008). HD, is a dominant autosomal disease caused by the repeats of the exon 1 of the gene that encodes for huntingtin (HTT) protein. The mutant protein contains a Polyglutamine (PolyQ) repeats, that results in neurotoxic aggregates with the potential to be cleared by autophagy or CMA. However, autophagosomes fail to recognize and recruit the cytosolic cargo due to an aberrant interaction between p62 with the mutant huntingtin that leads to a slower turnover, decay and increased accumulation of inefficient autophagic vesicles inside neurons (Martinez-Vicente et al., 2010).

Cancer

Defects in autophagy have been associated with an increased susceptibility to DNA damage and metabolic stress that may lead to aberrant mutations and ultimately cancer. In this field, autophagy has been shown to play a dual role in tumor suppression or tumor progression that might contribute to a variety of ways at different stages of tumorigenesis. This has been shown to be a complex process that is highly dependent on many factors including genetic predisposition and the type of cancer (Eisenberg-Lerner et al., 2009). In early steps of cancer development autophagy is considered as a cytoprotective tumor-suppressor mechanism, whereas during tumor progression autophagy act as the fuel, a nutrient supplier that fulfill the excessive metabolic demands of the tumor cells (Degenhardt et al., 2006; White, 2015;

White and DiPaola, 2009). For instance, some autophagy related genes, such as *Beclin1*, *Atg4*, *Atg5*, *Atg7* among others are considered as tumor suppressor genes. In fact, monoallelic deletion of *beclin1* in mice lead to spontaneous tumors. Also, *Atg5* and *atg7* specific hepatocytes deficiency in mice develop liver tumors due to an excessive increase of mitochondrial damage and oxidative stress (Komatsu et al., 2005; Qu et al., 2003).

During tumor growth, autophagy can act as an oncogenic factor, as its activation help the tumor to overcome stressful conditions. In fact, advanced pancreatic cancers show an increase of baseline autophagy to ensure a proper and sustained tumor growth (Yang et al., 2011). Suppression of autophagy by *beclin1* or *FIP200* depletion enhances cell death and reduces tumor progression in osteosarcoma and mammary tumors (Liang et al., 1999; Qu et al., 2003; Wei et al., 2011). Additionally, genetic depletion of *Atg* genes in tumor mouse models also exhibit a dramatic decrease of the tumor volume. Hence, autophagy may play an essential role as regulator of tumor promotion in advanced cancers. During tumor development, cells loss cell adhesion acquiring the ability to invade adjacent tissues and metastasize to other regions of the organism. In this stage, autophagy inhibition limits cancer to metastasizes stage and therefore the progression of cancer, thus high levels of autophagy were shown to correlate with a poor patient prognosis (Galluzzi et al., 2015) (**Figure 9**).

DUAL ROLE OF AUTOPHAGY DURING TUMORIGENESIS

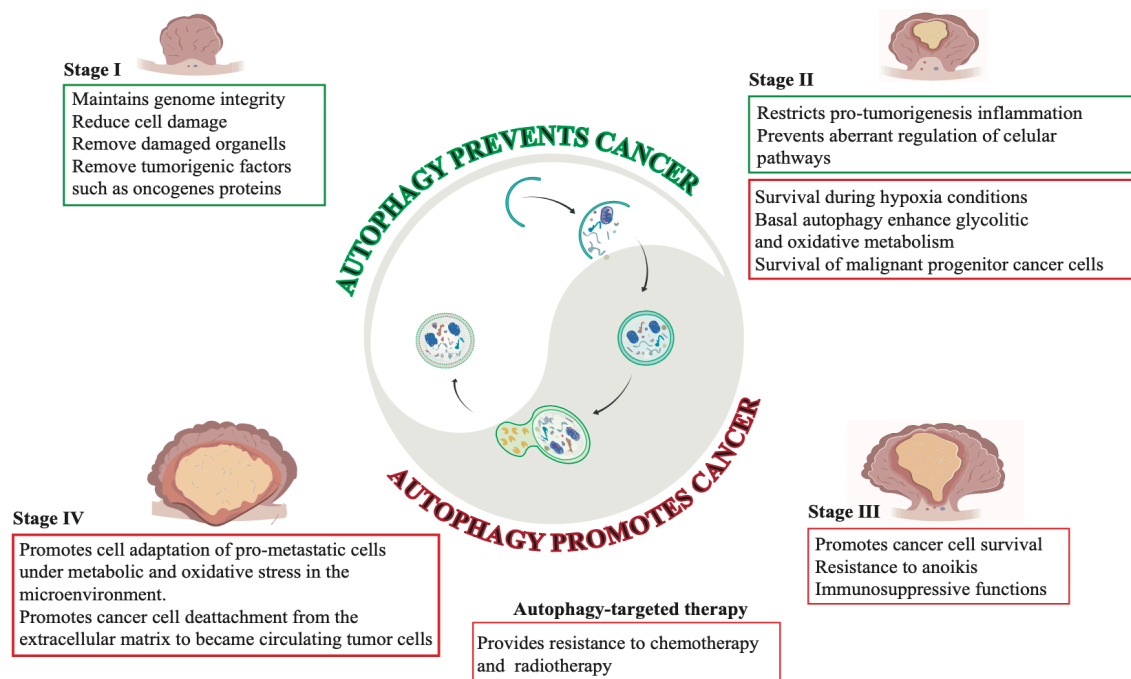


Figure 9. The dual role of autophagy as preventing or promoting cancer progression. Autophagy is considered the yin and yang of the cell as an increase controversy on the role of autophagy in the different stages of tumor development as either activation or inhibition of autophagy have different outcomes in the fight against cancer.

Based on the impact of autophagy in different stages of cancer, currently the field is focus on the a deeper understanding the interplay between autophagy and cancer to find the right target and drugs that can be used for chemotherapeutic purposes to defeat cancer.

1.3.3 AUTOPHAGY AS A THERAPEUTIC TARGET. *A Matter of Activation or Inhibition.*

The use of mTOR-dependent autophagy inducing agents, such as rapamycin has been proposed to treat neurodegenerative diseases in mouse models. Rapamycin is a selective inhibitor of mTORC1 used as a therapeutic treatment in transgenic mouse models of AD (APP mutants), prion diseases, PD (SNCA mutants) or HD (Htt mutant). Additionally, treatments that target autophagy in an mTOR-independent manner by inhibiting inositol monophosphate have also raised interest as a therapeutically approach. For instance, Carbamazepine (CBZ) or Valproic acid (VPA), enhances cellular clearance of huntingtin and α -synuclein (Lin and Qin, 2013; Xiong et al., 2011; Zhang et al., 2007), whereas, Trehalose, a disaccharide with pharmacological chaperone activity, acts as AMPK activation and enhance autophagy activity (Sarkar et al., 2007). Therefore, treatment with Trehalose enhances the removal of cytotoxic protein aggregates that have accumulated in the cell. However, it is still unclear whether enhanced autophagy is a beneficial treatment for neurodegenerative diseases. For instance, treatment with Astemizole, a lysosomotropic drug, similar to Hydroxychloroquine (HCQ), results in the inhibition of Autophagosome-Lysosome fusion but also inhibit prion infection between cells (Chong et al., 2006).

Moreover, many anti-tumoral chemotherapeutic drugs are used to treat cancer, by inhibiting or promoting autophagy depending on the tumor stage. For instance, as in other diseases, autophagy inducing treatments like rapamycin water-soluble derivatives including Temsirolimus or Everolimus are widely use as anticancer drugs (Yazbeck et al., 2008). For instance, Everolimus is used to treat pancreatic cancer and breast cancer, the latter in combination with an aromatase enzyme inhibitor, Exemestone (Amaral et al., 2012), whereas Temsirolimus is highly used to treat Glioblastoma when combined with Desatinib (another autophagy inducer)(Milano et al., 2009; Yan et al., 2016). However, recent studies show a negative clinical outcome on the use of rapalogs as treatment against glioblastoma due to the sole inhibition of the mTORC1 complex, thus activation of mTORC2 suggest glioma resistance to rapalog treatment and a need to change to potent dual inhibitors of both complexes, which shows a higher efficacy in counteracting glioblastoma growth, invasiveness and cell death of glioblastoma (Mecca et al., 2018). Other clinical trials suggest

a successful combination of histone deacetylases (HDAC) inhibitors and VPA with Temsirolimus as a treatment for hepatocellular carcinoma and Burkitt leukemia/lymphoma by inducing cell death (Ji et al., 2015). In overall, those compounds rarely work efficiently alone rather combination of compounds burst the effect and increase the cell response to fight against the disease.

1.4 THE NUCLEUS AS A MASTER REGULATOR OF AUTOPHAGY

Autophagy was considered to be purely regulated by cytosolic proteins as enucleated cells can undergo autophagy and display LC3 puncta (Joseph, 2015; Tasdemir et al., 2008). Nevertheless, over the past decades, the nucleus has taken special attention regarding autophagy regulation due to the identification of a wide number of transcription factors, histone post-translational modifications as well as microRNAs related to this process (Ozeki et al., 2017; Pietrocola et al., 2013).

1.4.1 TRANSCRIPTIONAL CONTROL OF AUTOPHAGY

The first evidence of transcriptional regulation of autophagy was revealed by a transcriptional response of Atg8-induced by nitrogen starvation in yeast (Kirisako et al., 1999). However, it was not until years later when the nucleus and transcription factors regulating ATG genes were considered as a major regulator of autophagy in coordination with the cytoplasm (Beck et al., 1999; Mammucari et al., 2007). In fact, aberrant expression of Autophagy-related genes by dysregulation of transcription factors due to mutations and/or post-translational modifications, triggers a signaling cascade that affects autophagy. For instance, disrupted transcriptional *BECLIN1* expression, due to deletion on its genomic sequence occurs in the 50-70% of breast cancer as well as downregulation of *WIPI* genes in 100% of the patients with pancreatic cancer (Aita et al., 1999). However, the upstream mechanisms upstream that leads to an aberrant transcriptional ATG genes regulation still remains to be elucidated.

FOXO transcription factors

The class O of forkhead box (FOXO) transcription factors are involved on autophagy by dependent or -independent transcriptional activation of *ATG* genes in a diverse cell types and organisms (Liu et al., 2014; Mammucari et al., 2007; Sanchez et al., 2012); (Matsuzaki et al., 2018). Activation of FOXO transcription factors are mainly regulated by nuclear/cytosolic shuttling upon stressful conditions such as nutrient deprivation. In these conditions, AKT-mediated phosphorylation of FOXO3 is inhibited, which promotes its translocation into the nucleus and the expression of *ATG*-targeted genes including *ATG4*, *ATG12*, *BECN1*, *BNIP3*,

LC3, *ULK1/2* and *VPS34* in neurons or cardiomyocytes (Audesse et al., 2019; Schips et al., 2011), whereas FOXO1, other member of the FOXO transcription factors family, also promotes transcription of other *ATG* genes such as *ATG5* in neurons or *ATG14* in cancer (Audesse et al., 2019; Xu et al., 2011). Moreover, reports show that cytosolic FOXO1 positively regulates autophagy in a transcriptional-independent manner, by its acetylation and direct binding to key components of the autophagy machinery as ATG7 (Liu et al., 2014). In fact, mutations of FOXO family members have been found to negatively affects autophagy and has been associated to neurodegenerative diseases and cancer (Pino et al., 2014).

TFEB, and its transcriptional counterpart ZKSCAN3

Transcription factor EB (TFEB) is considered the main regulator of most of the *ATG* genes. Following autophagy induction in response to stress, such as starvation, lysosomal stress or treatment with mTOR inhibitors (Torin1 or Rapamycin), TFEB is highly upregulated, dephosphorylated and translocated to the nucleus. Subsequently, TFEB undergo its transcriptional program by direct binding to TFEB target genes and increase its expression, that range from autophagy-related genes (*ATG4*, *ATG9B*, *MAP1LC3B*, *UVRAG* or *WIP1*) to CLEAR (Coordinated Lysosomal Expression and Regulation) genes that are involved in lysosomal biogenesis (Settembre and Ballabio, 2011). On the other hand, the zinc-finger protein with KRAB and SCAN domains 3 (ZKSCAN3) is considered to be the physiological TFEB counterpart, as inhibits the expression of multiple *ATG* genes, such as *ULK1* or *MAP1LC3* under nutrient replete conditions, whereas upon autophagy induction shuttles back to the cytoplasm from the nucleus, having the opposite cellular dynamics compared to TFEB (Chauhan et al., 2013). TFEB mutations or loss-of-function act as a main driver of multiples diseases associated with autophagy dysfunction such as neurodegenerative diseases and results in a metabolic imbalance of the lipid catabolism in the liver that leads to obesity in mouse models (Cortes et al., 2014; Settembre et al., 2013; Wang et al., 2016). These data suggest TFEB as a main regulator of *ATG* genes but also lysosomal genes, being a central component that define autophagy status, which eventually its dysregulation plays an essential role in various pathological disorders.

STAT transcription factors.

The signal transducers and activators transcription (STAT) proteins are a family of cytoplasmic transcription factors that translocate into the nucleus upon inflammatory challenge or oxidative stress. STAT3, a member of the STAT family, either enhances or inhibits autophagy. A constitutive basal level of cytoplasmic STAT3 regulates autophagy in

a transcriptional-independent manner as it recruits and sequester FOXO1, FOXO3 and Eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2) transcription factors under growth conditions (Mammucari et al., 2007). On the other hand, lack of nutrients STAT3 releases those transcription factors allowing them to undergo their transcriptional program, as previously described. On the other hand, nuclear STAT3 can either enhance or inhibit autophagy. For instance, STAT3 acts as negative transcriptional regulator of autophagy as enhance B-cell lymphoma 2 (Bcl-2) transcriptional levels (Shen et al., 2012; Tai et al., 2014; You et al., 2015). On the other hand, STAT3 also has the ability to promotes autophagy induction by activate transcription of the hypoxia-inducible factor α (HIF1A) and BNIP3. STAT3-mediated BNIP3 expression disrupts BECLIN1 and BCL-2 interaction which eventually promotes autophagy induction (Shen et al., 2012).

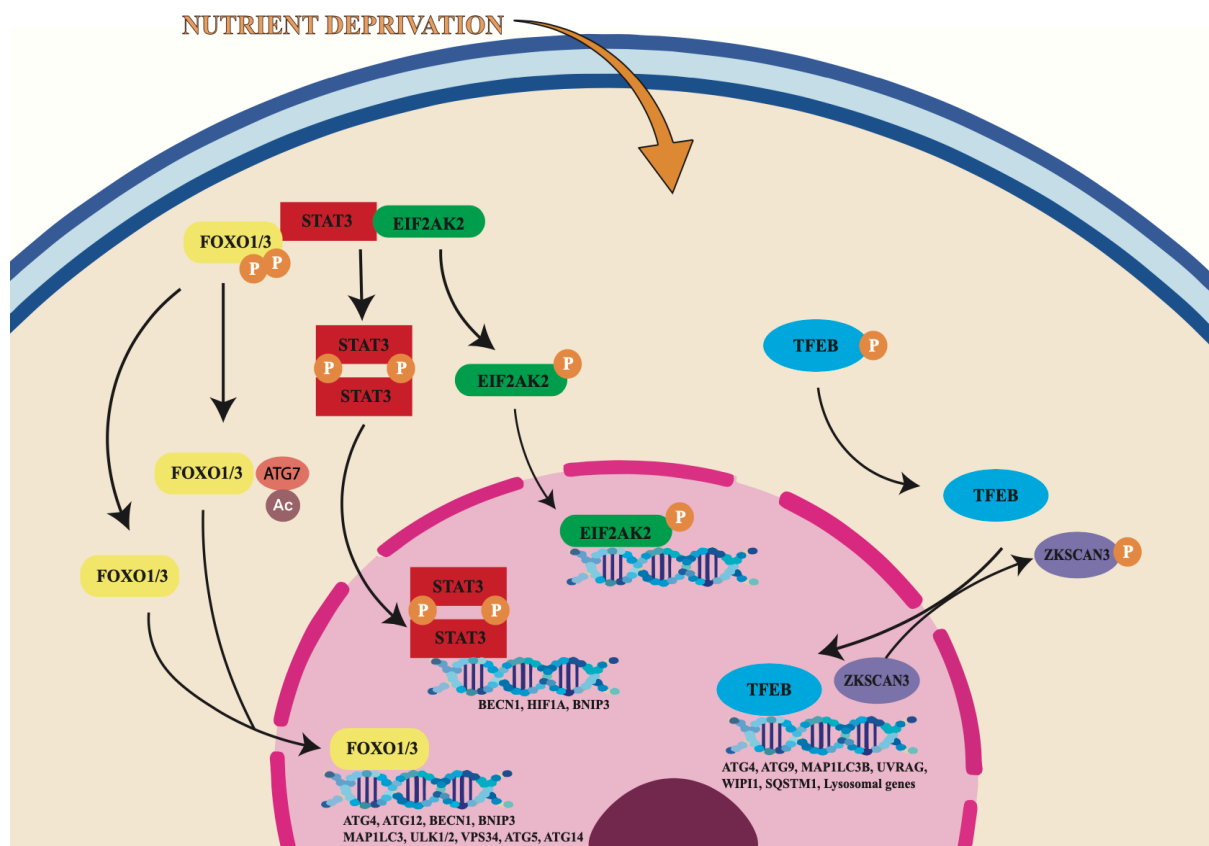


Figure 10. Simplification of the transcriptional regulation of autophagy-related genes during nutrient deprivation conditions. STAT3 regulates FOXO1/FOXO3, EIF2AK2 as well as TFEB *versus* ZKSCAN3 cellular dynamics and transcriptional regulation of autophagy-related genes. Whereas it is known that EIF2AK2 translocate towards the nucleus and enhance autophagy, it is still unknown specifically the autophagy-related genes that are targeted by this transcription factor. Of note, here we show the transcriptional master regulators.

Other transcription factors

Other transcription factors have been shown to be involved in the autophagy pathway such as Tumor protein 53 (TP53/p53) or TP63/p63, whom are activated by DNA damage or stimulated by oncogenes, are reported to inhibit mTORC1 via its transcriptional regulation of several ATG genes including ULK1, ATG2, ATG4, ATG7 and ATG10 (Huang et al.,

2012; Kenzelmann Broz et al., 2013). Under normoxia conditions, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is recruited to the promoter of the hypoxia-inducer activator of autophagy, BNIP3, by silencing its expression, while under hypoxia NF- κ B is released allowing E2-transcription factor 2 (E2F1), a transcriptional activator of ATG genes, to bind to the promoter of ULK1, ATG5 and LC3 and enhance their expression (Polager et al., 2008; Shaw et al., 2008).

1.4.2 EPIGENETIC REGULATION

The term epigenetics was first coined by Conrad Waddington in 1952 as “a definition of the branch of biology which studies the causal interactions between genes and their products which brings the phenotype into being”. However, that definition did not explain the transgenerational inheritance, mitotic inheritance or the long-term effects on gene activation/repression as well as chromatin states. Thus, years later Nanney, Riggs and Holliday, and further modified by Bird and others evolve the term “epigenetics” to a “mechanism that involves mitotically and meiotically heritable changes in the gene function that are not explained by changes on the DNA structure, as well as the cell property to remember an event in the past.”. As seen, the term “epigenetics” does not bear complete similarities with the previous descriptions. Nowadays, epigenetics refers to “the information of the DNA, that switch on/off genes, beyond of what it is encoded on the DNA sequence, with the potential of being heritable. As well as long-term alterations on the transcriptional cell potential that are not necessarily heritable” (defined by the NIH Roadmap Epigenomics Mapping Consortium(Cavalli and Heard, 2019). In fact, epigenetics is able to describe the mechanism behind the phenotypic differences present on tissue and cell types in the organism. As all the cells in the organism share the same genome, different cell types have different transcriptome and expression patterns, different proteome and thus different cellular functions, which are controlled by epigenetic modifications (Margueron and Reinberg, 2010). These modifications include DNA methylation, histone post-translational modifications, nucleosome positioning, RNA silencing and alternative splicing. In fact, these epigenetic marks are established during development in order to ensure cell identity and throughout life (Jones and Taylor, 1980). However, environmental cues can promote changes on some epigenetic modifications in order to ensure the transcription of genes required to undergo a specific and successful cellular response upon stimuli that otherwise would be repressed (Klosin et al., 2017).

1.4.2.1 DNA Methylation: principle, regulation and implications

DNA methylation is considered of being one of the main mechanisms that represses gene expression under different conditions (Compere and Palmiter, 1981; Holliday and Pugh, 1975; Weber and Schübeler, 2007). In fact, the methylation of the DNA has deep effects on the regulation of several cellular pathways including transcription repression, binding of transcription factors, modulation of the structure of the chromatin, preservation of the integrity of the genome, X-chromosome inactivation, genomic imprinting, embryogenesis and development (Baylin and Herman, 2000; Holliday and Pugh, 1975). The establishment and maintenance of the DNA methylation pattern is due to the action of DNA methyltransferase (DNMTs) enzymes, responsible for catalyzing the transfer of a methyl group to the fifth carbon of a cytosine residue localized on the CpG dinucleotides generating 5-methylcytosine (Bird, 2011; Moore et al., 2013)(**Figure 11**). Instead, demethylation is mediated by Ten-eleven translocation (TET) enzymes: TET1, TET2 and TET3. TET enzymes are responsible for converting 5-methylcytosine to 5-hydroxymethylcytosine in one DNA strand, thereafter, is removed by DNA repair or dilution during replication (Wu and Zhang, 2014).

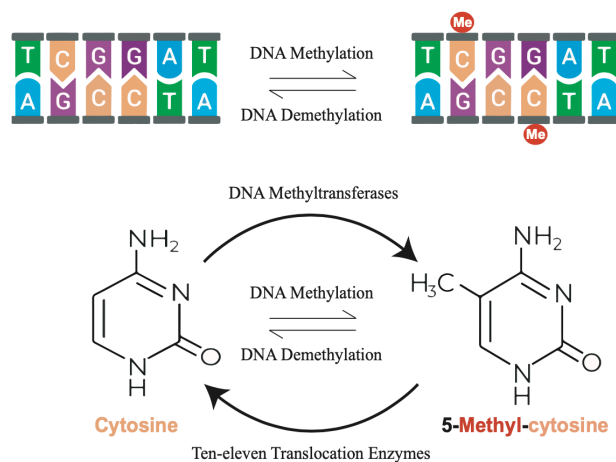


Figure 11. DNA Methylation mechanism. DNA methylation occurs on the fifth carbon of a cytosine, which is mediated by DNMTs enzymes, whereas DNA demethylation is carried out by TET enzymes.

The DNMTs family is composed of: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L enzymes. Although DNMT2 has been found to have conserved catalytic domains in prokaryotes and eukaryotes; its inactivation in embryonic stem cells does not perturb the maintenance or *de novo* methylation of the DNA, suggesting that DNMT2 is not required for the above-mentioned mechanisms (Okano et al., 1999). On the other hand, DNMT1 is the enzyme responsible for the maintenance of the methylation of the DNA following each cell mitotic division and consequently ensure the identity of the cell, whereas DNMT3A and

DNMT3B are crucial for the establishment of *de novo* methylation, particularly during embryonic development stages (Okano et al., 1999). Although, DNMT3A and DNMT3B share similarity in their structure and function, recent studies demonstrate that they differ on the preference for targeting DNA sequences *in vivo* by generating a different gene expression pattern upon different stimuli (Challen et al., 2014; Robertson, 2005). For instance, DNMT3B plays an essential role during early development, as *Dnmt3b* knockout mice are embryonically lethal whereas *Dnmt3a* knockout mice survive up to one month after birth suggesting that DNMT3A is required for a regular cell differentiation, indicating the distinct functions of both enzymes. DNMT3L lack of the DNA methyltransferase domains and therefore its ability to bind and methylate the DNA. More recent findings demonstrate that DNMT3L enhance DNA methyltransferase activity of DNMT3A and DNMT3B independent of the targeting sequence (Suetake et al., 2004).

One of the most characteristic features of DNA methylation is the fact that it can be an inherited mechanism over offspring generations, known as transgenerational epigenetic inheritance. In fact, mice that have been undergone to high-fat diet, diabetes or starvation is shown to reproduce the same alterations in the resulting progeny (Gapp et al., 2016; Wei et al., 2015). In human, epidemiological studies suggest a link between DNA methylation and environmental cues, such as starvation on the regulation of the organism and the ability to impact in the offspring generations (Bygren, 2013; Kaati et al., 2007). Disruption of this mechanism could be also considered as a hallmark of multiple pathologies. In fact, mutations in DNMT3A catalytic domain have been found to be mutated in the 20% of Acute Myeloid Leukemia patients (Yan et al., 2011), whereas mutations in DNMT1 and DNMT3B are rare, which suggest an important role of DNMT3A as tumor suppressor gene by preventing malignancies. DNMT3A has also been involved in the pathogenesis of autoimmune diseases, metabolic and neurologic disorders as well as aging (Dayeh et al., 2014; Jung and Pfeifer, 2015). Recent studies suggest DNA methylation, as an “epigenetic clock” of the cell, as the measurement of DNA methylation levels of CpG sites in blood cells acts as an estimator of the biological age and predictor of life expectancy (Bell et al., 2019; Horvath, 2013). However, the mechanism involving age-related DNA methylation patterns as well as the influence of lifestyle during life still need further investigations.

1.4.2.2 Epigenetic regulation by histones modifications

All the information required to keep cell homeostasis is written in the DNA. Hence it is important to keep a strict regulation of how and when this information is expressed. DNA is highly compacted as chromatin, which consists of repeating units of nucleosomes that are composed of a core set of two copies of four histones (H2A, H2B, H3 and H4) and 147 pair of bases of DNA. Single nucleosomes are connected by a piece of 20-80 base pairs of DNA and a linker histone H1 that stabilizes the structure (Kornberg, 1974; Kornberg and Lorch, 1999)(**Figure 12**).

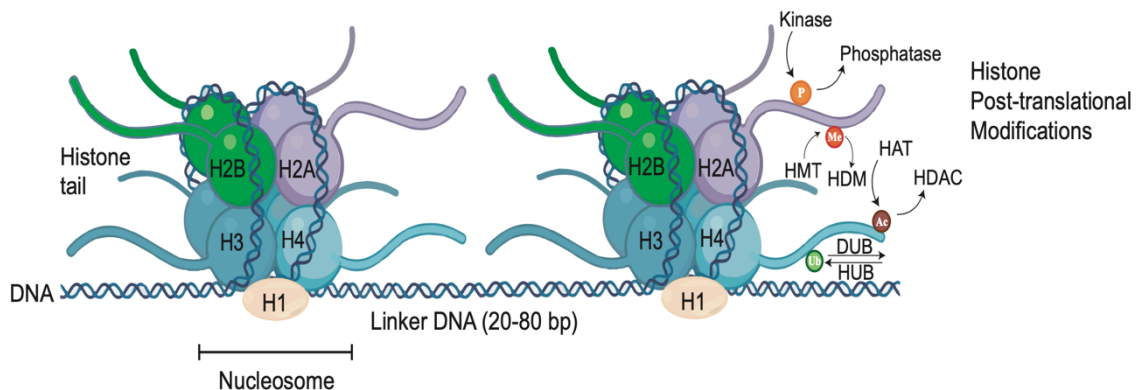


Figure 12. Structure of the nucleosomes. The nucleosomes consist of 147 base pairs of DNA that are wrapped around the histone core, composed by two copies of each of the histones H2A, H2B, H3 and H4. The histone H1, acts as a linker that stabilize the nucleosomes. The histone tails can undergo post-translational modifications the most common are phosphorylation, methylation, acetylation and ubiquitination, which are regulated by “writers” (add the mark) and “erasers” (remove the mark) and responsible for the specific addition or removal of a type of histone modification).

Histones are a family of proteins with a positive charge that wraps the negatively charged DNA that allows the compaction and regulation of the genetic material. Histones are able to undergo post-translational modifications (PTMs), playing an essential role in the condensation of DNA and transcription activation/repression. Histones post-translational modifications occur in the histone tails that protrude from the nucleosome, which involves from acetylation, methylation, ubiquitination, citrullination, ADP-ribosylation, SUMOylation to phosphorylation (**Figure 13**). These modifications are tightly regulated by chromatin modifying enzymes that in turn will either transfer or remove the modification. For instance, histone acetylation is carried out by the action of histone acetyltransferases (HATs) that add the acetyl group, while histone deacetylases (HDACs) are responsible for the deacetylation and thus removal of the acetyl group. Alike, the addition of the methyl group to the histones is regulated by histone methyltransferase (HMTs) and removed by histone demethylases (HDMs) (Bannister and Kouzarides, 2011)(**Figure 12-13**).

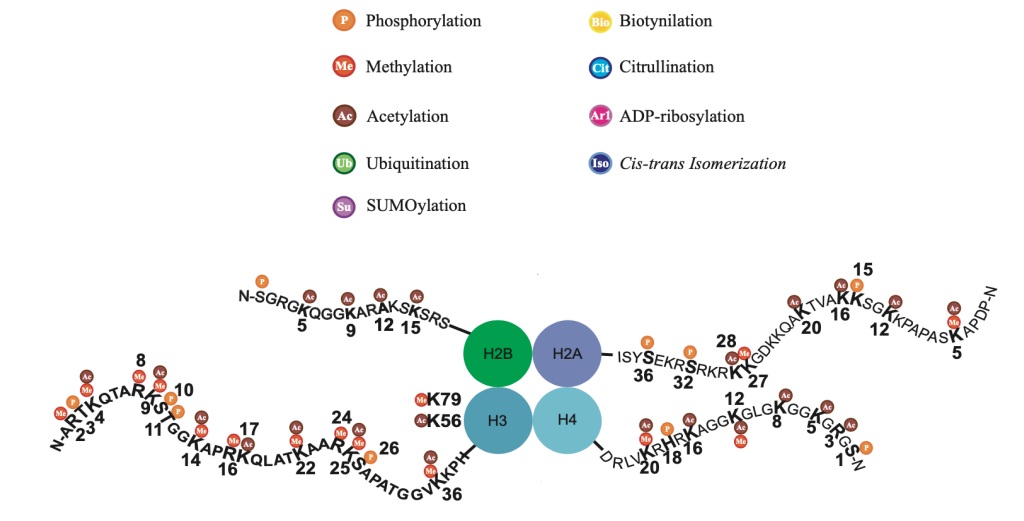


Figure 13. Histones post-translational modifications. Types of histone modifications and the most common ones that are studied.

For instance, histone 4 lysine 16 acetylation (H4K16ac) provides an open-like chromatin structure resulting in activation of gene transcription. On the other hand, methylation occurs specifically on an arginine or lysine residues mainly at gene promoter regions. Moreover, depending on where the methylation occurs will have a different outcome by either repressing or activating gene expression. In fact, Histone 3 lysine 9 and 27 trimethylation (H3K9me3/H3K27me3) are linked with repressed chromatin whereas histone 3 lysine 36 trimethylation (H3K36me3), H3K4me3 and H3K79me3 are found to be associated to transcriptionally active regions (Cavalieri, 2020)(**Figure 14**).

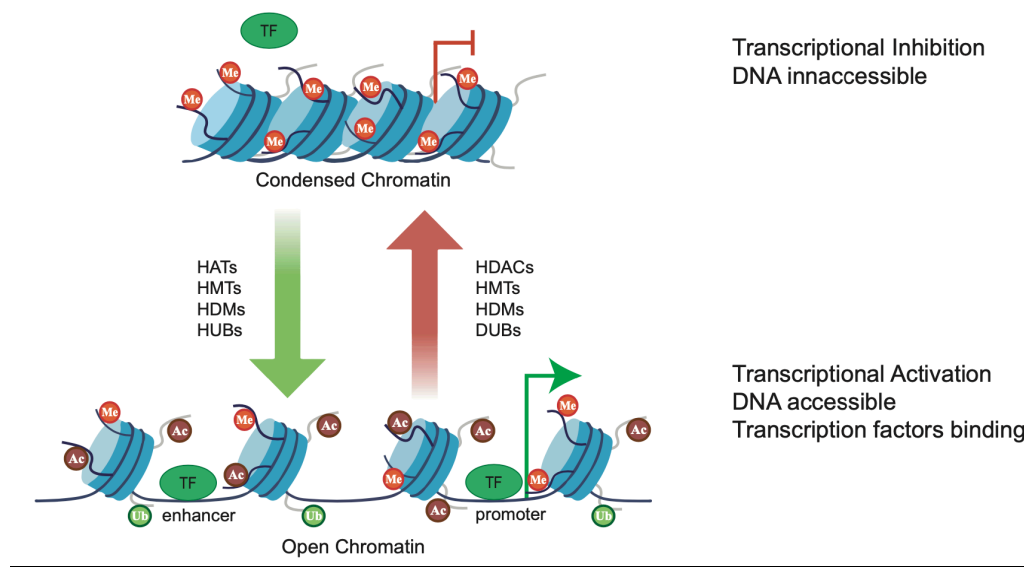


Figure 14. Histones dynamics and regulation of gene expression. Depending on the post-translational modification could either condense or open the chromatin allowing transcription factors binding and gene expression. Although it is not limited to transcriptional inhibition, histone mark methylation causes nucleosomes to pack tightly together. The DNA is inaccessible, transcription factors cannot bind and gene expression is repressed. On the other hand, histone marks such as acetylation and also methylation or ubiquitination results in loose packing of the nucleosomes, the DNA is accessible for transcription factors to bind and activate gene transcription of targeted genes.

However, histones marks do not work alone as the interplay with DNA methylation create a specific “epigenetic signature” on the DNA that dictate a specific biological outcome to define cellular identity, response to intracellular stimulus or extracellular stresses.

Set2/SETD2 and rph1/KDM4 regulates Histone 3 Lysine 36 Trimethylation

Of interest for this thesis, H3K36me3 is specifically mediated by the histone methyltransferase SET-containing domain 2 protein (Set2, in yeast; SETD2/HYPB/KMT3A, in the mammalian system), whereas demethylation is carried out by the regulator of PHR1 (Rph1, in yeast; KDM4A-C in mammals) and the nucleolar protein 66 (NO66) (**Figure 15**). The distribution of the H3K36me3 mark is observed at body regions of active genes, in exons rather than introns (Wagner and Carpenter, 2012).

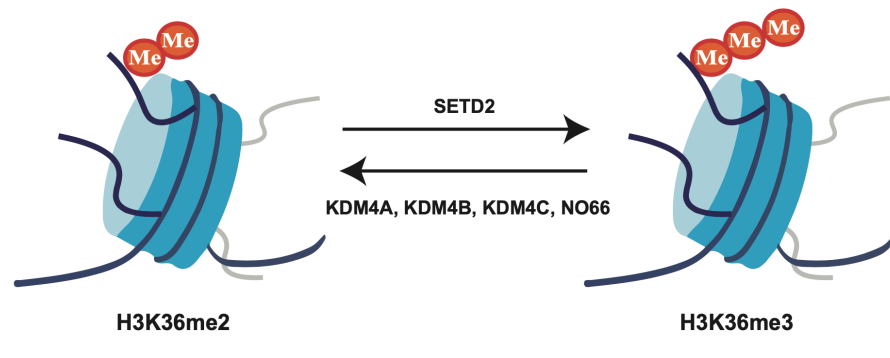


Figure 15. H3K36me3 regulation. H3K36me3 is modulated by the HMT, SETD2, and HDMs such as KDM4A-C and NO66.

SET-domain containing histone 3 lysine 36 trimethyltransferase, was firstly introduced by Edmunds *et al.* whom studies made a detailed analysis of the global distribution of H3K36 trimethylation over the genome in mammalian cells. However, it was Faber and colleagues who isolated and identified SETD2 from hematopoietic stem cells as a huntingtin interacting protein, a key protein linked to the pathogenesis of Huntington disease (Faber et al., 1998). At the same time, studies on *Saccharomyces cerevisiae* identified γ SET2 interaction with the RNA polymerase II (RNA pol II) in its serine2 phosphorylated C-terminal domain (CTD), suggesting the link of γ SET2/SETD2 on transcription elongation (Strahl et al., 2002). One of the main differences between yeast and mammals is the fact that in yeast SET2 catalyzes the addition of the methyl group in all the methylation status of H3K36 whereas in mammals it is the unique enzyme responsible for the trimethylation of H3K36 (Sun et al., 2005).

The main functions for SETD2-mediated H3K36me3 is to regulate RNA pol II, nucleosome positioning, alternative splicing and DNA repair (McDaniel and Strahl, 2017;

Tiedemann et al., 2016). SETD2 mediated H3K36me3 mark is associated with nucleosome dynamics and transcription initiation by direct binding with FACT and the CTD domain of the RNA pol II (Carvalho et al., 2014). In addition, SETD2 plays also an essential role in murine embryonic stem cell differentiation as *Setd2* deficiency in mouse embryonic stem cells promotes a deep effect on endoderm differentiation by downregulation of endoderm-related genes through SETD2-mediated mitogen-activated protein kinase (MAPK) signaling pathway (Zhang et al., 2014). Furthermore, it has been confirmed that SETD2 acts as a main regulator of DNA repair, as H3K36me3 is required for the interaction with hMutS α in order to start the DNA mismatch repair response in several kinds of cancer cell types (Li et al., 2016). In fact, mutations in SETD2 have been highly associated with several types of cancer such as clear cell renal cell carcinoma, gliomas, acute leukemia, breast and lung cancer (Fontebasso et al., 2013; Morris and Latif, 2017; Zhu et al., 2014). For instance, it is common that patients with clear cell renal cell carcinoma (ccRCC) harbor mutations or loss of function on SETD2 gene leading to a global decrease of DNA methylation, increased genome instability, aberrant splice variants and defective cell cycle arrest (Morris and Latif, 2017).

On the other hand, demethylation of H3K36me3 can be carried out by the histone demethylase enzymes, KDM4A-C and NO66 (Labbé et al., 2014; Sinha et al., 2014). The lysine (K)-specific demethylase 4 (KDM4) enzymes and the nucleolar protein 66 (NO66) are members that belong to the KDM4/JMJD2 histone demethylase subfamily within the family of Jumonji-C domain containing (JMJD) enzymes. Among the proteins able to demethylate H3K36, KDM4A is the most studied one. KDM4A is able to demethylates di- and trimethylated lysine by targeting H3K9me2/me3 and H3K36me2/me3. However, the efficiency is higher on demethylating H3K9me3 rather than H3K36me3 (Klose et al., 2006). In addition, depletion of KDM4A and KDM4C leads to an aberrant vascularization in zebrafish and spurious differentiation of endothelial cells of mice embryonic stem cells (Wu et al., 2015).

1.4.2.3 Epigenetic regulation of alternative splicing – A potential regulation of ATG alternative splice forms

Alternative splicing stands on controversy to the central dogma of molecular biology that states that one gene generates one protein. Alternative splicing is a mechanism to generate from a single gene to multiple proteins, known as isoforms, which structure and function are different (Gilbert, 1978). The main function of the splicing of RNA is to process the pre-mRNA into mature mRNA. An event that can occur in different ways: the removal of non-

coding introns, inclusion of introns, exon skipping or exon retention (Beyer and Osheim, 1988). The most common studied mechanism on the regulation of alternative splicing is the recruitment of the spliceosome, a complex composed by small nuclear ribonucleoproteins (snRNPs), and a large number of protein splicing factors that allows the recognition of the 5' and 3' splice sites on the immature pre-mRNA that eventually generates the mature mRNA (Wahl et al., 2009). Alternative splicing can also be regulated co-transcriptionally by the involvement of the transcriptional machinery such as RNA pol II in coordination with transcription factors. In fact, a decrease on the rate of transcription elongation by RNA pol II in *Drosophila melanogaster* might enhance the recognition and inclusion of alternative exons, whereas higher processivity of RNA pol II promotes the skipping of the alternative exon (de la Mata et al., 2003).

Recent studies underline the connection between histone modifications and the regulation of alternative splicing (Brown et al., 2012; Kim et al., 2011). In fact, the enrichment of H3K36me3 on the exons is higher as compared to introns within the same gene in both *Caenorhabditis elegans* and humans, suggesting the link between chromatin-mediated alternative splicing (Kolasinska-Zwierz et al., 2009). Therefore, based on the fact that SETD2 regulates RNA pol II transcription elongation by H3K36me3 recognition and the enrichment of H3K36me3 in exon-intron boundaries, it is not surprisingly to suggest the potential role of H3K36me3 histone mark on the regulation of alternative splicing. If so, exons susceptible to be spliced would have an increased H3K36me3 enrichment on exons excluded by alternative splicing. Other studies show the impact of SETD2 downregulation on the differential expression of PKM2, TPM1 and TPM2 variants promoted by alternative splicing (Luco et al., 2010). Furthermore, latest studies show the involvement of other proteins on the regulation of chromatin-mediated splicing. For instance, the histone-lysine N-methyltransferase SMYD3, a histone modification reader also recognizes H3K36me3 and is suggested to play a role on promoting intron retention (Guo et al., 2014) or the effect of SPOP, a ubiquitin E3-ligase, on regulating alternative splicing through the direct interaction with SETD2 ensuring its stability and therefore modulating H3K36me3 (Zhu et al., 2017). These studies suggest that SETD2 mediated H3K36me3 define exons rather than the frequency of alternative splicing. Although SETD2 has been linked to alternative splicing, how it is fully regulated still remains to be elucidated, as there is still controversy on the understanding of the molecular machinery behind SETD2- mediated H3K36me3 alternative splicing and the influence of potential interaction partners.

1.4.3 Short-term transcriptional regulation of autophagy

Moreover, recent studies revealed the role of several histone modifying enzymes linked to histone modifications on the regulation of autophagy, which offers an epigenetic explanation on the short-term regulation of autophagy (**Table 1**). Recent studies elucidate how H4K16 deacetylation is mediated by KAT8/hMOF downregulation resulting on a feedback regulatory loop by a reduced expression of *ATG* genes (Fullgrabe et al., 2014). H3K4me3 decreases in parallel with H4K16ac downregulation upon autophagy induction, suggesting a coordinative work between two histone marks in the control of autophagy, and therefore preventing from cell death (Fullgrabe et al., 2013). These findings described one of an increased number of histones marks that are linked to the regulation of autophagy.

Additionally, G9A/EHMT2, leaves the promoter of the core *ATG* genes, which promotes a decrease of the repressive demethylated Histone 3 Lysine 9 (H3K9me2) histone mark and of H3K9 acetylation resulting in an increase of *ATG* genes expression upon short autophagy stimuli (Artal-Martinez de Narvajás et al., 2013). Other studies revealed an mTOR-mediated positive regulation of H3K56ac, which is downregulated upon rapamycin treatment, thus mTOR inhibition promotes the expression of *ATG* genes. In the same lines, the enhancer of Zeste 2 polycomb repressive complex 2 subunit (EZH2) is histone methyltransferase for H3K27me3 that represses the expression of mTOR-related genes (Wei et al., 2015).

Interestingly, some histone modifying enzymes have a dual role on the regulation of histone modifications but also regulation of autophagy via interaction with other components. However, whether those two roles are interconnected still requires further investigation. For instance, downregulation of the histone deacetylases 1 (HDAC1) due to mutations or treatment with HDAC inhibitors are found to induce autophagy (Oh et al., 2008). Under nutrient deplete conditions the Glycogen Synthase Kinase-3 (GSK3) it is activated, thus enhances the activity of the histone acetyltransferase TIP60 through phosphorylation, which results on the acetylation of ULK1, required for autophagy initiation (Lin et al., 2012).

Taken together, these studies suggest an orchestrated mechanism to keep basal levels of autophagy, mediated by histone modifying enzymes, histones modifications and transcription factors. During autophagy induction, autophagy-regulated cytoplasmic proteins are the first step of action, followed by a quick induction of transcription factors that allows a sustain autophagy. However, uncontrolled autophagy induction could lead to cell death if it is

prolonged over time. Hence, in order to avoid autophagy-mediated cell death a decrease of active histone marks regulate ATG genes is found after autophagy induction.

Table 1. Histone modifications involved in short-term regulation of autophagy

Histone Mark	Regulator	Impact on Autophagy	References
H3K4me3 ↓	WNT/β-catenin pathway	Inhibition by WNT degradation	(Wang et al., 2017)
H3K9me2 ↓	G9A/EHMT2	Inhibition	(Artal-Martinez de Narvajas et al., 2013)
H3K9ac ↑	SIRT6	Activation	(Artal-Martinez de Narvajas et al., 2013; Jin et al., 2011)
H3K27me3 ↑	EZH2	Inhibition	(Wei et al., 2015)
H4K20me3 ↑	SETD8/SUV420	Inhibition	(Kourmouli et al., 2004)
H3K56ac ↓	EP300 and KAT2A	Inhibition	(Chen et al., 2012)
H4K16ac ↓	KAT8/hMOF	Inhibition	(Fullgrabe et al., 2013)
H3K14ac ↑	HDAC9	Activation	(Zhang et al., 2019)
H3K18ac ↓	P300/HDAC9	Activation	(Zhang et al., 2019)
H3R17me2 ↑	CARM1	Activation	(Shin et al., 2016)
H2BK120 ↓	USP44	Activation	(Chen et al., 2016)

* ↓, downregulation upon autophagy induction; ↑, upregulation upon autophagy induction

1.5 CELULAR MODELS

Different cellular models are commonly used by scientists in the field to reveal the role of autophagy in physiological and pathological contexts, as well as epigenetics. In fact, cell culture is normally the first experimental set up, moving next to animal models or primary cell types to further validate the findings obtained in cell lines. Here, it is stated the cellular models that are of interest for this thesis.

1.5.1 Mouse cell lines

Mouse cell lines are established from mouse tissue from different organs, such as the liver, neurons and fibroblasts. The fact of using one cell line or another depends on the downstream application such as protein assessment of retroviral vector production or feeders. In particular, Mouse Embryonic Fibroblasts (MEF) are widely used in the autophagy field as represent a powerful tool to test gene function of ATG genes on different cellular pathways.

For instance, innumerable studies using MEF Knockout cells for *Atg* genes such as *Atg7*^{-/-}, *Atg5*^{-/-}, *Ulk1*^{-/-}, *FIP200*^{-/-} or for the gene of interest for the different studies, thus allowing the comparison of the indicated effect between deficient cells and Wild-type MEF cells. For instance, ATG7 is essential for autophagy induction, autophagosome biogenesis and therefore the formation of a functional autophagosome, hence genetic depletion lead to the incapacity to undergo autophagy (Komatsu et al., 2005; Tanida et al., 2001).

Studies in humans always raise a special interest since the research performed has the goal of understanding the human body and thus, how to overcome specific diseases. Therefore, in most of the studies the use of human cell lines, primary cells or even patient cells from a biopsy or blood are an important model to the studies in every field, of course among those the field of autophagy and transcriptomic. As occurs in mice, the use of specific cell line depending on the downstream procedure or hypothesis it is to choose a proper cell line. For instance, Human Embryonic Kidney 293 (HEK-273) cells are used widely use to knockout a gene of interest as the cells respond very well to treatment. However, they might be suitable to validate findings or a signaling pathway but HEK cells lack of the desired phenotype, which offers a limitation of this model leading to the use of other cell lines such as SH-SY5Y cell lines (derived from neuroblastoma cells), if the study will be performed in neurons (Xiong et al., 2011). In other fields like cancer, cancer cell lines have been broadly used to study how dysregulation of autophagy, mutation of a transcription factor or histone modifying enzymes or disruption of the splicing machinery that are commonly downregulated in different types of cancer and contributes to tumor development (Baylin and Herman, 2000; Ellis et al., 2009; Robertson, 2001; Wible et al., 2019).

Renal cell carcinoma

Renal cell carcinoma (RCC) cell lines are of interest to this thesis, which study on their cell biology allowing the finding of treatments that are applied later to the clinic (Hsieh et al., 2017b). RCC are primary tumors originating in the tubules. Considered a heterogeneous group of tumors, they can be classified depending on their histology into different subtypes as chromophobe RCC, papillary RCC and clear cell renal cell carcinomas (ccRCC). Among these tumors, the latter is the most predominant subtype. One of the main features of this type of tumor is the inactivation of the tumor suppressor gene, von Hippel-Lindau (VHL) is present in most of the tumors. VHL is a E3 ubiquitin ligase that targets both HIF1 α and HIF2 α transcription factors for its degradation under normoxia. However, in hypoxia conditions,

lack of VHL promotes the activity of these transcription factors, and the activation of targeted genes that have an impact in a wide number of cellular pathways involved in angiogenesis, glycolysis, and apoptosis (Dalglish et al., 2010; Network, 2013; Sato et al., 2013). Recent high-throughput sequencing studies brought new genes into, beyond the *VHL* mutation, that are frequently mutated and contribute to the development of the disease (Maxwell et al., 1999). In fact, genes located also on chromosome 3p have been found to be downregulated including the components of the SWI/SNF chromatin remodeling complex (*PBRM1*) and the *SETD2* (Hsieh et al., 2017a; Sato et al., 2013). The latter, has the highest frequency in ccRCC subtypes as it is observed in approximately 10% of ccRCC primary tumors, increasing to 30% in metastatic ccRCC patients, which is associated with SETD2 inactivation. These observations suggest a role of SETD2 as a tumor suppressor gene, highly involved in driving tumor development, progression and metastasis on ccRCC tumors (Fahey and Davis, 2017). There are different types of cells that are established for all the different subtypes of RCC, papillary, clear cell or chromophobe. Although the scientific community refer to all of the subtypes as Renal Cell Carcinoma, it is important to characterize the cells regarding their genetic profile and markers that differs between the subtypes. For instance, 769-P and CAKI-1 cell lines are both considered as the clear cell type but have different genetic profiles as CAKI-1 harbors a *SETD2* mutation whereas is wild-type for *VHL*. On the other hand, 769-P has the opposite genetic profile being *SETD2* wild-type, but defective for *VHL*, which suggests that an essential characterization of RCC cell lines, such as their genetic background is essential for proper understanding of the underlying treatments as well as novel discoveries of new drugs (Brodaczewska et al., 2016).

1.6 ANIMAL MODELS

A wide number of animal models are used to study epigenetics and autophagy. However, *Saccharomyces cerevisiae* and *Dario Rerio* are of interest for this thesis.

1.6.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, also known as yeast, is a well-studied model system employed to study conserved molecular mechanisms involved in a wide range of cellular pathways in multiple fields due to the high degree of conservation with higher eukaryotes as well as cellular pathways. In the 1980s, yeast research on the field of autophagy became fundamental, enhancing the discovery and characterization of the pathway as well as the molecular roles of *ATG* genes. An interesting fact of this unicellular eukaryotic organism is its rapid response to stimuli upon short exposure to stimuli and adaptation to different

environments. Moreover, *S. cerevisiae* is a model system that offers the capability to do *in vivo* genetics in a flexible and quick manner for the study of cellular pathways compared to other model systems. Another advantage and useful approach is the efficient homologous recombination system, which allows the alteration of any selected chromosome sequence. Sections of the chromosomes can be manipulated by and reintroduced in plasmids that can be maintained through cell division. Taken together, these techniques make this model essential to many research fields, such as cancer and neurodegenerative diseases. Specific for the autophagy field, yeast offers specific autophagy assays to monitor different steps of the pathway such as the Pho8Δ60 assay, which relies on the measurement of alkaline phosphatase activity. This assay is a quantitative approach to monitoring autophagy and autophagic flux based on the activity of the enzyme from vacuolar delivery of zymogen (Klionsky, 2007; Klionsky et al., 2012; Noda and Klionsky, 2008)

1.6.2 Danio Rerio

Danio Rerio, commonly known as Zebrafish, have many characteristics that made of them a good animal model to study autophagy and epigenetics (Cavalieri, 2020; Klionsky et al., 2016). Zebrafish are translucent to microscopic observation, which allows to monitor live imaging of cellular changes, especially during development, as well monitoring autophagosomes formation *in vivo* using transgenic GFP-Lc3 fish. Besides of its characteristic of being a translucent animal, it is also an easy model to knockdown a gene function of *atg* genes (Hu et al., 2011; Kyöstiä et al., 2015). Additionally, zebrafish are relatively permeable to water-soluble molecules, thus it makes an ideal animal model to monitor pharmacological drugs and for drug discovery *in vivo*. Interestingly, due to zebrafish genome has a 70% of homology with the human as well as almost the 99% of the genes involved during development (Howe et al., 2013). Moreover, epigenetics and transcriptional mechanism are also conserved from in higher organisms, thus makes of this animal model excellent to explore changes on the epigenetic state such as DNA methylation or histone modifications, on normal physiological events as well as under exposure to environmental stressors (Ali et al., 2014; Rai et al., 2008).

2 AIMS OF THE THESIS

This thesis aims to increase the knowledge on how the nuclear and cytosolic mechanisms regulates the outcome of autophagy in two lines of research: first, gaining more insight on the transcriptional mechanism that regulates short-term response to autophagy, and second extend further this research on the mechanism behind the long-term regulation of this process.

The specific aim for each particular study are:

- **Paper I.** Previous studies investigated the potential involvement of histones modifications on the regulation of autophagy. In **paper I**, our focus is to determine the role of the histone demethyltransferase for H3K36me3, Rph1/KDM4A, as a potential transcriptional regulator of autophagy in yeast and mammals.
- **Paper II.** As previously reported in **paper I**, the role of Rph1/KDM4A as a negative regulator of autophagy. In **paper II**, we aimed to uncover the role of SETD2, the histone methyltransferase with an opposite function of Rph1/KDM4A on the regulation of H3K36me3, as a regulator of the autophagy pathway.
- **Paper III.** Renal Cell Carcinoma (RCC) has a high frequency of SETD2-inactivating mutations. In **Paper III**, our goal was to determine how the loss of SETD2 observed in a RCC subtype, clear cell renal cell carcinoma, might impact the autophagy machinery and therefore bring light on the possible therapeutic interventions when targeting this process in this type of cancers.
- **Paper IV.** The identification of histone modifications and histone modifying enzymes are associated with short-term transcriptional regulation. In **paper IV**, we aim to decipher the impact of a brief autophagy stimuli can promote a epigenetic mechanism responsible for a long-term regulation of autophagy.
- **Paper V.** Here, we aim to identify the upstream signaling pathway that could mediate the regulation of DNMT3A upon short autophagy induction that contribute to MAP1LC3 genes downregulation and thus responsible for the long-lasting responses to autophagy, described in **paper IV**.

3 RESULTS AND DISCUSSION

3.1 PAPER I. Rph1/KDM4A mediates nutrient-limitating signaling that leads to the transcriptional induction of autophagy

The expression of most ATG genes and their consequent proteins are dramatically increased under stress conditions such as starvation (Jin et al., 2014). Recent studies in yeast and mammals show the important role of transcription factors as well as histone modifying enzymes and histones modifications on the short-term regulation of autophagy (Fullgrabe et al., 2013). However, very few transcription factors that contribute to the induction of specific ATG genes have been identified. Here we identify the involvement of Rph1/KDM4 as transcriptional regulator of autophagy (**Figure 16**).

3.1.1 Rph1 acts as transcriptional repressor on the expression of autophagy related genes

Firstly, with the aim of identifying new transcriptional regulators of autophagy we screened over 150 yeast mutant strains. Furthermore, the analysis of the expression of a set of *ATG* genes in the library created previously lead to the identification of Rph1, as a candidate to be a transcriptional repressor of autophagy. Rph1 is the only known histone demethylase enzyme for H3K36me3 in *Saccharomyces cerevisiae* (Tu et al., 2007), and has a paralog protein, Gis1, that shares high similarity in the JmjC-domain in the N-terminus but only has a modest histone demethylase activity due to a mutation in the cofactor binding site (Klose et al., 2006). With the purpose of analyzing the impact of Rph1 as a transcriptional regulator of autophagy we analyzed the expression level of a set of *ATG* genes in *rph1Δ* strains compared to wild-type strains. Our results show that the *ATG* selected genes were found to be up-regulated in *rph1Δ* in growing conditions. The paralog *Gis1Δ* also increased, albeit to lower extend, the expression of *ATG* genes, and the double mutant *Rph1Δ gis1Δ* results to enhance even more the expression of those genes compared to *RPH1* deletion. Additionally, in line with the changes found at mRNA level, the *rph1Δ* mutant also promoted an increase at protein level of the selected genes compared to wild-type cells.

3.1.2 Rph1 acts as a negative regulator of autophagy and this is not dependent on its histone demethylase activity

The previous results rendering Rph1 as repressor of *ATG* genes suggest it is a negative regulator of autophagy. For this propose, we made used of the Pho8 Δ 60 assay and the GFP-ATG8 processing assay to measure autophagic flux. Our results showed in opposition to the wild-type cells that under nitrogen starvation at different timepoints, autophagic flux was highly increased in *rph1 Δ* and *rph1 Δ gis1 Δ* cells, whereas the short-term induction was attenuated after a prolonged time of starvation. This data unravels that lack of *RPH1* contributes to an immediate increase of transcription of *ATG* genes and response to nitrogen starvation with the aim ensure a proper autophagy induction, while prolong stimulation abolishes the differences found in shorter timepoints, which may be regulated by other mechanism. Moreover, we aimed to elucidate whether the JmjC-containing histone demethylase domain or the DNA-binding domain were responsible for its activity to regulate autophagy. Rph1^{H235A} mutant for the histone demethylase domain exhibit no differences compared to wild-type, whereas overexpression of Rph1 and the Rph1^{H235A} mutant lead to a reduction of autophagy activity compared to wild-type cells. On the other hand, Rph1 mutant that prevents DNA-binding (Rph1 ^{Δ z}) exhibit an increase of autophagy activity as *rph1 Δ* yeast strains. Taken together these data suggest that the effects found are independent of the histone demethylase activity and is not required for its function on the regulation of autophagy, rather the DNA-binding ability, so may contribute to the recruitment of other transcription factors that could mediated the epigenetic regulation on the expression of *ATG* genes.

3.1.3 Autophagy induction promotes Rim15-mediated phosphorylation of Rph1 required for proper initiation of autophagy

As previously uncovered, depletion of *RPH1* during nutrient rich conditions induced the expression of *ATG* genes, but no effects were found after nitrogen starvation. Recent studies, reveal a similar mechanism describing the impact of oxidative stress or DNA damage as a trigger of Rph1 phosphorylation and dissociation from the promoter of target genes allowing gene expression to cope with cellular stress (Liang et al., 2013). Additionally, we found that Rph1 phosphorylation is essential for autophagy induction. Therefore, we aimed next to identify the phosphatase or kinase responsible for this effect. Rim15, showed to be a good candidate as it has been linked previously to the regulation of transcription factors upon nitrogen starvation by mediating Ume6 phosphorylation and inhibition and therefore upregulation of Atg8 (Bartholomew et al., 2012). In fact, *RIM15* deletion partially blocked the expression of the *ATG* genes and ATG proteins previously analyzed accompanied by a

reduction of the autophagy activity due to the lack of regulation of Rph1 in those conditions. These findings strongly depict Rim15-mediated Rph1 phosphorylation as an upstream regulation and Rph1 role on the regulation on *ATG* genes such as *ATG7* expression, the most upregulated gene on *rph1Δ* yeast strains upon autophagy induction.

3.1.4 The function uncovered by Rph1 in yeast is evolutionary conserved by its homolog KDM4A in the mammalian system

To further test whether the previous mechanism was conserved in higher organisms, we studied the role of the Rph1 homolog in mammalian cells, KDM4A. The analysis of several *ATG* genes involved in different steps, such as *ATG7* and *ATG14* suggest an increase on the expression of those genes in HeLa cells with a reduced expression of KDM4A, whereas *MAP1LC3B* had no major effect. Furthermore, the loss of KDM4A suggested an increase in baseline autophagy and enhance autophagic flux compared to the control cells, while overexpression of KDM4A act as a repressor of autophagy. Finally, similar to Rph1 findings, we found that KDM4A increased phosphorylation might act as a button that allows autophagy induction.

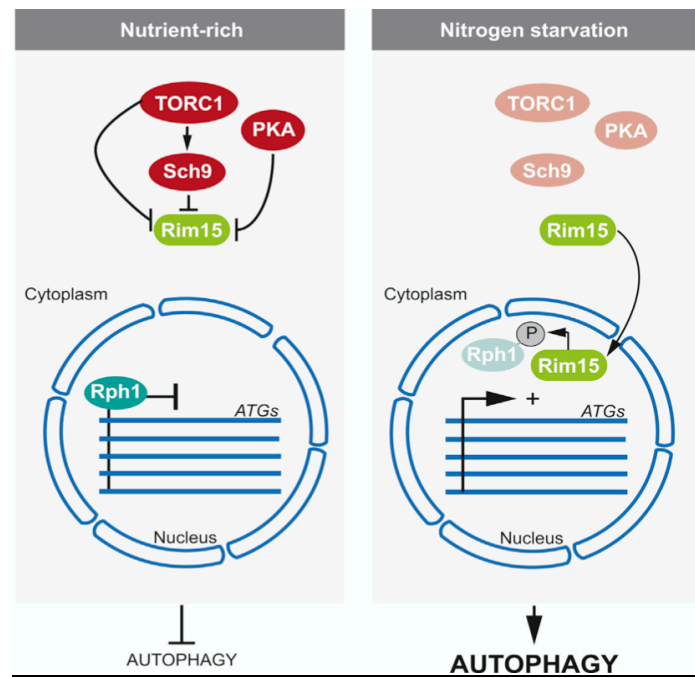


Figure 16. Role of Rph1 on the regulation of autophagy. Rph1 is a negative transcriptional repressor of *ATG* genes in nutrient rich conditions, while under nitrogen starvation Rim15 inhibited by phosphorylation promotes *ATG* genes expression.

3.2 PAPER II. Set2/SETD2 regulates the expression of ATG14 splice forms and impacts on autophagosome-lysosome fusion

Here, we extended our investigation on the role of the sole histone methyltransferase for Histone 3 Lysine 36 trimethylation, Set2/SETD2, on the regulation of autophagy. We wondered whether Set2/SETD2 would also impact autophagy as we found for Rph1/KDM4A in **Paper I**.

3.2.1 Set2/SETD2 positively regulates autophagy in yeast and mammals

In order to understand the impact of Set2 on autophagy, we firstly analyzed in *set2Δ* yeast strain the autophagy levels in comparison to wild-type cells. Our analysis showed a decrease on Atg8 lipidation in cells that lack of Set2 expression only under nitrogen starvation whereas not big changes were found at baseline conditions. Additionally, we wondered whether the decrease found on autophagy was linked to a regulation on the transcription of *ATG* genes regulating this pathway. For this propose, we analyzed the mRNA levels of several *ATG* genes and found a global decrease in the expression of most of the *ATG* genes analyzed under starvation conditions, with the exception of *ATG1* that remained unchanged.

Furthermore, we wanted to validate whether our findings in yeast were evolutionary conserved in higher eukaryotes. For this propose we either decreased or increased the expression of the mammalian SETD2 in HeLa Cells. Our data showed that SETD2 downregulation promotes a decrease in LC3-II levels, whereas its overexpression lead to a higher increase compared to their respective control cells at baseline levels. These data suggest that SETD2 positively enhance autophagy, which is further validated with Bafilomycin A1 treatment, a late inhibitor of autophagy. Interestingly, SETD2 OE followed by Bafilomycin A1 did not show a further increase in autophagy suggesting that this process might be tightly regulated with the aim of avoiding an oversaturation of the system that could lead to cell death. As shown in yeast, we also aimed to analyze a set of *ATG* genes in cells that lack SETD2 expression. Results shown a significant downregulation of several genes such as *MAP1LC3B*, *ATG7* and *ATG14*. Among these, *ATG14* appeared to be the most regulated gene by SETD2, results that were further validated by overexpressing SETD2. As a whole, these results highlight several observations. We can conclude that Set2, as in the mammalian system, is a positive transcriptional regulator of autophagy. Although, this mechanism is evolutionary conserved from yeast to mammals, there are minor differences between both systems. For instance, in the yeast system decrease of Set2 expression results in a global downregulation of *ATG* genes, while in the mammalian system this regulation

seems to be more specific for certain genes, in particular for *ATG14*. These findings might bring the possibility that in lower eukaryotes requires a wider transcriptional regulation of *ATG* genes occurs by Set2, whereas in the mammalian system SETD2 might be more targeted on the regulation of specific *ATG* genes in order to display a similar or more specific effect. However, in both systems Set2/SETD2-mediated gene transcriptional regulation of *ATG* genes enhance autophagy.

3.2.2 In mammals, SETD2 is a transcriptional regulator of ATG14 isoforms

Next, our aim was to elucidate the impact of ATG14 in both systems. In *set2Δ* yeast strains, its deficiency decreased Atg14 levels in both at protein and mRNA levels under starvation conditions. Interestingly analysis on mammalian cells revealed two ATG14 bands at protein expression in SETD2 deficient cells, which might suggest that SETD2 mediates the expression of ATG14 isoforms. In fact, research for ATG14 on Uniprot database provided the existence of two distinct isoforms (**Figure 17**).

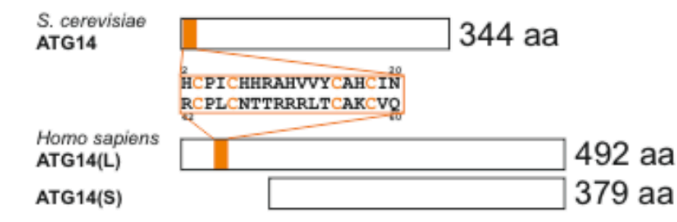


Figure 17. ATG14 sequence in yeast and ATG14 isoforms in the mammalian system. Similarity of ATG14 amino acidic sequence between *S. cerevisiae* and *Homo Sapiens*, as well as the differences on the different ATG14 variants. In orange is displayed the conserved cysteine-rich domain responsible for homo-oligomerization that allows autophagosome-lysosome fusion. The canonical protein, corresponds to the long isoform, whereas the short isoform, lack the cysteine-rich domain and the ability to homo-oligomerize.

These findings, were further validated by the analysis of both isoforms by RT-qPCR, suggesting in the mammalian system that SETD2 transcriptionally regulates the differential expression of ATG14 isoforms.

3.2.3 Set2/SETD2 decreases autophagic flux and inhibit autophagosome-lysosome fusion

Based on the fact that Set2 positively regulates autophagy in yeast but also in mammals by specifically regulating ATG14 isoforms, we wanted to determine whether this might impact autophagy activity with the assays previously used in **paper I**. Taken together those results indicate that lack of Set2 results in a decrease of autophagy activity after short-term induction. On the opposite, Set2 overexpression appeared to show no differences, suggesting that overexpression of Set2 might be subjected to a more restrictive regulation that could

result to be deleterious for the cell. In fact, previous studies report that SETD2 upregulation correlates with an increase in apoptotic-related genes expression and contributes to cell death (Xie et al., 2008). Thus, the previous report and our findings suggest that the amplitude of the signal by Set2/SETD2 overexpression might be tightly regulated to ensure a quick and efficient induction of autophagy in a controlled manner that avoided the saturation of the system and allowed the cell to return to its constitutive basal level of autophagy. Similar analysis to monitor autophagic flux with the mRFP-GFP-LC3 reporter assay in mammalian cells revealed that SETD2 deficiency is associated with a decrease of LC3 punctae, while autophagy induction for 24 hours resulted in a decrease on the number of autolysosome but an accumulation of autophagosomes. This data brings light on the specific step of the pathway that it is affected by SETD2 modulation, which is the fusion of the autophagosome and lysosome, thus leading to an accumulation of autophagosomes that are not degraded (**Figure 18**). Additionally, analysis of p62 degradation with the purpose to monitor autophagic flux showed an accumulation of p62 in cells that lacked SETD2 expression, validating upon autophagy induction, which corroborates that Set2/SETD2 deficiency decreases autophagic flux. In the mammalian system not only decreases autophagic flux but also SETD2 deficiency impaired autophagosome-lysosome fusion. This effect suggests that the lack of ATG14 long isoform in these conditions might contribute to autophagy inhibition by preventing the formation of the STX17-SNAP29 complex with VAMP8, thus lead to an unsuccessful completion of the pathway by abrogating autophagosome-lysosome fusion (**Figure 18**).

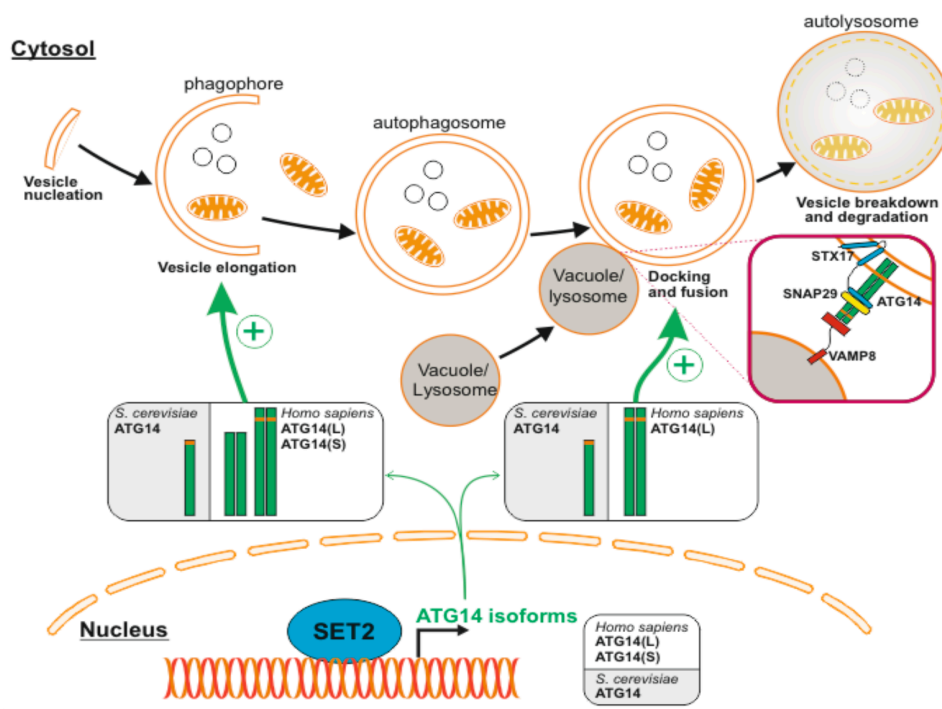


Figure 18. Summary of the mechanism uncover by Set2/SETD2 regulation on the differential expression of ATG14 isoforms and its impact on the autophagosome-lysosome fusion.

3.3 PAPER III. SETD2 mutation in renal clear cell carcinoma suppresses autophagy via regulation of ATG12

As reported in **paper II** the role of SETD2 on the regulation of autophagy, here we report whether the deficiency of SETD2 in the context of ccRCC and how might impact the autophagic core machinery.

3.3.1 SETD2 mutation in clear cell renal cell carcinoma cell lines promotes a reduced autophagic flux

Firstly, we aim to determine the impact of SETD2 loss of function in ccRCC on autophagy we monitored the occurrence of autophagy, by an increase lipidation of LC3, in a set of RCC cells that were either *SETD2*-competent or *SETD2*-deficient. Of note, these RCC cell lines were also selected taking into consideration whether they are wild-type for *VHL* or not, which could impact on the interpretation of the unique effects caused by SETD2 deficiency. Analysis of LC3 expression in RCC cells with SETD2-inactivating mutations exhibited a decrease in autophagy level when comparing with RCC competent cells, whereas mRNA expression levels of the *LC3B* gene displayed a small increase in SETD2 deficient cells suggesting that the decrease in LC3 at protein levels is not a due to a transcriptional regulation in these cells. Furthermore, we aimed to acquired more knowledge on the impact of SETD2 deficiency on autophagy.

For this propose, we analyzed autophagic flux, as we performed in **paper II**. In addition to mRFP-GFP-LC3 and p62 accumulation treatment we analyzed p62/LC3 co-localization to monitor occurrence of autophagosome formation in ACHN and CAKI-1 cells. Altogether, these results indicated a reduced autophagic flux in RCC cells that lack SETD2, as compared to SETD2-competent RCC cells.

3.3.2 SETD2 deficient RCC cells accumulate free-ATG12 and ATG12 associated complexes

Next, we aimed to get further explanation on how the decrease in autophagic flux by SETD2 deficiency might be due to impairment of the autophagy machinery that are involved in LC3 lipidation in ccRCC cells. For this propose, we analyzed the protein levels of the two ubiquitin-like protein conjugation systems, which catalyze ATG12-ATG5 and forms a multimeric complex with ATG16L1 that allows the addition of the phosphatidylethanolamine group to LC3B. The other ubiquitin-like conjugation system involves the activity of ATG7, which also facilitates the previous reaction. Analysis of ATG7 protein expression showed no major differences, whereas striking differences were found

when analyzing ATG12 protein level in SETD2-deficient *versus* SETD2-competent RCC cells. In fact, we found that SETD2-inactivating mutations lead to a global increase of ATG12 protein expression levels but also an increase of free-ATG12 and the accumulation of an additional ATG12-associated complexes, which might explain the observed decrease in LC3 lipidation, autophagosome formation and reduced autophagic flux, that promotes cell migration.

3.3.3 Loss-of-function of SETD2 promotes a differential expression of ATG12 isoforms and impacts on their migration capability

As found in **paper II**, where we uncover SETD2-mediated alternative splicing regulation of ATG14, we wondered whether this regulation could occur on RCC SETD2-deficient cells and control the expression of different *ATG12* variants by RT-qPCR. In fact, it has been reported the existence of two ATG12 isoforms that are generated by alternative splicing. Therefore, we sought to uncover how SETD2 might play a role in the expression of ATG12 isoforms in SETD2-competent and SETD2-deficient RCC cells. Our results indicate that SETD2 loss-of-function is correlated with an increase of *ATG12* short isoform compared to the long isoform, which might contribute to the abnormal formation of additional ATG12-containing complexes.

3.3.4 SETD2 mutation in patients is accompanied by a decrease in H3K36me3 expression and it is associated with increased ATG12 expression and worse prognosis

Furthermore, we extended our efforts to investigate the correlation between the increased ATG12 gene expression levels with SETD2 expression and thus if it could be used as a survival prognostic marker on patients that suffer from ccRCC carcinomas. To further test this hypothesis, we analyzed the expression levels of H3K36me3 and ATG12 expression in ccRCC tumor biopsies, which exhibited that SETD2-deficient tumor correlates with an increase in ATG12 expression in these patients. Thereafter, we analyzed gene expression data sets available at the Human Protein Atlas to test if SETD2 and ATG12 expression levels could be linked with survival of these patients. Analysis performed was based of the fragment per kilobase of transcript per Million mapped reads (FPKM) for both genes in ccRCC patients and the correlation between expression levels and patient survival was assessed. Analysis of this data supports that low expression of SETD2 is associated with high expression level of ATG12 and should be considered as an unfavorable prognostic factor for ccRCC patients.

3.4 PAPER IV. The DNA methyltransferase DNMT3A contributes to long-term memory of autophagy

Histones modifications or histone modifying enzymes, which are considered to promote reversible changes on the expression of specific genes offers an epigenetic explanation for short-term regulation of autophagy (Described in **paper I** and **paper II**). Here, in **Paper IV**, we aimed to investigate long-term responses to autophagy that could be mediated by long-lasting epigenetic effects such as DNA methylation.

3.4.1 Brief autophagy induction is associated with a persistent downregulation of MAP1LC3 at protein and transcriptional level

To investigate this possibility, we generated a panel of cells that were previously exposed to an autophagic stimulus with either mTOR-dependent (starvation or treatment with Torin1) or mTOR-independent (carbamazepine, Trehalose or Clonidine) inducers for 4 hours and then left to recover under normal cell culture conditions from 1 to 4 weeks. Thereafter, baseline autophagy by monitoring LC3 expression was analyzed in these cells previously exposed to autophagy as well as their parental counterparts (or control cells) (**Figure 19**).

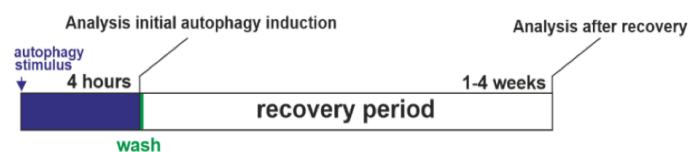


Figure 19. Scheme of the *in vitro* model used in the study.

Analysis of the latter, revealed that these cells previously exposed to an autophagy stimulus showed a decrease at baseline level of LC3 after the recovery time. This data was further confirmed by Bafilomycin A1 treatment, which confirms a total and persistent reduction of LC3 protein level. Subsequently, we wanted to elucidate if this reduction is linked to a transcriptional downregulation of *MAP1LC3* isoforms. For this purpose, using the same model displayed in **Figure 19**, we monitored *MAP1LC3* isoforms gene expression level after a recovery period in a panel of cell lines. We found that the *MAP1LC3B* mRNA expression was reduced in those cells previously exposed to starvation, Torin1, Trehalose or Clonidine compared to their respective controls, whereas *MAP1LC3B2* expression decreased on starvation, Torin1 and Trehalose. Moreover, we analyze the expression of 84 genes either directly involved in the autophagy machinery or the regulation of this process and found that *MAP1LC3A* and *MAP1LC3B* were the most downregulated genes after a recovery period. Collectively these data reveal that a brief and transient autophagy induction is coupled with

a long-lasting transcriptional downregulation of *MAP1LC3*, which corresponds to the decrease found in LC3 protein expression levels.

Interestingly, the found effect was found to be abrogated in cells that lack ATG7. In fact, we made use of Wild-type MEF as well as *Atg7*-deficient MEF cells in order to compare whether autophagy induction is required to the DNMT3A-mediated long-term signature. After a recovery period, we found that autophagy-deficient *Atg7*^{-/-} MEF cells shows no effect on LC3B either at protein (by monitoring LC3-I) and mRNA levels when compared to the autophagy-proficient wild-type cells.

3.4.2 DNMT3A is upregulated and recruited to MAP1LC3 promoters and leads to DNA methylation upon autophagy induction

Furthermore, we ought to develop our investigations on the understanding on the mechanism that explains the persistent transcriptional repression of *MAP1LC3* isoforms after recovery in those cells previously exposed to an autophagy stimulus. Firstly, we performed a re-analysis of previous data obtained from Global Run-On sequencing (GRO-seq) of cells treated by rapamycin for a short time and looked to the gene expression of DNMT3A under these conditions. Deep analysis of GRO-seq data supported with protein expression analysis, revealed an increase in DNMT3A under autophagy induction. With the purpose to demonstrate whether DNMT3A is behind the effect observed, we analyzed DNMT3A recruitment on the potential CpG sites on the promoter regions of the *MAP1LC3* isoforms upon short autophagy induction and therefore, analyzed DNA methylation in these regions after a recovery period. Our results revealed an increase of DNMT3A recruitment on *MAP1LC3* promoters upon short autophagy induction associated with an increase of DNA Methylation at these CpG sites after a 4-week period of time. These results, support that DNMT3A mediates DNA methylation of *MAP1LC3* loci upon a short autophagy stimulus that could explain an epigenetic and heritable mechanism for the long-term and persistent downregulation of *MAP1LC3*.

Intriguingly, WT MEF cells also lead to DNMT3A upregulation upon autophagy induction, whereas the lack ATG7 or ATG5 (either *atg7*^{-/-} or *atg5*^{-/-} MEF cells or HeLa cells transfected with siRNA against *ATG5* or *ATG7*) exhibit a dramatic decrease on DNMT3A expression at both protein and mRNA level, which abrogate the ability in these cells to acquire the described autophagy long-term epigenetic memory.

3.4.3 Long-lasting decrease of MAP1LC3 levels impacts the cells response to a second autophagy stimulus as well as sensitizing these cells to an apoptotic stimulus

Given the fact that LC3 downregulation has a major impact on the autophagy pathway, we further checked the impact of a second autophagy stimulus on the previous autophagy exposed cells compared to those that have not been exposed and therefore exposed for the first time to an autophagy stimulus. Our data showed that cells previously exposed to autophagy inducers were able to induce autophagy, but showed a reduced LC3 protein level compared to the parental cells. We further extended our investigation to check whether a second stimulus has an impact on the transcriptional expression of *MAP1LC3B* and *MAP1LC3B2* isoforms, previously found to be a target of DNMT3A upon the first autophagy stimulus. Our findings reveal a decrease on the expression of both isoforms upon the second stimulus in the “previously autophagy-exposed” cells when compared to the untreated ones after a recovery period. These findings suggest that DNMT3A-mediated methylation of *MAP1LC3* isoforms does not completely suppress the expression of these genes but rather decreases their expression, allowing the cells to respond to a second autophagy stimulus although with less amplitude in comparison to the non-stimulated ones.

Moreover, due to the well-known interplay between autophagy and apoptotic pathways, we speculated whether those cells previously exposed to an autophagy stimulus differed when were stimulated after a recovery period with an apoptotic stimulus. In order to address the impact of a short autophagy stimulus on the way the cells respond to a stimulus that triggers apoptosis, cells previously exposed to autophagy were treated with Staurosporine (STS) as well as the untreated counterparts after a recovery period. Interestingly, these cells that were exposed initially to an autophagy stimulus display an increase of Caspase-3 processing and cleaved-PARP, as well as a quicker response to STS treatment after recovery, as compared to control cells. In overall, those results suggest that those cells that were previously challenged to an autophagy stress and left to recover show an increased sensitization to an apoptotic stimulus but also seems to undergo cell death faster.

3.4.4 Short autophagy induction leads to a persistent *MAP1LC3* downregulation *in vivo*

Next, we aimed to demonstrate, our previous findings in cell lines, using an animal model, such as *Danio Rerio* (Zebrafish). Therefore, larvae were exposed to clonidine or DMSO (as control) 4 hours and either collected to monitor autophagy induction or left to recover for 3 days. After recovery, Lc3-II protein expression of those zebrafish larvae previously exposed

to clonidine showed a decrease compared to the control ones, which correlated with a significant decrease in *map1lc3b* mRNA levels. Additionally, we analyzed available genome datasets that monitor the impact of the placenta withdrawn after birth. Analysis of those datasets revealed that among the analyzed genes involved in the autophagy pathway, a sustained downregulation of *Map1lc3b* was found right after birth. These results bring an additional demonstration of *Map1lc3b* downregulation over time after autophagy induction *in vivo* and validates our previous findings *in vitro*.

3.5 PAPER V. ULK3-dependent activation of GLI1 promotes DNMT3A expression upon autophagy induction

Here, in **paper V**, we identify the upstream signaling pathway involved in the regulation of *DNMT3A* gene expression upon the initial autophagy stimulus, which was found to be recruited to the promoters of MAP1LC3 isoforms and leads to a sustained downregulation after a recovery period.

3.5.1 Autophagy induction promotes GLI1 expression and recruitment to the DNMT3A promoter

Here, we aimed to uncover the transcription factor behind DNMT3A regulation upon short autophagy induction by recapitulating the transcription factors that have an impact on DNMT3A expression based on literature research. Among the tested candidates, GLI1 was the one that showed an increase in affinity and impact on DNMT3A expression upon autophagy induction. In fact, GLI1 increased at both protein and mRNA level when treated with autophagy inducers, either with mTOR-dependent (Torin1 or Rapamycin) or mTOR-independent (Carbamazepine, Trehalose or Clonidine). Moreover, this increase of GLI1 expression was associated with an increase in its phosphorylation status on serine residues, leading to its translocation into the nucleus and recruitment to the DNMT3A promoter after autophagy induction for 1 and 2 hours.

3.5.2 ULK3-mediated phosphorylation of GLI1 promotes its activation and translocation into the nucleus and impacts on the long-term regulation of MAP1LC3 expression upon autophagy induction

Thereafter, we wondered what signaling pathway was responsible for triggering GLI1 phosphorylation, activation and translocation into the nucleus after autophagy induction. Recent reports, linked ULK3-mediated activation and translocation of GLI1 but also ULK3 upregulation and role on autophagy initiation by serum starvation (Kasak et al., 2018; Maloverjan et al., 2010a; Maloverjan et al., 2010b). These observations bring light to build a

hypothesis if ULK3-mediates GLI1 activation by phosphorylation upon autophagy induction. In order to address and demonstrate such statement, we firstly performed proximity ligation assays (PLA) experiments to examine the interaction between ULK3 and GLI1, as well as whether the lack of ULK3 impacts on the phosphorylation of GLI1 upon autophagy induction with Torin1. In fact, a significant increase on ULK3/GLI1 interaction was found under autophagy induction. Moreover, repression of ULK3 expression by using a small hairpin RNA (shRNA) successfully repressed GLI1 phosphorylation of Serine residues under autophagy stress conditions compared to the cells infected with shRNA control, whereas autophagy induction lead to an increase of GLI1 phosphorylation and thus activation.

Finally, in order to prove that GLI1 might have an impact on the mechanism uncovered on **paper IV** by using the same experimental set up, we aimed to analyze whether GLI1 deficiency affects autophagy long-term memory. For this propose, we made use of *Gli1*^{-/-} deficient MEF cells and WT MEF cells. After one-week recovery, LC3 expression was monitored at protein and messenger level. Interestingly, WT MEF cells showed the expected reduction in LC3 levels, whereas *Gli1*^{-/-} deficient cells were unsuccessful on the acquisition of the mentioned phenotype.

4 CONCLUDING REMARKS

An increasing number of transcription factors and histone modifying enzymes revealed a complex network of components bringing the nucleus into the picture on the regulation of the autophagy process. This thesis highlights novel and additional evidences for the role of the nucleus on the regulation of autophagy by uncovering mechanisms involved in the short-term and long-term regulation of this process. These studies are based on the regulation of Autophagy-related genes by histone modifying enzymes as well as the differential expression of *ATG* genes splice isoforms. Additionally, this thesis uncovers a heritable epigenetic mechanism and the upstream signaling pathway responsible for long-lasting effects of autophagy.

The main findings of the present studies are summarized below:

- The histone demethyltransferase Rph1/KDM4A is a negative transcriptional regulator of *ATG* genes in nutrient-rich conditions. A mechanism conserved from yeast to mammals.
- Rph1/KDM4A histone demethylase activity is not crucial on the regulation of autophagy, while the DNA binding ability is strictly necessary. Depletion of *RPH1/KDM4A* enhance autophagy activity and increases autophagic flux in basal conditions. Whereas its overexpression promotes cell death and inhibit autophagy.
- Rim1-mediates Rph1 inhibition by phosphorylation under nitrogen starvation conditions and therefore autophagy induction.
- Set2/SETD2 promotes autophagy induction in yeast and mammals. Set2 positively regulates autophagy by induction of *ATG* genes in yeast. However, in the mammalian system lack of SETD2 regulates the differential expression of *ATG14* splice variants in mammals, which downregulates autophagy by inhibiting autophagosome-lysosome fusion.
- ccRCC progression and recurrence in patients is associated with a decrease in SETD2 expression and a reduced autophagic flux.

- *SETD2* loss-of-function mutation, but not *VHL* mutation, in ccRCC cell lines impacts on the expression of different *ATG12* splice variants that lead to the occurrence of additional ATG12-containing complexes and accumulation of free ATG12, which enhances cancer migration of ccRCC and it is associated with unfavorable prognosis in patients.

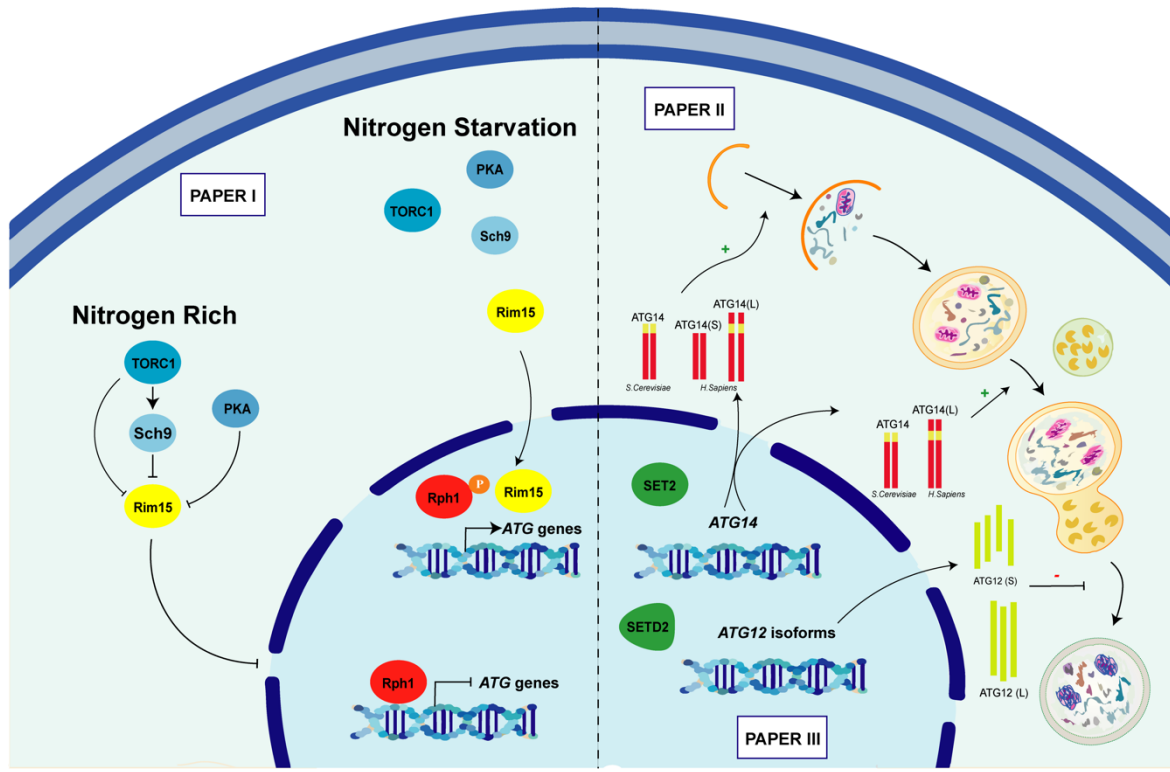


Figure 20. Schematic overview of Paper I, II and III.

- A short autophagy stimulus is associated with an upregulation of DNMT3A expression, recruitment and DNA methylation that leads a sustained decrease of *ATG* genes expression in these cells previously autophagy-exposed cells, after a recovery period *in vitro* and *in vivo*.
- Cells have acquired this “autophagy epigenetic memory”, exhibit the ability to respond to a second autophagy stimulus but to a lower extent due to the presence of a lower baseline as compared to control cells that has been exposed for the first time to autophagy, but are also sensitized to an apoptotic stimulus.
- ULK3-mediated GLI1 phosphorylation and transcriptional activation followed by subsequent DNMT3A upregulation upon short autophagy induction, which eventually leads to the observed effect described in paper IV.

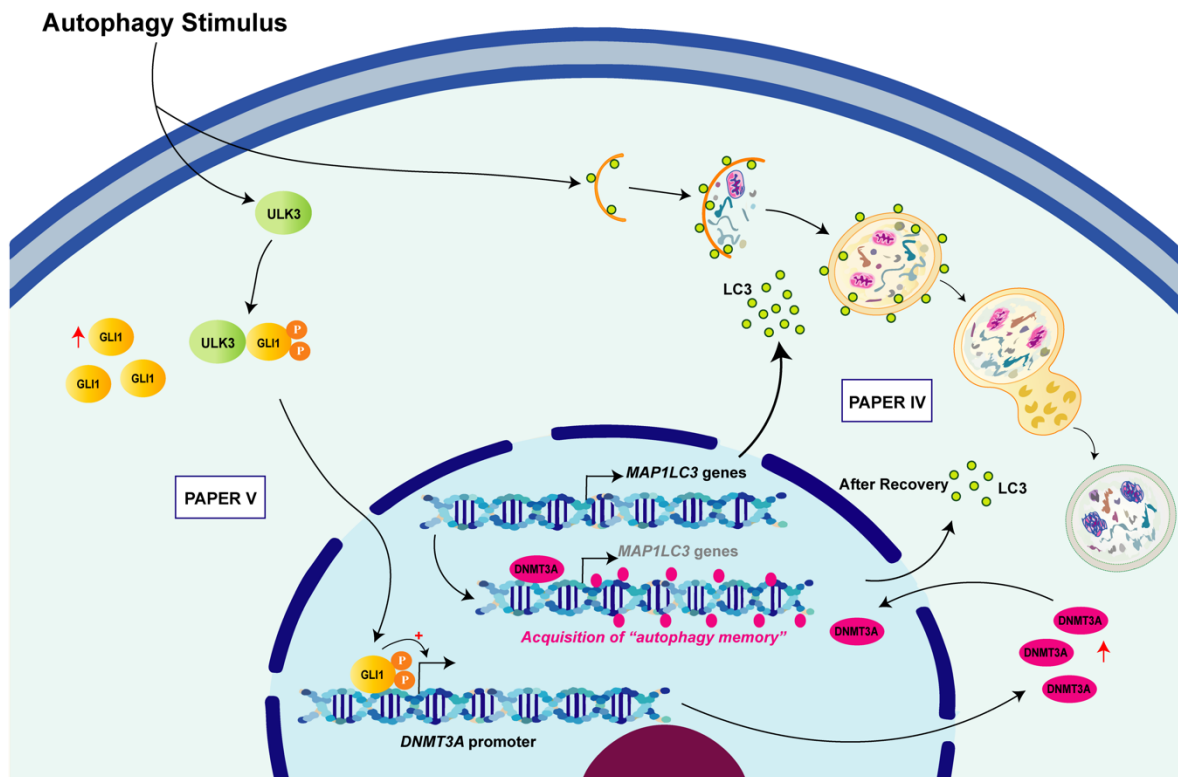


Figure 21. Schematic of the findings of Paper IV and paper V.

5 FUTURE PERSPECTIVES

During the past years, emerging studies have focused on the understanding and crosstalk between the nucleus and the cytoplasm in the regulation of autophagy. In fact, dysfunction of autophagy-relevant transcription factors and histone modifying enzymes are linked to the onset of a broad range of diseases (Di Malta et al., 2019; Levine and Kroemer, 2008). This field is evolving dramatically and the mechanisms already known might only represent small pieces of a large puzzle. However, we still have the need to find and place new pieces in this puzzle that will offer the complete picture.

Although this thesis offers new insights on novel epigenetic and transcriptional mechanisms and signaling pathways that control autophagy, it would be interesting to address several questions that our findings bring to the field.

For instance, when we uncovered the mechanism behind Rph1-mediated phosphorylation by Rim15, there is no clear evidence on the mechanism behind Rph1 degradation. In humans, the ubiquitin ligase complex SCF (FBXO22) targets KDM4A for proteosomal turnover (Tan et al., 2011). However, it would be of interest to analyze whether this complex also regulates KDM4A activity and degradation upon autophagy induction, or if not, which components are responsible. KDM4A, in the mammalian system has other homologs that also regulates H3K36me3 demethylation. Thus, analysis on the impact on autophagy of the other KDM4 isoforms might help in the understanding of the mechanism and interplay of KDM4 demethylases as therapeutic targets and may lead to the development of therapies to combat cancer and other diseases. In fact, ER- α positive breast tumors show an upregulation of KDM4A and KDM4B levels (Kawazu et al., 2011), that based on our findings would inhibit autophagy and eventually promote tumor progression. However, it might be also suitable to provide a co-treatment of a combination of KDM4 inhibitors with autophagy inducers such as Everolimus or Temsirolimus, among others, which might be more successful as a therapeutic treatment in these types of cancers.

According to new and interesting data published from Jacomin and colleagues, the homolog of KDM4A in *Drosophila*, Sequoia, interacts directly with Atg8a in the nucleus via its LIR-motif in nutrient rich conditions and prevents autophagy to occur (Jacomin et al., 2020). In line with this data, it would be valuable to further demonstrate the role of Atg8a on the nucleus and if it acts as a regulator of other histone modifying enzymes, such as Set2/SETD2.

Indeed, unraveling the mechanisms between histone modifications with alternative splicing machinery as well as DNA methylation has been a target of multiple studies (Luco et al., 2010; Rahhal and Seto). In fact, it has been reported that SETD2 is involved in the occurrence of both alternative splicing as well as DNA methylation. Thus, high-throughput screening to discover the impact of SETD2 on the generation of other alternative splice isoforms of *ATG* genes would be of interest on the context of autophagy. As well as whether autophagy induction regulates alternative splicing. In line with others and our findings on SETD2-mediated differential expression of ATG14 isoforms might have an implication on Huntington diseases. In fact, SETD2 interacts with the Hh protein through its WW domain, which eventually inhibits SETD2 function (Passani et al., 2000). For instance, it would be worthy to demonstrate in further studies if Hh-mediated sequestration of SETD2 might lead to an aberrant expression of ATG14 isoforms as well as other ATG splice variants that would inhibit autophagy. This mechanism would explain the reason behind patient's disease worsen and how treatments targeting autophagy that ameliorate the symptoms fail over time. The same could be applied for different types of cancer that carry SETD2-inactivating mutations. Here, treatments whose main target is to enhance autophagy, might be inefficient to be used in the clinic. The lack of specific isoforms like ATG14(L) that inhibit autophagosome-lysosome fusion and thus autophagy, even though if the treatment used enhances autophagy the completion of the process would still be ineffective, suggesting the need of new treatment or a combination of treatments.

SETD2 also interacts with DNMT3A and DNMT3B, suggesting the role of H3K36me3 as a trigger to recruit these DNA methyltransferases to a specific genomic region that undergoes active transcription (Neri et al., 2017). Moreover, DNMT3A carries the ability to read H3K36me3 and H3K36me2 (with more affinity) and be recruited to these sites (Xu et al., 2020). These results are highly relevant to our findings on DNA methylation mediated long-term epigenetic memory of autophagy, as might bring some light on what triggers or which factors are responsible for the specific targeting of DNMT3A to ATG genes upon autophagy induction.

Additionally, it might be of need to check how long and sustainable this epigenetic memory is *in vitro* and *in vivo*. This, it will be surprising as results published by Bygren and colleagues show that starvation affects your lifespan and your progeny, suggesting that starvation leads to stable and heritable changes in our genome (Bygren, 2013). Moreover, as we found in cell lines that two autophagy stimuli impact on the way the cells respond it would be of

interest to check whether differences on the number of starvation cues might also impact on the longevity of the whole organism. There is supporting data that autophagy declines with aging, whereas the mechanism responsible for it is still unknown. Our results bring that DNA methylation induced by short autophagy stimulus could explain the natural mechanism of aging. Therefore, this research could be extended to analyze whether short autophagy stimuli as well as intermittent autophagy cues might impact not only the longevity but also on the onset of age-related diseases.

During the past decade, many anticancer drugs (like rapamycin) act by inducing autophagy and provide direct advantage on inhibiting tumor growth in several types of tumors such as colorectal cancer, breast cancer and children with high-grade gliomas (Meng and Zheng, 2015). For instance, Temsirolimus (a rapamycin analogue) is the first drug used in clinical trials in pediatric patients with these tumors. Whereas in the phase I the treatment resulted effective and was well tolerated on the phase II the treatment resulted less effective than expected. Suggesting the combination of Temsirolimus with other chemotherapeutic drugs like Erlotinib.

Understanding the reason why cancer cells are able to adapt to clinical treatments will improve autophagy-based chemotherapeutic approaches. Our new findings shed light on how new nuclear mechanisms regulates long-term autophagy and how cancer cells stimulated with an autophagy inducer may adapt over time. The fact that tumors show a decreased basal level of autophagy compared to the non-treated cells might be due to an acquisition of an epigenetic memory of autophagy that make the tumor less responsive, being able to remember the initial and following treatments. In order to provide more translational evidence of our project, analysis of patient samples treated with autophagy inducers and analyze whether they have acquired this epigenetic memory, might bring valuable results and hint to which type of treatment or combination of treatments would be more suitable in order to avoid resistance to it.

In conclusions, this is very promising time for the field of autophagy. New insights on the mechanisms involving the nucleus on the regulation of autophagy that brings light and hope on the understanding of the biology of age-related diseases, cancer, among others. Hopefully, we can move one step forward on the treatment and cure of these diseases and increase the treatment opportunities.

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