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TIPPING THE SCALES IN CANCER: NOVEL MECHANISMS OF INHIBITING PROTEIN DEGRADATION

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Tipping the scales in cancer: novel mechanisms of inhibiting protein degradation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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It's that dream that we carry with us
that something wonderful will happen,
that it has to happen,
that time will open,
that the heart will open,
that doors will open,
that the mountains will open,
that wells will leap up,
that the dream will open,
that one morning we'll slip in
to a harbor that we've never known

Det er den draumen – Olav V. Hauge
(It's the dream)
Translated by Robert Fly

POPULAR SCIENCE SUMMARY OF THE THESIS

Do you feel that many people around you are being diagnosed with cancer? This wouldn't be surprising: the risk of cancer development increases with age, and the years we are expected to live (the 'life expectancy') is steadily increasing over the world. Therefore, there is a need to invest in biomedical research, so we can understand how to treat cancer better. Cancer is not a single disease, but a term that encompasses the many types of tumors that can develop in our bodies. The work in this thesis seeks to find new ways to kill cancer cells within those tumors.

In a way a cell can be compared to a car: they are made of pieces that, in conjunction, allow them to work. In a cell, we call these components proteins. To keep the car in good condition, we visit the mechanic occasionally to change the pieces that get worn out over time. Those old pieces are then discarded. Cells also replace malfunctioning proteins to keep cellular functions up-and-running. However, nothing in biology goes really into waste, and therefore cells have waste management systems that are amazing at recycling.

Cells depend on proteins for their normal functioning, such as growing and dividing to produce new cells. Cancer cells grow and divide way faster compared to normal cells, leading to tumor growth. To sustain this accelerated growth, cancer cells produce a lot more proteins than normal cells – which in turn means they depend greatly on their waste management system, since accumulation of waste can lead to cell disease and, eventually, to make them die. This means that if we find ways to reduce the efficiency of the cell's garbage collection, we could effectively kill cancer cells without damaging healthy cells.

The ubiquitin-proteasome system (UPS) and autophagy are the waste management systems of the cell. The UPS detects worn out proteins and tags them with a chain of small ubiquitin proteins (also called the death tag) so they can be transported to the proteasome, a shredding machine that tears the proteins apart. Autophagy breaks down proteins by isolating them within a small space with acids and protein degraders (a bit like a 'cellular stomach') that destroy it. After going through the UPS and autophagy, all that's left of proteins are their building blocks - amino acids. These can be reused by the cell to generate new proteins, closing the cycle.

Chemotherapy that kills cancer cells by reducing the efficiency of protein recycling systems is already in use to treat blood cancers. However, those drugs don't seem to work very well to treat other types of cancers. That is why in the work presented in the thesis we focus in finding new ones. The work is divided into three academic papers. In papers **I** and **II**, we

found new chemicals that can block the waste management systems of the cell, leading to a dirty environment that intoxicates them and leads to cell death.

In paper **I**, we found a potential drug we call CBK77, that is harmless on its own, but inside the cell a protein called NQO1 can transform it and, unfortunately for the cell, turn it into a toxic substance. This protein is commonly found in cancer cells, which makes it an interesting drug target for cancer therapy. We also show that giving CBK77 to mice with cancer reduces tumor size. Usually, the ubiquitin death tag needs to be cut off before degradation because it can otherwise block the access to the proteasome, the protein shredder. We found that CBK77 binds to ubiquitin and propose that this can be the cause for the decrease in protein recycling in cells exposed to this compound.

In paper **II**, we found another potential drug we call CBK79, that is toxic to cells on its own. It reduces the capacity of both the UPS and autophagy, leading to the piling up of damaged proteins. Usually, when one of the waste management systems does not work, cells can survive by using the other one. Since CBK79 blocks both systems at the same time, the drug could make this type of therapy potentially more effective.

In paper **III** we focused on proteins called shuttle factors that are part of the UPS. These are responsible to recognize proteins tagged with ubiquitin and carry them to the proteasome for recycling. In this study, we discovered a previously unknown way in which the cell affects the behavior of one of these shuttle factors.

Future research with CBK77 and CBK79 can lead to new insights about the waste management operations in cancer cells. Also, to understand their potential as anti-cancer agents, further studies are needed to understand how well tolerated they are in animals, and to get a more detailed picture on how they work. The findings in paper III provide a new strategy that can be explored in new research projects aiming to block protein degradation in cancer cells.

RESUMEN DIVULGATIVO DE LA TESIS

¿Tienes la impresión de que a muchas personas a tu alrededor se les está diagnosticando con cáncer? Esto no sería sorprendente: el riesgo de desarrollar cáncer aumenta con la edad, y los años que se espera que vivamos (la "esperanza de vida") están aumentando en todo el mundo. Por lo tanto, es necesario invertir en investigación biomédica para que podamos comprender cómo tratar mejor el cáncer. El cáncer no es una sola enfermedad, sino un término que abarca los muchos tipos de tumores que pueden desarrollarse en nuestro cuerpo. El trabajo en esta tesis busca encontrar nuevas formas de matar las células cancerosas dentro de esos tumores.

En cierto modo, una célula se puede comparar con un coche: ambos están hechos de piezas que, en conjunto, les permiten funcionar. En una célula, llamamos proteínas a esas piezas. Para mantener el coche en buen estado, visitamos al mecánico de vez en cuando para cambiar las piezas que se desgastan con el tiempo. Luego, esas piezas viejas se descartan. Las células también reemplazan a las proteínas que funcionan mal para mantener la eficiencia de las funciones celulares. Sin embargo, nada en biología se desecha y, por lo tanto, las células tienen sistemas de gestión de residuos que son remarcablemente eficientes reciclando.

Las células dependen de las proteínas para su funcionamiento, como crecer y dividirse para producir nuevas células. Las células cancerosas crecen y se dividen mucho más rápido en comparación con las células normales, lo que conduce al crecimiento del tumor. Para mantener este crecimiento acelerado, las células cancerosas producen muchas más proteínas que las células normales, lo que a su vez significa que dependen en gran medida de su sistema de gestión de residuos, ya que la acumulación de basura puede provocar enfermedades celulares y, finalmente, hacer que mueran. Esto significa que, si encontramos formas de reducir la eficiencia de la recolección de basura de las células, podríamos matar de manera efectiva las células cancerosas, en principio, sin afectar a las células normales.

El sistema de ubiquitina-proteasoma (SUP) y la autofagia son los sistemas de gestión de residuos de la célula. El SUP detecta las proteínas gastadas y las etiqueta con una cadena de ubiquitina (una “etiqueta de la muerte”) para que puedan ser transportadas al proteasoma, una máquina trituradora que degrada las proteínas. La autofagia descompone las proteínas aislándolas en un espacio pequeño con ácidos y degradadores de proteínas (un poco como un "estómago celular") que las destruyen. Después de pasar por el SUP y la autofagia, lo único que queda de las proteínas son sus componentes básicos: los aminoácidos. Éstos podrán ser reutilizados por la célula para generar nuevas proteínas, cerrando el ciclo.

Quimioterapia que mata células cancerosas reduciendo la eficiencia de los sistemas de reciclaje de proteínas ya se utiliza para tratar ciertos tipos de cáncer en la sangre. Sin

embargo, esos medicamentos no parecen funcionar muy bien para tratar otros tipos de cánceres. Por lo tanto, en el trabajo presentado en la tesis, nos enfocamos en encontrar otros nuevos. El trabajo se divide en tres artículos académicos. En los artículos I y II, encontramos nuevos compuestos químicos que pueden bloquear los sistemas de gestión de residuos de la célula.

En el artículo I, encontramos un compuesto que llamamos CBK77. Éste es inofensivo por sí solo, pero dentro de la célula una proteína llamada NQO1 puede transformarlo y convertirlo en una sustancia tóxica. Esta proteína se encuentra comúnmente en las células cancerosas, lo que la convierte en una diana interesante para la terapia del cáncer. También mostramos que administrar CBK77 a ratones con cáncer reduce el tamaño del tumor. Por lo general, la etiqueta de ubiquitina debe cortarse antes de la degradación porque, de lo contrario, puede bloquear el acceso al proteasoma, la trituradora de proteínas. Encontramos que CBK77 se "pega" a la ubiquitina y proponemos que esta puede ser la causa de la disminución del reciclaje de proteínas en las células expuestas a este compuesto.

En el artículo II, encontramos otro compuesto al que llamamos CBK79, que es tóxico para las células por sí solo. Éste reduce la capacidad tanto del SUP como de la autofagia, lo que provoca la acumulación de proteínas dañadas. Por lo general, cuando uno de los sistemas de gestión de residuos no funciona, las células pueden sobrevivir utilizando el otro. Dado que CBK79 bloquea ambos sistemas al mismo tiempo, el fármaco podría hacer que este tipo de terapia sea potencialmente más eficaz.

En el artículo III nos centramos en las proteínas llamadas "factores lanzadera" que forman parte del SUP. Estos son responsables de reconocer las proteínas etiquetadas con ubiquitina y llevarlas al proteasoma para su reciclaje. En este estudio, descubrimos una forma previamente desconocida en la que la célula afecta el comportamiento de uno de estos factores lanzadera.

Investigando más estos compuestos puede conducir a nuevos conocimientos sobre las operaciones de gestión de residuos en las células cancerosas. Además, para comprender su potencial como agentes contra el cáncer, se necesitan más estudios para comprender qué tan bien son tolerados en modelos animales y obtener una imagen más detallada de cómo funcionan. Los resultados del artículo III proporcionan una nueva estrategia que podría explorarse en nuevos proyectos de investigación destinados a bloquear la degradación de proteínas en las células cancerosas.

ABSTRACT

Protein homeostasis (in short, 'proteostasis') requires the timely degradation of proteins to retain control on protein quality, amount and function. Two main proteolytic systems, the ubiquitin-proteasome system (UPS) and autophagy, complement each other to fulfill this regulatory role. Defective proteostasis is linked prominently to age-related disease, including neurodegenerative disorders and cancer.

Components of the UPS and autophagy are often mutated or dysregulated during cancer progression, a phenomenon linked to sustained cell proliferation, tumor growth and resistance to therapy. Hence, cancer cells display an increased sensitivity towards drugs that reduce the function of these proteolytic systems, as illustrated by the clinical success of inhibitors of the proteasome, and the clinical trials of lysosome neutralizers to inhibit autophagy in cancer. However, severe side effects, therapy-induced resistance and a lack of efficacy hamper their use, underscoring the need for more effective and tumor-selective compounds blocking the UPS and autophagy.

The work presented in this thesis set out to identify novel inhibitors of the UPS. Despite being rich in potentially druggable proteins, it is challenging to identify, *a priori*, a suitable target for drug development due to extensive functional redundancy across the pathway. Thus, we took two different approaches to find novel mechanisms for inhibition of protein degradation. In papers I and II, we used a cancer cell line stably expressing a fluorescent UPS reporter and employed a forward chemical genetic screening approach to interrogate the pathway in an unbiased manner, seeking new targets and/or new therapeutics to inhibit proteolysis in cancer. In paper III, we explored whether inhibiting the turnover of specific UPS reporters can be achieved by modulating the delivery of proteins to the proteasome.

In **paper I**, we characterized CBK77, a first-in-class UPS inhibitor that requires the enzymatic activity of the oxidoreductase NQO1 to be activated in cells. CBK77 impairs the degradation of ubiquitin-dependent substrates, leading to the accumulation of ubiquitylated proteins followed by caspase-mediated cell death. We found that activated CBK77 binds to ubiquitin and hinders deubiquitylating activity *in vitro*, providing a plausible mechanism for CBK77-induced UPS impairment. We propose that bioactivation can be exploited as a new means to increase cancer selectivity of UPS inhibitors.

In **paper II**, we describe CBK79, a promising novel small molecule inhibitor of proteostasis that simultaneously impairs both the UPS and autophagy and induces profound proteotoxic stress in cancer cells. Whilst this leads to the activation of several stress responses to counteract the disruptive effect of CBK79 on proteolysis, these are not successful in restoring

homeostasis or preventing cell death. This work shows the potential of dual targeting of the UPS and autophagy for the development of inhibitors that can overcome cellular compensatory mechanisms and could therefore result in more efficient targeting of cancer cells.

In **paper III**, we studied the mechanisms controlling the delivery of proteins to the proteasome through the ubiquitin-dependent Cdc48/VCP/p97 pathway. We used the turnover of Cdc48-dependent fluorescent reporters in yeast to study these processes and found that the ubiquitin shuttle protein Rad23 is itself ubiquitylated prior to substrate delivery. Modulating this step affected the degradation of Cdc48 model substrates. Overall, our findings reveal an additional layer of regulation in the UPS that could be explored for pharmacological intervention.

In conclusion, by employing reporter substrates of the UPS, we have uncovered new compounds and highlighted novel regulatory processes amenable to targeting with the ultimate goal of expanding the army of proteolysis inhibitors with anti-cancer properties.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which are referred to throughout by their roman numerals.

I. **Giovannucci, TA.,** Salomons, FA., Haraldsson, M., Elfman, LHM., Wickström, M., Young, P., Lundbäck, T., Eirich, J., Altun, M., Jafari, R., Gustavsson, AL., Johnsen, JI., Dantuma, NP.
Inhibition of the ubiquitin-proteasome system by an NQO1-activatable compound. *Cell Death Dis,* 12, 914 (2021).

II. **Giovannucci, TA.**, Salomons, FA., Stoy, H., Herzog, LK., Xu, S., Qian, W., Merino, LG., Gierisch, ME., Haraldsson, M., Lystad, AH., Uvell, H., Simonsen, A., Gustavsson, AL., Vallin, M., Dantuma, NP.
Identification of a novel compound that simultaneously impairs the ubiquitin-proteasome system and autophagy. *Autophagy,* DOI: 10.1080/15548627.2021.1988359 (2021).

III. Gödderz, D., **Giovannucci, TA.**, Laláková, J., Menéndez-Benito, V., Dantuma, NP. **The deubiquitylating enzyme Ubp12 regulates Rad23-dependent proteasomal degradation.** *J Cell Sci,* 130 (19): 3336-3346 (2017).

RELATED SCIENTIFIC PAPERS

Gierisch, ME.*, **Giovannucci, TA.***, Dantuma, NP. **Reporter-based screens for the ubiquitin/proteasome system**. Review. *Front Chem,* 8:64 (2020).

* These authors contributed equally to this work

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LIST OF ABBREVIATIONS

3-MA	3-methyladenine
ABPP	affinity-based protein profiling
ACTB/β-actin	beta actin
APC/C	anaphase-promoting complex/cyclosome
ATG	autophagy related
ATG16L1	autophagy related 16 like 1
BafA1	bafilomycin A1
CDKN1A/p21	cyclin-dependent kinase inhibitor 1A ($p21^{Cip1}$)
CDKN1B/p27	cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$)
CMA	chaperone-mediated autophagy
CQ	chloroquine
DMSO	dimethyl sulfoxide
DUB	deubiquitylating enzyme
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FBXW7	F-box and WD repeat domain containing 7
FDA	U.S food and drug administration
G3BP1	G3BP stress granule assembly factor 1
GABARAP	γ-aminobutyric acid receptor associated protein
GFP	green fluorescent protein
GOF	gain of function
HCQ	hydroxychloroquine
HDAC	histone deacetylase
HIF1A/HIF-1α	hypoxia inducible factor 1 subunit alpha
HSF1	heat shock transcription factor 1
HSP	heat shock protein
HSPA1A/Hsp70	heat shock protein family A (Hsp70) member 1A
HSPA5	heat shock protein family A (Hsp70) member 5
HSR	heat shock response
IAP	inhibitor of apoptosis
LAMP1	lysosomal associated membrane protein 1
LC3	microtubule-associated proteins 1A/B light chain 3
MAP1LC3B/LC3B	microtubule associated protein 1 light chain 3 beta
MDM2	mouse double minute 2
MHC-I	major histocompatibility complex class I

mRFP	monomeric red fluorescent protein
MTOC	microtubule organizing center
MTOR	mechanistic target of rapamycin kinase
NQO1	NAD(P)H Quinone Dehydrogenase 1
ODC1	ornithine decarboxylase 1
PE	phosphatidylethanolamine
PQC	protein quality control
PS	phosphatidylserine
PtdIns3K	phosphatidylinositol 3-kinase
PtdIns3P	phosphatidylinositol-3-phosphate
PTM	post-translational modification
ROS	reactive oxygen species
SAR	structure-activity relationship
SCF	SKP1-Cullin1-F-box protein
SKP1	S-phase kinase-associated protein 1
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SQSTM1/p62	sequestosome 1
Suc-LLVY-AMC	Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin
TP53	tumor protein p53
Ub	ubiquitin
UBA	ubiquitin-associated domain
UBL/UbL	ubiquitin-like
UCHL5	ubiquitin C-terminal hydrolase L5
ULK1	unc-51 like autophagy activating kinase 1
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
USP	ubiquitin specific peptidase
v-ATPase	vacuolar-type H+-translocating ATPase
VCP/p97	valosin containing protein
VIM	vimentin
WIPI2B	WD repeat domain, phosphoinositide interacting 2 beta
YFP	yellow fluorescent protein

1 INTRODUCTION

Maintaining a functional proteome (the collection of all proteins in a cell) is paramount to cell fitness. Accumulation of non-functional, damaged, or mutated proteins, can compromise cellular functions and form insoluble aggregates, leading to cell death. Protein homeostasis (or proteostasis in short) is achieved by maintaining a constant state of self-renewal: protein synthesis rates are tightly balanced with those of protein degradation to control protein quality, amount and function [1]. The cellular systems that safeguard the proteome's integrity comprise the protein quality control (PQC) network. These include the chaperone system, aiding the folding of newly synthesized or damaged polypeptides, and the main proteolytic systems: the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal pathway (the latter referred to as 'autophagy').

Dysregulation of protein homeostasis is prominently linked to diseases associated with aging, as is the case of many neurodegenerative disorders and cancer. In cancer, proteolytic machineries are essential for maintenance of the malignant state. In addition to their role in PQC, these systems regulate many physiological processes that go awry during malignant transformation, such as cell-cycle progression and cell division. Thus, the UPS and autophagy can sustain tumor progression and have therefore gained attention as therapeutic targets.

The work presented in this thesis aimed to find novel inhibitors of the UPS and understand their mechanism of action, with the final goal of blocking proteolysis in cancerous cells. As the UPS is comprised by hundreds of proteins interacting in many different (and sometimes unexpected) ways, the outcome of targeting specific proteins in the system can be difficult to predict. In addition, its extensive crosstalk with other degradation pathways adds to this uncertainty. Together, these factors complicate selecting a protein to inhibit with small molecules, limiting the potential to develop successful targeted screens for developing new drugs that target proteolysis. We therefore used phenotypic screens to interrogate protein degradation in a holistic manner, as an unbiased approach to finding novel compounds with cytotoxic activity and with potentially unexplored mechanisms of inhibiting protein degradation.

The strength of phenotypic screens can also become their limiting factor. Identification of the molecular targets responsible for the phenotypic effect observed of the compounds is a common bottleneck in the development of new therapeutics. Hence, efforts to understand the detailed molecular mechanisms of protein degradation are required to inform targeted screens

for specific targets. Therefore, we studied the post-translational regulation of Rad23, a protein required for the delivery of specific substrates to the proteasome, providing new knowledge for the study of potential new targets for the development of future modulators of proteolysis.

2 LITERATURE REVIEW

In this section, I present an overview of the molecular mechanisms underlying the main degradative pathways of the cell, as well as anti-cancer treatments targeting these processes to contextualize the research presented in this thesis.

2.1 Ubiquitin-proteasome system

The lifespan of a protein varies widely, from minutes to days, depending on the role it plays in the cell: proteins required for activating a specific function, like cyclins in cell division, have generally shorter half-lives than, for example, structural proteins. This constant turnover has three main goals: (i) timely degradation of regulatory proteins, required for the progression of key cellular processes such as cell division; (ii) ensure the removal of damaged proteins and substitution by newly-synthesized ones; and (iii) the recovery of amino acids that can be reutilized for protein synthesis. In cells in culture, the ubiquitin-proteasome system (UPS) is estimated to degrade 70% of all proteins [2]. This essential proteolytic task largely depends on ubiquitin – a multitasker also involved in endocytosis, DNA damage repair, amongst others. An overview covering the biochemical aspects of the pathway follows to answer the question: how can a single protein regulate so many different and essential processes?

2.1.1 Ubiquitin and ubiquitin-like proteins

After their synthesis at the ribosome, proteins are often subjected to post-translational modifications (PTM). These can modulate the protein's level, activity and/or localization, and therefore work as regulatory switches that quickly activate or deactivate cellular signaling to respond to external and internal cues.

Most of these modifications involve attachment of small chemical groups, such as the addition of phosphates during phosphorylation. In contrast, ubiquitin is a small protein (76 amino acids) that can be attached to other proteins to modify their function, localization, or abundance. Ubiquitin received its name owing to its ubiquitous expression in all eukaryotic cells and extreme abundance compared to most other proteins. It is encoded by four genes, either as single moieties fused to ribosomal proteins (*UBA52* and *RPS27A*), or as multimeric ubiquitin precursors (*UBB* and *UBC*) containing three or nine ubiquitin moieties in tandem, respectively, which will be cleaved to free single ubiquitin entities by deubiquitylating enzymes. This pool of free ubiquitin will be available to all processes depending on ubiquitylation [3].

An important structural feature of ubiquitin is the conformation it acquires when folded, defined by five anti-parallel β-strands and a single helical segment. This characteristic β-grasp fold renders the protein very resistant to pH, temperature, oxidation and degradation [4], a particularity shared by the members of the ubiquitin-like protein family comprising other small proteins like SUMO1/2, NEDD8, UFM1, FAT10 and ISG15 [5]. These are also attached to substrate proteins by enzymatic cascades that resemble those of ubiquitin, resulting in modulation of the target protein's properties and ultimately the regulation of diverse cellular functions such as DNA repair pathways, autophagy, proteasome-mediated proteolysis and signal transduction.

2.1.2 *Ubiquitylation*

Degradation signals, so-called degrons, are conserved motifs that target proteins for degradation. The first identified degrons were the N-terminal amino acid of the protein itself (now known as the N-end rule pathway) and the requirement of a lysine residue for 'tagging' the protein for degradation via ubiquitylation [6].

Similar to passing through modules in a factory assembly line, ubiquitin will need to pass through three different enzyme classes in order to be conjugated to a substrate protein. The first enzyme class, **E1**, activates ubiquitin by forming a thioester linkage between its catalytic cysteine and the carboxy (C-) terminus of ubiquitin (E1∼Ub) in a reaction dependent on ATP. Only two E1 enzymes, UBA1 and UBA6, have been described in humans [7]. Recent studies suggest that UBA1 and UBA6 have overlapping but also distinct substrates [8], consistent with the dual capacity of UBA6 to activate both ubiquitin and the Ub-like protein FAT10. UBA1 is generally more abundant and is the canonical ubiquitin activator in the UPS.

Subsequently, a transthiolation reaction transfers the ubiquitin moiety to the cysteine in an **E2**-conjugating enzyme, forming again a thioester intermediate (E2∼Ub). Finally, the E2 works together with an **E3** ligase to form an isopeptide bond between the ubiquitin C-terminal glycine (G) and, typically, an ε-amino group in a distinct lysine (K in the one-letter code) residue in the substrate protein [9].

The E3 ligases can be divided into three families characterized by their conserved structural domain and the mechanism by which the transfer of ubiquitin from the E2 occurs. The largest group is the RING (Really Interesting New Gene) finger family of ligases, which lack intrinsic catalytic activity but function as scaffolds that facilitate the interaction between the E2∼Ub and the substrate [10]. A subfamily of particular interest due to their prominent role in regulating the cell cycle are the Cullin-RING E3s (CLR). These ligases form multimeric

complexes consisting of a cullin scaffold, a RING-domain containing protein that binds to the E2, and an adaptor protein that links the complex to an F-box protein, which is the subunit that recognizes the substrate. Second, the HECT (Homology to E6-AP C Terminus) family, which are *bona fide* enzymes that catalyze the transfer of ubiquitin from E2 to E3 first (forming an intermediate E3∼Ub) and then to the substrate [11]. Lastly, the RBR (RING-between-RING) ubiquitin ligases, representing hybrid forms that first bind the E2∼Ub to then catalyze an E3∼Ub intermediate [12]. Since E3 ligases are the enzymes recognizing the substrate and catalyzing the attachment of ubiquitin, they determine the substrate specificity and the rate of the ubiquitylation reaction. This critical function explains the need for the more than 600 E3 ligases encoded by the human genome [13], while the number of E2 enzymes is much lower. However, E2s are not simply carriers of ubiquitin as these enzymes can determine the type of ubiquitin chain and extent of substrate ubiquitylation [14].

Since ubiquitin itself contains seven exposed K residues (K6, K11, K27, K29, K33, K48 and K63), its repeated ubiquitylation can generate a wide variety of polymeric structures. The ubiquitin moieties in the chain can either be linked all through the same K residue (homotypic) or through different residues (heterotypic chains). Canonical ubiquitylation in residues other than lysine occurs due to the activity of the linear ubiquitin chain assembly complex (LUBAC), an E3 ligase that assembles linear ubiquitin chains via its methionine at the N-terminus [15]. Since ubiquitin contains seven lysine residues itself, iterative rounds of ubiquitylation can occur, leading to the formation of ubiquitin chains in the substrate [16]. In some instances, **E4** enzymes are required to elongate short ubiquitin chains to reach their optimal length; for proteasomal degradation, a chain of minimum four ubiquitin moieties is preferred [17]. The first identified E4 was UFD2 in yeast [18, 19], constituting a family with several human homologs. E4 enzymes have also been recently found to mediate a switch in polyubiquitin chain topology, forming K29-K48 branched chains that target for degradation [20].

Recent findings have revealed ubiquitylation of a non-peptidic substrate, lipopolysaccharides in the bacteria *Salmonella*, which are ubiquitylated by the E3 ligase RNF213 [21]. These findings raise the intriguing possibility that there are other, perhaps intrinsic, non-protein substrates undergoing ubiquitylation in cells with yet-to-be known functions.

2.1.3 *The ubiquitin code*

The variety of ubiquitin chains constitute a complex ubiquitin code that is interpreted by proteins containing ubiquitin-binding domains, triggering different outcomes. The best understood role of ubiquitin chains is the canonical targeting of proteins to the proteasome,

achieved primarily by K48-linked polyubiquitin chains, which are also the most abundant in the cell, although K11 and K29 homotypic and heterotypic chains have also been reported [22]. Ubiquitin also signals proteolysis by selective autophagy (see section 2.2.2), such as K27 chains in the clearance of damaged mitochondria [23].

Ubiquitin serves as a PTM in many cellular processes beyond protein degradation, with ubiquitylation modulating protein-protein interactions and protein localization. For instance, mono- and multi-monoubiquitylation regulate diverse processes such as receptor internalization [24], endocytosis [25] and gene transcription [26]. Homotypic K63 chains are known to regulate, among others, activation of NF-κB [27] and DNA repair [28]. To add further complexity to this intricate code, ubiquitin can undergo PTMs itself [29]. For example, the kinase PINK1 phosphorylates ubiquitin at Ser65 to activate Parkin-mediated mitophagy [23, 30]. There are many more ubiquitin linkages and chain types, the functions of which are just starting to be understood [22, 31].

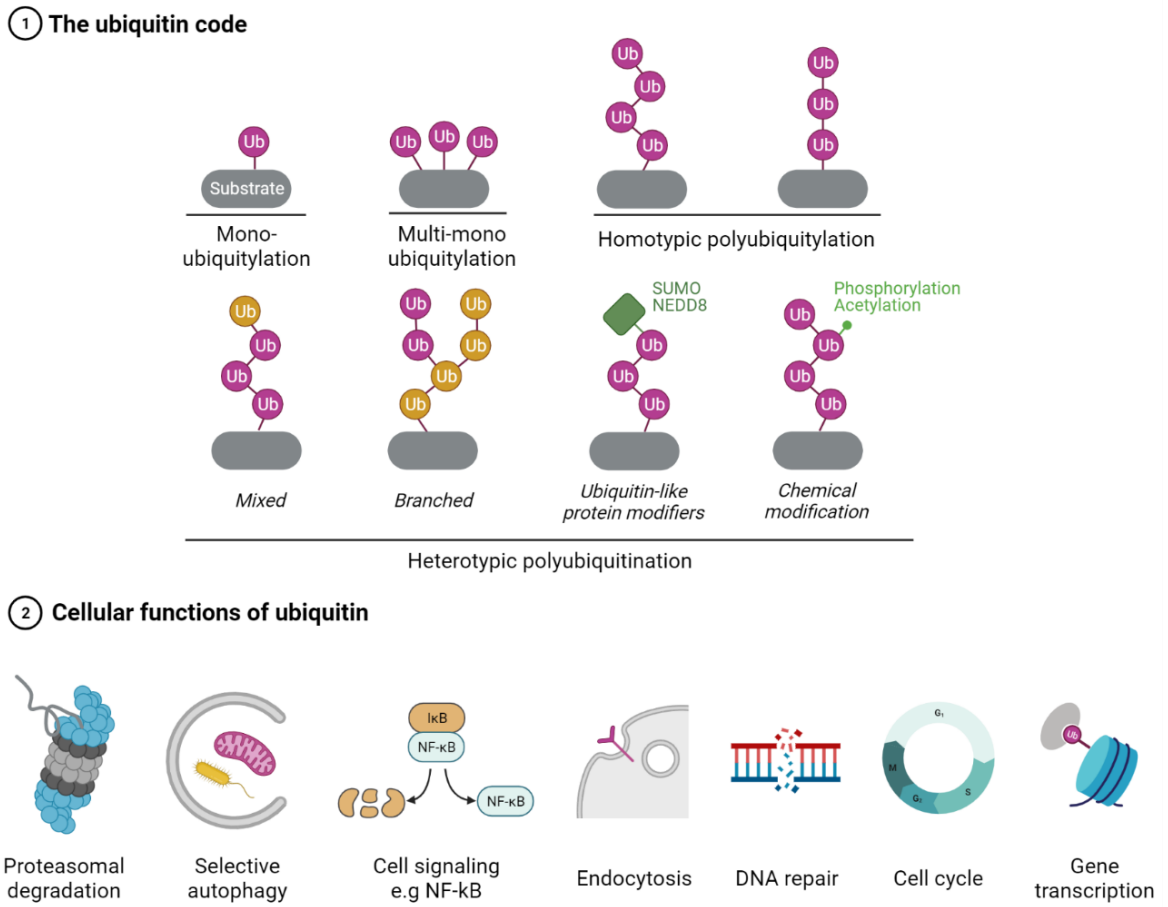

Figure 1. The ubiquitin code and cellular functions of ubiquitin. Schematic representation and examples of the ubiquitin modifications that constitute the 'ubiquitin code' and cellular functions that are regulated by ubiquitylation. The figure has been created with BioRender and is based on the figure in [32].

2.1.4 *Deubiquitylating enzymes*

Like most PTMs, ubiquitylation is a reversible process. Deubiquitylating (DUB) enzymes are responsible for catalyzing this process, and they act by cleaving ubiquitin-linked molecules after the last residue of ubiquitin (G76). DUBs can be divided into two main classes depending on their catalytic site: cysteine proteases and a smaller group of metalloproteases. These groups can be further subdivided into families: the cysteine DUBs can be ubiquitin-specific peptidases (USPs), ubiquitin C-terminal hydrolases (UCHs), Machado-Josephin domain proteases (MJDs) and ovarian tumor proteases (OTU), while the metalloprotease group is only composed of Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM) domain proteases [33]. Recently, two more DUB families have been identified: ubiquitin-containing novel DUB family (MINDY) [34] and the zinc-finger and UFSP domain protein proteases (ZUFSP) [35].

Eukaryotic cells encode approximately hundred different DUBs, which means that they are much less abundant than the E3 ligases. Hence, most DUBs show promiscuity in the types of ubiquitin linkages they process (as is the case for most USPs), providing a high degree of redundancy in substrate deubiquitylation. This apparent promiscuity is, in turn, regulated via protein interactions since DUBs are often inactive or not efficient until they are part of a complex.

DUB activity is required at several steps in the UPS. To start with, DUBs process newly-synthesized, inactive ubiquitin precursors, important for maintaining the equilibrium between free and conjugated ubiquitin, itself essential for the maintenance of the ubiquitin pool and all ubiquitin-dependent activities in the cell [36]. DUBs may be associated to specific proteins and have a ubiquitin chain-editing function that can result in promoting or inhibiting substrate degradation, or in non-proteolytic outcomes such as regulation of protein-protein interactions and protein activity. Lastly, DUB activity at the proteasome is required for the substrate to access the proteolytic core (see section 2.1.7) [37].

A recent study showed that DUBs from different families can also cleave ubiquitin moieties linked to threonine and serine residues instead of lysine [38]. Both viral and human E3 ligases capable of ubiquitylating the same amino acids have already been described [39-41]. These findings suggest that ubiquitylation of amino acids other than lysine and methionine contribute to the ubiquitin code.

2.1.5 *VCP/p97*

VCP/p97 (Cdc48 in yeast) is an evolutionary conserved AAA^+ ATPase that functions as a complex, composed of two stacked hexameric rings, each possessing ATPase activity. VCP/p97 is associated with two main processes: the extraction and the unfolding of proteins from protein complexes, membranes and chromatin [42] to facilitate their degradation, mainly by the UPS [43] but also by autophagy [44, 45]. This activity is illustrated well by endoplasmic reticulum (ER)-associated degradation (ERAD). In this process, misfolded proteins present in the ER lumen are retrotranslocated to the cytosol by the activity of a ternary complex formed by VCP/p97 together with the cofactors UFD1 and NPL4. Specifically, VCP/p97 binds Derlin-1 at the ER membrane and mediates the extraction of substrates from the ER lumen to the cytosol, where it facilitates substrate ubiquitylation and delivery to the proteasome [46].

The large interactome of VCP/p97, comprised of E3 ligases, DUBs and many ubiquitin-binding proteins that function as adaptors, direct the protein's activity to the required subcellular localization and to specific substrates. Illustrating its central role in proteostasis, knockdown of VCP/p97 leads to the accumulation of polyubiquitylated substrates, protein aggregates and cancer cell death [47]. Moreover, mutations in VCP/p97 are causative of human diseases characterized by loss of PQC and accumulation of protein aggregates, including IBMPFD (Inclusion Body Myopathy associated with Paget's Disease of the bone and Frontotemporal Dementia) and amyotrophic lateral sclerosis (ALS) [48].

Work presented in this thesis further uncovers the regulatory roles of the yeast orthologue Cdc48 in the UPS by binding and regulating the activity of ubiquitin shuttle factors, such as Rad23 (HHR23A/B in mammals; see section 5.3).

2.1.6 *Protein shuttle factors*

How ubiquitylated proteins reach the proteasome is far from a stochastic process, expected given the level of regulation present in the cell's proteolytic machinery. Several so-called shuttle proteins containing ubiquitin-binding domains have been identified, some of which are discussed here due to existence of compelling evidence for their role in the UPS. In mammals, HHR23A and HHR23B (Rad23 in yeast), the family of ubiquilin proteins, UBQLN1-4 and UBQLNL (with only one protein, Dsk2, in yeast) and DDI1/2 (Ddi1 in yeast) can recruit polyubiquitylated substrates and guide them to the proteasome [49]. These scaffold proteins have the ability of binding ubiquitin and the proteasome simultaneously through ubiquitin-associated (UBA)- and a ubiquitin-like (UBL) domains, respectively [50].

While yeast strains lacking all these proteins were viable, HHR23A/B knockout mice are embryonic lethal, suggesting that these factors play crucial roles in higher organisms. Moreover, both overexpression and down-regulation of shuttle factors can lead to the impairment of ubiquitin-dependent degradation, suggesting that their levels need to be tightly regulated in order to maintain proteostasis [51, 52].

These protein shuttles have overlapping but also distinct substrate specificities. Since a conserved hydrophobic patch on the surface of ubiquitin, formed by residues Leu8, Ile44 and Val70, is required for the interaction with ubiquitin-binding domains, the differential specificities are most likely defined by the different binding affinities to ubiquitin polymers of their UBA domains [53] and/or their interaction with other PQC components [43]. The regulation of substrate delivery to the proteasome by protein shuttle factors can substantially influence proteostasis by different means, such as by prioritizing certain substrates over others in different cellular contexts or by avoiding oversaturation at the proteasome.

In the case of budding yeast, the C-terminal UBA domain of Rad23 acts as an intrinsic stabilization signal that allows it to interact with the proteasome without being itself degraded, allowing for multiple reiterations of substrate delivery that seems to be also conserved in other shuttle factors and in human HHR23A/B [54].

2.1.7 *The proteasome*

The 26S proteasome is a large (2.5-MDa), multi-subunit, ATP-dependent proteolytic complex that degrades proteins into small peptides. Due to its broad peptidase activity, the proteasome restricts access to its active core, the 20S proteolytic core, to avoid unsolicited protein degradation. It does so by (i) ‘gate’ closing and (ii) capping the core by binding to either one (26S proteasome) or two (30S proteasome) regulatory particles (RPs). Since the proteasome is largely conserved from yeast to humans [55], the yeast nomenclature is used throughout unless stated otherwise.

Ubiquitylated protein substrates can access the 20S proteolytic core only after unfolding, deubiquitylation and translocation by the 19S/PA700 RP, which consists of the base and the lid. The base has six AAA+ ATPases (Rpt1-6), which mediate the unfolding and translocation of substrates, essential for globular structures to enter the proteasome [56], whilst the ATPases Rpt2, -3 and -5 operate the opening of the 20S entrance [57]. The RP base also contains non-ATPase regulatory particles or Rpn proteins: Rpn10 and Rpn13 bind polyubiquitin chains, serving as integral substrate receptors, whilst Rpn1, Rpn2 and Rpn13 have been shown to coordinate binding to ubiquitin shuttle factors [58]. Interestingly, Rpn10

can also be found in a proteasome-free form that binds to the UBL domain of Dsk2, presumably acting as part of a complex in a regulatory step ensuring that only Dsk2-bound substrates with adequate ubiquitin chain lengths are delivered to the proteasome [59].

The RP lid has nine Rpn subunits (Rpn3, 5-9, 11-12 and 15), with substrate deubiquitylation orchestrated by the DUB enzyme Rpn11, activity that is essential for protein degradation [60]. This metalloprotease removes entire ubiquitin chains from substrates committed to be degraded, as it is located right above the translocation channel [61].

There are two other proteasome-associated DUB enzymes: Ubp6 (USP14 in mammals) and UCHL5 (also called UCH37 in mammals, it is not present in budding yeast). These are not stoichiometric partners of the proteasome but are instead transiently recruited by Rpn1 and Rpn13, respectively [58, 62]. These DUBs disassemble the ubiquitin chain from the distal tip, having more of a 'chain-editing' or 'trimming' function. It has been proposed that this activity can rescue substrates from degradation, functioning as an extra exclusion criterion for discarding poorly ubiquitylated substrates bound to the proteasome [62].

The 20S proteolytic core consists of four rings, each of which is made up by seven subunits. The outer rings are made of α-subunits, which enable docking of the 19S RP(s) and form a 'gate' that occludes access to the β inner rings. Passing through this gate is the rate-limiting step in proteasome-mediated proteolysis [63]. In the inner rings, each β ring contains three peptidase activities that can cleave different types of peptide bonds to achieve processive proteolysis. Two display chymotrypsin-like (β5), two trypsin-like (β2) and two caspase-like (β1) activities [64]. After proteolytic cleavage, most of the remaining small peptides are further processed by cytosolic peptidases to generate free amino acids, which will be repurposed into the formation of new proteins.

More regulatory caps exist beyond the 19S RP: 11S/PA28 family (α,β,γ) and Blm10/PA200 [37]. As these open the gate, they accelerate protein degradation when bound to the 20S, yet don't have unfoldase activity or require ATP and as such, are thought to be involved in proteasomal degradation in a ubiquitin-independent manner (see also section 2.1.8). Heterogeneity also exists in the constituents of the 20S core. Immunoproteasomes are formed by incorporating specialized β subunits (β1i, β2i, β5i) that have different cleavage preferences. For example, the β1i subunit does not cleave after acidic residues but rather attacks hydrophobic ones. This shift in cleavage preference generates peptides that are optimized for major histocompatibility complex class I (MHC-I)-dependent antigen presentation, linking proteasome activity with the immune response [57]. Thus, these specialized proteasome subunits are abundant in cells of the hematopoietic system, while in

other cell types they are only upregulated upon certain conditions like oxidative stress and exposure to interferon gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α) [65].

Overall, the pool of proteasomes in the cell is dynamic, with proteasomes assembled with constitutive and immuno-specialized subunits, and different RP particles, depending on the tissue and specific cellular context [66].

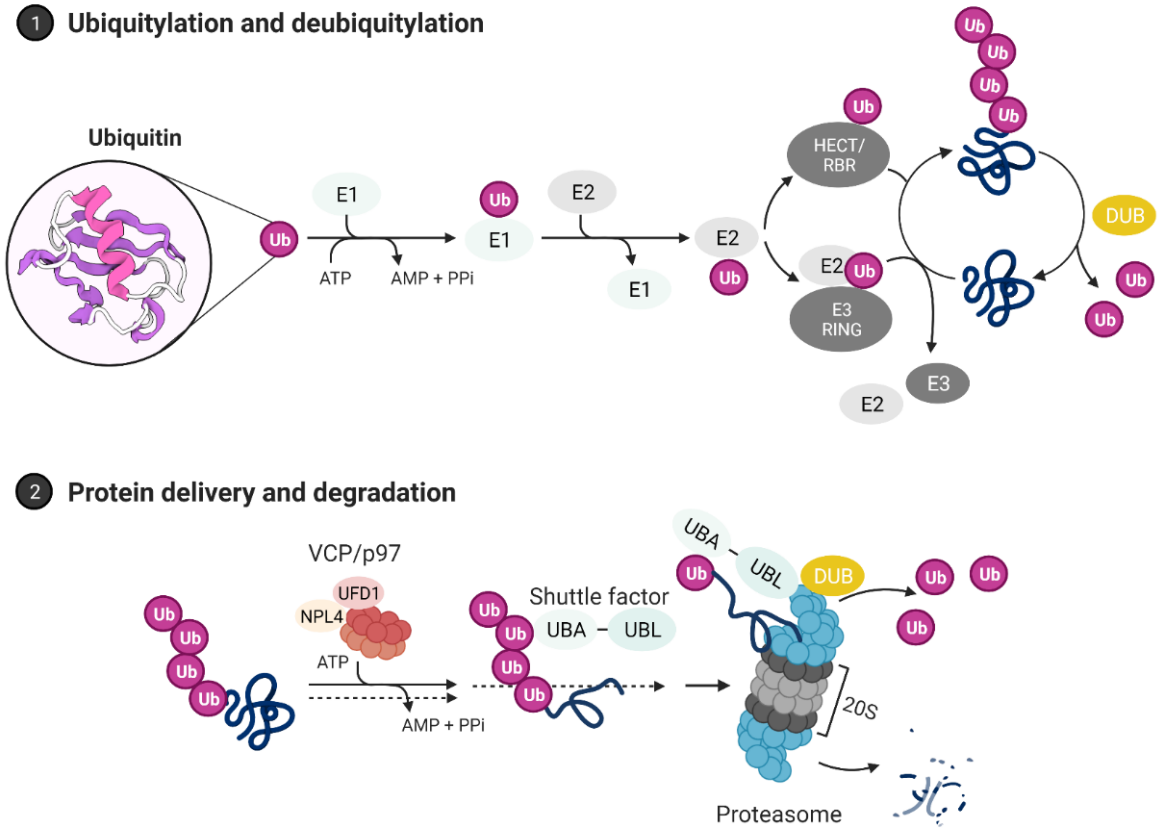

Figure 2. The ubiquitin-proteasome system (UPS). Schematic representation of key steps in the UPS. **1)** Ubiquitin (Ub) is first 'activated' in an ATP-dependent reaction to form a high energy bond (~) with the catalytic cysteine of the E1-activating enzyme, forming an E1~Ub conjugate. Then Ub is transferred to the cysteine in a E2-conjugating enzyme that will act coordinately with an E3-ligating enzyme to mediate the formation of an isopeptide bond between ubiquitin and the substrate protein (depicted as a folded protein in dark blue). The mechanism responsible of the final conjugation to the substrate depends on the type of E3 ligase involved: RING ligases act as scaffolds that bring the substrate and the E2~Ub conjugate in close proximity, whilst HECT and RBR ligases from a E3~Ub conjugate before transferring Ub to the substrate. Deubiquitylating enzymes (DUB) are peptidases that cleave ubiquitin modifications. **2)** VCP/p97 complexed with UFD1 and NPL4 can recognize ubiquitylated substrates and unfold them to prepare them for degradation. Ubiquitin shuttle factors can interact with Ub through their ubiquitin-associated domain(s) (UBA) and with the proteasome via ubiquitin-like domain(s) (UBL) to deliver substrates to the proteasome, degraded by the proteasome catalytic core (20S). DUBs associated or intrinsic to the proteasome release ubiquitin from the substrate prior to its degradation. Substrates that may be independent of VCP/p97 and/or shuttle factors are represented by dashed lines. Figure created with BioRender.

2.1.8 *Ubiquitin-independent protein degradation*

Proteins can also be degraded in a process independent of ubiquitin. This is not mutually exclusive for ubiquitylation; some proteins can be degraded by both ubiquitin-dependent and –independent mechanisms depending on the cellular context. One such context is during oxidative stress, when 26S proteasomes disassemble to increase the pool of 20S cores, which are themselves more resistant to oxidation and can therefore handle the acute burden of oxidized, damaged proteins [67]. In agreement, NRF2, a transcription factor playing a pivotal role in the antioxidant response, mediates the increase of 20S proteasome subunits under oxidative stress [68].

Proteins containing poorly folded stretches have been proposed as the main substrates of 20S proteasomes under physiological conditions. Intrinsically disordered regions are naturally unstructured domains and are contained within several regulatory proteins, including tumor suppressors such as TP53, cell cycle regulators (CDKN1A/p21, CDKN1B/p27), transcription factors (HIF1α, PGC-1α) and metabolic rate-limiting enzymes (ornithine decarboxylase, ODC) [69]. Many of these substrates contain a PEST sequence (named by the one-letter code for amino acids that it is enriched with), a highly hydrophobic patch that has been widely used for the generation of short-lived protein reporters [70] .

Similar to the regulatory units that bind the 20S core to form the 26S, there are other regulatory proteins that modulate the accessibility and activity of the uncapped 20S. A relatively well studied example is the NRF2-regulated protein NAD(P)H Quinone Dehydrogenase 1 (NQO1), which regulates ubiquitin-independent degradation of TP53 by the uncapped 20S core [71].

2.2 Autophagy

In autophagy, the lysosome (or the vacuole in yeast) is the site of protein degradation. To date, three types of autophagy have been described: microautophagy, where the lysosome engulfs a small part of the surrounding cytosol; chaperone-mediated autophagy (CMA), with the chaperone Hsc70 mediating the targeting of substrates to the lysosome, where the protein LAMP-2A serves as substrate receptor; and macroautophagy (see section 2.2.1).

Compared with the UPS, microautophagy and CMA share the characteristics of only being able to degrade single, unfolded peptides, and of performing degradation under basal conditions. On the other hand, macroautophagy operates at low rates under basal conditions, but is heavily upregulated in conditions of nutrient shortage. Albeit low, the autophagic flux under basal conditions is important because it mediates the degradation of larger cargo (organelles, protein aggregates), and extends to substrates of non-proteinaceous nature, such as lipids and nucleic acids.

In paper II, we found that CBK79 impaired the degradation of long-lived proteins under basal conditions, the main substrates of macroautophagy [72]. Hence, the following chapter will focus on the description of the molecular mechanisms governing macroautophagy.

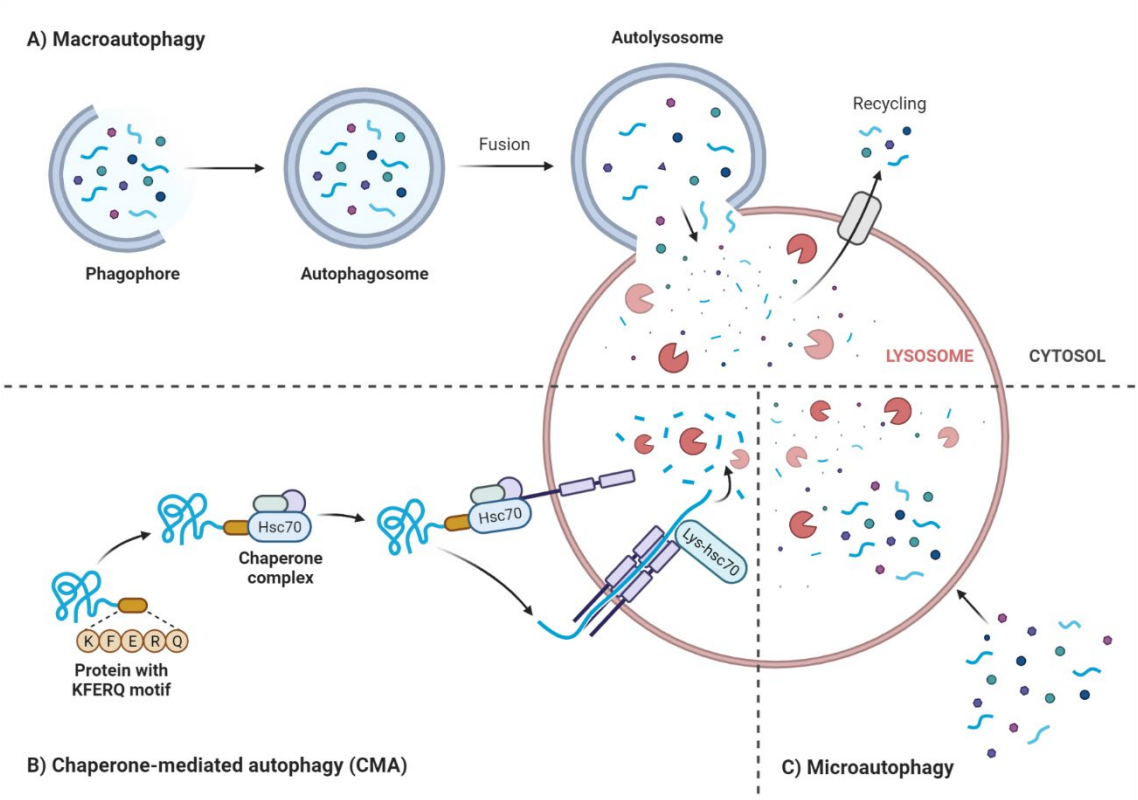

Figure 3. The main types of autophagy. Schematic representation of A) autophagosome formation during macroautophagy and fusion with the lysosome; B) the chaperone Hsc70 recognizes a dedicated degron motif (KFERQ) in the substrate protein and deliver it to the lysosome via LAMP-2A; C) components of the cytosol are engulfed during microautophagy. Figure created with BioRender.

2.2.1 *Macroautophagy*

Macroautophagy (from here on simply denoted as ‘autophagy’) involves the sequestration of cytoplasm by double bilayer membranes called autophagosomes. These ultimately fuse with lysosomes (forming the autophagolysosome), where their contents are degraded and recycled as free amino acids and other molecules [73].

Autophagy operates at basal levels in cells, but can be strongly induced under stress conditions, particularly during nutrient deficiency via inhibition of the kinase mammalian target of rapamycin complex 1 (mTORC1) [74]. In this context, autophagy is believed to be a non-specific catabolic process aimed at maintaining metabolic balance in conditions of scarcity. On the other hand, selective types of autophagy, only operating to degrade specific substrates and often depending on ubiquitylation like in the UPS, seems to be more relevant to PQC under basal conditions and is crucial for the fitness of postmitotic cells, such as neurons [75].

In the following section, the steps leading to lysosomal degradation by starvation-induced autophagy will be introduced, followed by a brief description of the unique characteristics of selective autophagy.

2.2.1.1 *Molecular mechanisms of autophagy*

The formation of the autophagosome is dictated by the hierarchical recruitment of 'autophagy related' (ATG) proteins to the initial membrane that will be used to engulf the substrates. The complex chain of events leading to the formation of the autophagosome in mammalian cells can be summarized in the following, simplified, steps [76]:

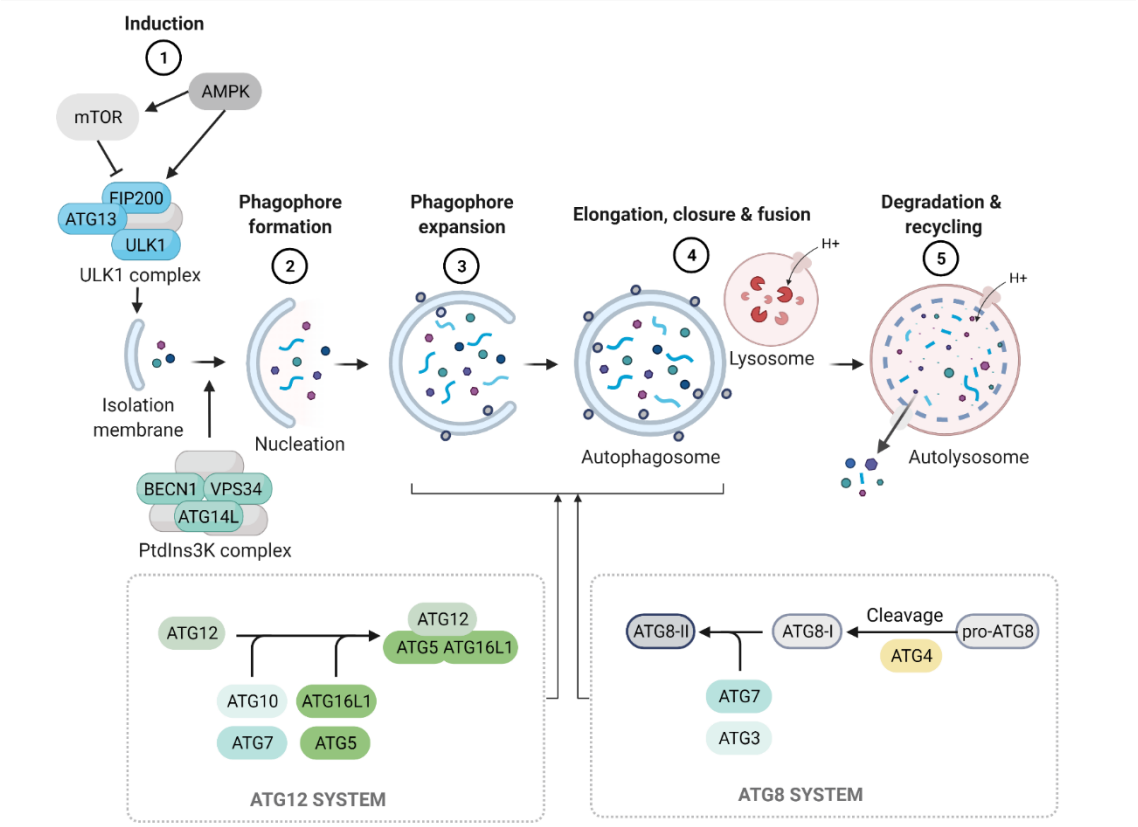

Figure 4. Molecular mechanisms of autophagy. Schematic representation of the steps involved in autophagy. See the main text to follow the main processes and proteins involved in each step of the pathway. Figure created with BioRender.

1. **Induction**: Under nutrient-rich conditions, mTORC1 binds to the autophagy-initiating kinase complex unc-51-like kinase (ULK1; comprised of ULK1, ATG101, ATG13, FIP200) and, by phosphorylating ULK1 and ATG13, inhibits its activity. Upon nutrient scarcity, mTORC1 is phosphorylated by the AMP-activated protein kinase (AMPK), inhibiting its activity and leading to a reduction in its phosphorylation of ULK1. Further dephosphorylation of the mTORC1 phospho sites on the ULK1 complex follows, and phosphorylation by (i) ULK1 itself and (ii) by AMPK serve to activate the ULK1 complex, initiating autophagosome formation [77].

2. **Initiation and nucleation (formation of the phagophore):** The ULK1 complex is recruited to the phagophore assembly site in the 'isolation membrane' [78]. This small

membrane structure requires a source of lipids to grow bigger and encapsulate the substrate(s). In yeast, $Atg9^{+}$ vesicles act as membrane seeds for phagophore formation, whilst the lipid transfer protein Atg2 provides the lipids required for the expansion of the phagophore membrane [79]. The mammalian homologues ATG9A and ATG2A are likely to operate in a similar manner [80]. The PtdIns3K class III complex I (PtdIns3KC3; composed of VPS34, BECN1, ATG14L, AMBRA-1, PIK3R4 and NRBF2) is a prominent ULK1 substrate that phosphorylates phosphatidylinositol (PtdIns) to produce phosphatidylinositol-3-phosphate (PtdIns3P) [81]. The PtdIns3KC3 complex II (containing UVRAG instead of ATG14L) regulates other processes such as endosome and autophagosome maturation [82] and LC3-associated phagocytosis (LAP) [83].

3. **Phagophore expansion:** PtdIns3P clusters recruit WIPI proteins and DFCP1 via their lipid binding domains, which will promote the expansion of the early autophagosome membrane by recruiting further interactors. WIPI2 recruits the ATG16L1 complex, essential to the expansion phase due to its E3-like ligase activity on the human ubiquitin-like ATG8 (hATG8; Atg8 in yeast) proteins [84]. These ubiquitin-like conjugation systems are discussed separately in the next section (see 2.2.1.2).

4. **Elongation, closure and fusion with the lysosome:** Phagophore elongation, closure to form the spherical autophagosome, and fusion with the lysosome are promoted by the hATG8 proteins. The hATG8-recruited Pleckstrin homology domain-containing family M member 1 (PLEKHM1) functions as a scaffold to mediate binding of the autophagosome to the lysosome via the homotypic fusion and vacuole protein sorting (HOPS) complex and the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. The fusion depends on the GTPase Rab7 [85].

5. **Degradation:** Active breakdown of substrates then proceeds within the lysosome by glycosidases, proteases and sulfatases, and the export of degraded materials into the cytoplasm for their recycling (including sugars, amino acids and lipids) is carried out by lysosomal permeases [86].

2.2.1.2 Ubiquitin-like conjugation systems in autophagy

Both the UPS and autophagy share key mechanistic features, such as the ATG12 and the hATG8 conjugation systems in autophagy functioning analogously to ubiquitylation. The former is required for the formation of the ATG16L1 complex needed for autophagosome expansion. In this instance, the E1-like enzyme ATG7 activates the ubiquitin-like protein ATG12, which forms a thioester bond with ATG10 and, independently of an E3-like protein, is conjugated to ATG5 via an isopeptide bond to form the intermediary complex ATG12-ATG5 [87]. ATG16L1 then associates to this complex and oligomerizes, forming the E3-like ligase complex ATG12-ATG5-ATG16L1 required for the final step of the second ubiquitin-like system, ATG8.

The human homologues of the yeast Atg8 protein are a family of ubiquitin-like proteins that includes microtubule-associated proteins 1A/B light chain 3A (MAP1LC3A; referred to as LC3A), LC3B and LC3C, γ-aminobutyric acid receptor associated protein (GABARAP), GABARAP-like protein 1 (GABARAPL1), and GABARAP-like protein 2 (GABARAPL2; also referred to as Golgi-associated ATPase enhancer of 16kDa [GATE-16]) [88]. Genetic deletion of all six proteins leads to superfluous formation of small autophagosomes that cannot fuse with lysosomes, impairing autophagic flux [89]. The same study conclusively showed that hATG8 proteins likely cooperate with each other to drive autophagy, but also have distinct functions, in that GABARAP proteins, but not LC3s, are required for starvation-induced autophagy [89].

hATG8 proteins are all first synthesized in a precursor form (‘prohATG8’) that requires the protease activity of the protein ATG4B to cleave at their C-terminus and produce their soluble mature forms (hATG8-I) [90-92]. Again, the E1-like enzyme ATG7 activates these mature forms in an ATP-dependent manner, and these are transferred to the specific E2-like enzyme of this pathway, ATG3. Finally, the aforementioned ATG12-ATG5-ATG16L1 complex catalyzes the conjugation of hATG8 to phosphatidylethanolamine (PE) in the autophagosome membranes, forming the hATG8 lipidated forms (hATG8-PE or hATG8-II) [93]. As hATG8 proteins are present both in the inner and outer membranes of the mature autophagosome, those at the inner will be degraded together with the cargo upon fusion with the lysosome. However, those at the outer remain or are slowly deconjugated, and therefore are commonly used to identify mature autophagosomes and monitor autophagy [94]. As with ubiquitylation, lipidation is also a reversible process and deconjugation can be mediated by any members of the ATG4 family (ATG4A-D) [95].

2.2.2 Selective autophagy

The core molecular machinery required for starvation-induced autophagy and selective autophagy is largely identical with the distinguishable feature of the latter being the use of cargo receptors. While some types of selective autophagy are ubiquitin-independent, most of these processes are dependent on ubiquitin signaling. As such, proteins with ubiquitin-binding domain(s) and LC3-interacting regions (LIRs) function as ubiquitin receptors that directly link the cargo with the autophagosome membrane through docking to the ATG8 proteins, as is the case of SQSTM1/p62, NBR1, OPTN or TOLLIP [96]. This was the preferred model to explain selective autophagy initiation; however, the discovery that ATG8 proteins are not required for mitophagy initiation [89] indicated that alternative means to recruit the initiation machinery existed. Indeed, some of the receptors can bind the ULK1 complex, as is the case of ubiquitin-positive aggregates degraded via SQSTM1/p62 [97].

Most intracellular organelles are degraded by these means, giving rise to degradation pathways named after them (e.g, ERphagy for the ER and mitophagy for mitochondria) [96]. In addition, large protein complexes, such as the ribosome and the proteasome are degraded by ribophagy and proteophagy, respectively. Of particular interest for the work presented in this thesis, the degradation of protein aggregates also occurs through a selective pathway named aggrephagy [98].

While the focus of study on selective autophagy has been on the degradation of large substrates and organelles, recent studies show selective degradation of single proteins by these means as well [99]. Starvation can even induce a selective type of degradation of the receptors required to link cargo in selective autophagy via microautophagy, likely protecting certain components of the cell from degradation in these conditions [100]. Indeed, mitochondria and other organelles are not degraded efficiently upon starvation [101]. These findings suggest an unappreciated level of selectivity in starvation-induced autophagy.

2.2.3 Noncanonical autophagy

The essential functions of hATG8 proteins in autophagy are known as the canonical functions of these proteins. However, hATG8 proteins are present and play roles in other membranous compartments involved in several pathways. The umbrella term ‘noncanonical autophagy’ encompasses all hATG8 functions distinct from the canonical setting, which are summarized on the table presented below.

LC3-associated phagocytosis (LAP) provides an illustrative example of noncanonical autophagy. In this process, single-membrane vacuoles originate from the engulfment of

extracellular material (pathogens or even living cells in a form of cell-cannibalism called 'entosis') [102] at the plasma membrane, independently of the canonical autophagy initiation factors (ULK1 and the PtdIns3KC3 complexes). The cytosolic side of the membrane is then conjugated to LC3 depending on ATG16L1 [83].

Functions related to ATG8	Roles of ATG8	Characteristics	Reference
Established functions			
Autophagosome formation	Degradative. Required for cargo recruitment (some types of selective autophagy) and autophagosome maturation	Vesicles with double bilayer membranes. ATG8 is associated with the inner and outer membranes of the autophagosome. Canonical initiation factors. Canonical ATG8 conjugation	[103]
'Emerging' functions			
LC3-associated phagocytosis (LAP)	Degradative. Transport of phagocytosed material (bacteria, apoptotic bodies, cells) from the plasma membrane to the lysosome	Conjugation of ATG8 to Single Membranes (CASM) ATG8 is associated only with the cytosol-facing side	[104],[105]
LC3-associated endocytosis (LANDO)	Degradative. Transport of endocytosed material from the plasma membrane to the lysosome. Recycling of membrane receptors	Bypasses canonical initiation factors Noncanonical ATG8 conjugation	[106],[107]
Endocytic microautophagy (eMI)	Degradative. May be required for cargo recruitment	Vesicles within multivesicular bodies ATG8 is associated on the inner side	[83]
LC3-dependent extracellular vesicle loading and secretion (LDELS)	Extracellular secretion. May be required for vesicle formation and cargo recruitment	Bypasses canonical initiation factors Noncanonical LC3 conjugation	[108]
Unconventional secretion	Extracellular secretion. Required for transport and fusion	Vesicles with double bilayer membranes LC3 is associated with the inner and outer membranes Uses the canonical LC3-conjugation machinery	[109]
Autophagosome formation	Degradative (?)	Vesicles with double bilayer membranes Can bypass canonical initiation factors Noncanonical LC3 conjugation or no LC3 at all	[110, 111]

Table 1. Functions related to hATG8 proteins. Based on [83].

The formation of degradative autophagosomes through noncanonical pathways has been described mostly in relation to the treatment of cancer cell lines with pro-apoptotic compounds [111]. Most compounds inducing these autophagosomes bypass the need for one

or both of the canonical initiation complexes. On the other hand, prolonged treatment with etoposide, a DNA damaging agent, induces a type of double-layered autophagosomes that emerge from the Golgi through the canonical initiating complexes, but in an ATG5/ATG7-independent manner [110]. These vesicles were decorated with the GTPase Rab9 instead of hATG8, and could fuse with the lysosomes - a surprising finding that contradicts the requirement of hATG8 proteins for autophagosome-lysosome fusion [110]. While these types of autophagosomes have also been observed in *in vivo* models, their physiological significance remains elusive.

Tools to discriminate between different types of vesicles associated with autophagy are expected to expand and clarify the molecular signature of each pathway, and their functional significance. A few defining features exclusive to noncanonical autophagy pathways are starting to emerge, including the requirement for the WD40 domain in ATG16L1 (dispensable for canonical autophagy) [112], and the conjugation of LC3B to phosphatidylserine (PS) [105].

2.3 UPS-autophagy crosstalk

Despite distinctive differences existing between the UPS and autophagy, extensive crosstalk links both cellular degradative machineries. Functionally, both pathways are involved in the regulation of similar processes, especially in PQC but also in others, such as cell cycle progression, apoptosis and antigen presentation. Mechanistically, both systems share common degradation signals (ubiquitin) and shuttle factors (like the UBQLN family) [113]. They can also share substrates, both short-lived and long-lived, regulated by the proteasome or autophagy, respectively (as is the case of IκB [114]) or whose fate can be determined by the specific chaperone or shuttle factor they bind to [115].

A proteasome-to-autophagy switch mediated by the levels of two co-chaperones of the BAG family has been proposed as a model to counteract the increase of aggregation-prone proteins in aged cells, dependent on the levels of BAG1 and BAG3. Levels of BAG1, an Hsp70 co-chaperone that directs substrates to the proteasome via a ubiquitin-like domain, fall with increasing cellular age whilst elevated BAG3 directs substrates towards autophagy [116]. This mechanism has also been observed in conditions of proteotoxic stress [117], a condition when aggregation-prone proteins are more susceptible to accumulate than soluble proteins [118] and cause an impairment in the degradation of other ubiquitin-dependent proteins [119]. Moreover, both pathways regulate each other's components: for example, autophagy initiation can be regulated by the ubiquitin-dependent degradation of WIPI2 [120], while whole 26S proteasomes undergo degradation via the selective proteophagy pathway [121].

This coordination between the two pathways allows compensation between each other, with these compensatory mechanisms offering an advantage for cells challenged with stress conditions when one of the pathways is either saturated or inhibited. Indeed, upon inhibition of the proteasome, autophagy is upregulated [122-124]. Conversely, inhibition of autophagy can result in UPS upregulation [125]. Given the interplay between- and the complexity of these pathways, predicting the outcomes of therapeutic interventions targeting either can be challenging. For example, chronic inhibition of autophagy can unexpectedly lead to inhibition in the degradation of ubiquitin-dependent substrates due to the accumulation of SQSTM1/p62, which ultimately results in their sequestration [126].

Collectively, these findings highlight the importance of careful consideration for the possible cross-over effects of modulating the activity of either proteolytic system.

2.4 Cellular response to misfolded proteins

Proteins have a defined 3D structure that they must adopt in order to be functional. Molecular chaperones aid polypeptides to reach their native state, a crucial task within the PQC network. When the cellular load of misfolded proteins exceeds the capacity of the proteolytic systems available, several stress responses are activated to mitigate their damaging effects and restore protein homeostasis.

2.4.1 The unfolded protein response

It is estimated that one third of the genome encodes for proteins that pass through the endoplasmic reticulum (ER) [127]. One of the main tasks of this tubular organelle is to post-translationally modify nascent transmembrane or secreted proteins, an essential step for their folding into mature, functional conformations.

Intricate mechanisms have evolved to sense the protein load of the ER, allowing the cell to adjust the ER's folding capacity to its needs. This is termed the 'unfolded protein response' and the chaperone HSPA5 (also known as BiP/GRP78), a member of the Hsp70 family, is the master regulator of the process. HSPA5 is highly abundant in the ER lumen, where it binds both to hydrophobic patches of polypeptides whilst they fold, and to bind to the luminal domains of IRE1α, PERK and ATF6, the unfolded protein response (UPR) sensors [128]. If the level of misfolded proteins increases, HSPA5 is titrated away from these sensors, resulting in the activation of signal transduction pathways through all three UPR sensors [129-131].

The general outcomes of the UPR help to reduce the burden at the ER through three specific mechanisms. PERK kinase activity drives the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α), leading to attenuation of protein translation. IRE1α dimerization activates its RNAase activity, leading to unconventional mRNA splicing of a unique transcript, the unspliced X box-binding protein 1 (XBP1u), to produce spliced XBP1 (XBP1s) protein, a transcription factor that then translocates to the nucleus to promote production of proteins involved in ERAD and PQC. Lastly, ATF6 is translocated to the Golgi and processed by proteases, releasing a fragment (ATF6f), which functions as a transcription factor that subsequently initiates expression of ERAD components.

The relative contributions of each branch are context and cell type-dependent. Underscoring the importance of ER homeostasis in the hyperactive translational landscape of cancer cells, inhibition of ERAD by proteasome inhibition leads to unresolvable UPR that activates c-JUN kinase and results in apoptosis [132]. Therefore, targeting the UPR represents a promising opportunity for treating malignant cells that produce high amount of proteins.

2.4.2 The heat shock response

Despite its name, the heat-shock response (HSR) is activated not only upon thermal stress, but also in response to other stimuli that induce the accumulation of misfolded or mislocalized peptides in the cytosol, such as oxidative stress and the presence of heavy metals [133]. It constitutes a cytoprotective response to stress that mediates the transcription of genes containing heat shock elements (HSE) in their promoter, of which HSF1 (heat shock transcription factor 1) is the central transcriptional regulator. The transcriptional targets of HSF1 are typically chaperones of the heat shock protein (HSP) family, including Hsp70, and the polyubiquitin precursor genes [134].

In an elegant autoregulatory model, Hsp70 and Hsp90 bind to HSF1 in basal conditions, inhibiting its gene regulation potential. Under unfolded protein stress in the cytosol, the chaperones are titrated away, freeing HSF1 to dramatically increase the levels of HSPs and ubiquitin, amongst other gene products [135]. Phenotypically, HSF1 forms foci in the nucleus termed ‘nuclear stress bodies’, which represent a space for HSF1-dependent transcription of non-coding transcripts [135]. In contrast to the upregulation of these specific sites, heat-shock conditions cause a transient repression of transcription that is restored once the stress is relieved [136].

Altogether, the HSR is a fast response to proteotoxic stress that promotes the re-folding of damaged proteins or facilitates their degradation when their state is beyond repair, protecting the cell from the deleterious effects of protein aggregation. Cancer cells from several tumor types seem to depend on the HSR for survival [137]; hence, there is an interest in developing chemical inhibitors of HSF1 and chaperones as anti-cancer therapeutics [138].

2.4.3 Aggresome formation

When the load of faulty proteins exceeds the capacity of the degradative systems, aggregated proteins are actively transported to the microtubule organizing center (MTOC) and form the aggresome, a membraneless structure characterized by a vimentin ‘cage’ [139]. This phenomenon has been hypothesized as a cellular strategy to constrict the cytotoxic properties of misfolded polypeptides to a defined space, thereby limiting the deleterious consequences of misfolded proteins. These include the sequestration of functional proteins, the formation of insoluble aggregates, and the eventual collapse of proteostasis due to inhibition of PQC components [140].

The deacetylase HDAC6 interacts with ubiquitylated proteins in the cytosol through its ubiquitin-binding BUZ finger and to dynein motors on microtubules to mediate the active transport of cargo into the aggresome. Cells deficient in HDAC6 neither form aggresomes

nor accumulate autophagosomes with ubiquitylated material, highlighting the essential role HDAC6 plays in the selective removal of ubiquitylated material by aggrephagy [141]. However, not all aggregation-prone proteins require ubiquitylation to be transported to the aggresome [142]. The chaperone BAG3, also involved in the switch from proteasomal to autophagic degradation, mediates the active transport of these substrates. Yet, despite transporting non-ubiquitylated cargo, this process is dependent on Hsp70 and the Hsp70-associated E3 ligase CHIP, core components of the UPS [98].

Lysosomes and components of the UPS are commonly detected in the vicinity of aggresomes [143]. In the case of lysosomes, the broad consensus across different studies holds that the receptor SQSTM1/p62 is enriched to mediate the breakdown of the aggresome by aggrephagy [98]. On the other hand, whether proteasomes are actively recruited there to achieve a physical crowding of proteolytic enzymes for degrading protein aggregates, or whether they are trapped, has been a question of debate. A recent report studying the contribution of vimentin to aggresome formation found that vimentin interacts with proteasomes and is required for their translocation to the aggresome [144]. This study showed that vimentin, despite having been used as an aggresome marker for more than two decades, is not required for aggresome formation. However, cells lacking vimentin showed a reduced ability to recover from proteotoxic insults, suggesting that proteasome localization in close proximity to the aggresome is an active process aimed at restoring proteostasis [144]. Whether proteasome activity is required for degrading substrates at the aggresome, or to promote aggresome clearance by other means, remains to be clarified.

2.5 Cancer

'Cancer' is not a single disease, but a term that includes more than 100 types of tumors that can develop in tissues across the body. The formation of a tumor is a multistep process in which a cell acquires, due to genetic and/or epigenetic changes, new characteristics that provide an advantage (most commonly, in growth) over other cells – what is known as malignant transformation. Those traits are, over time, selected for and passed along to new cells in a microscale evolutionary process. The iterative cycle of selecting for new traits ultimately leads to clonal expansion of transformed cells and tumor growth. Tumors are disorganized tissues that impair the function of the organs in which they arise, and in addition to inducing local pathology, cancer cells in many types of tumors acquire the ability to invade other organs and form new tumor sites called metastases.

Following cardiovascular disease, cancer is the second leading cause of death globally, responsible for one in every sixth death [145]. Since age is one of the main prediction factors for developing- and dying from cancer, and the average world's life expectancy is increasing, the number of patients and burden of this disease are only expected to rise [145, 146]. Better treatment options are key to the gradual improvements in 5-year survival rates across most cancers, but patients suffering from some types of cancers, such as pancreatic cancer, face very limited therapeutic options and poor prognosis [147].

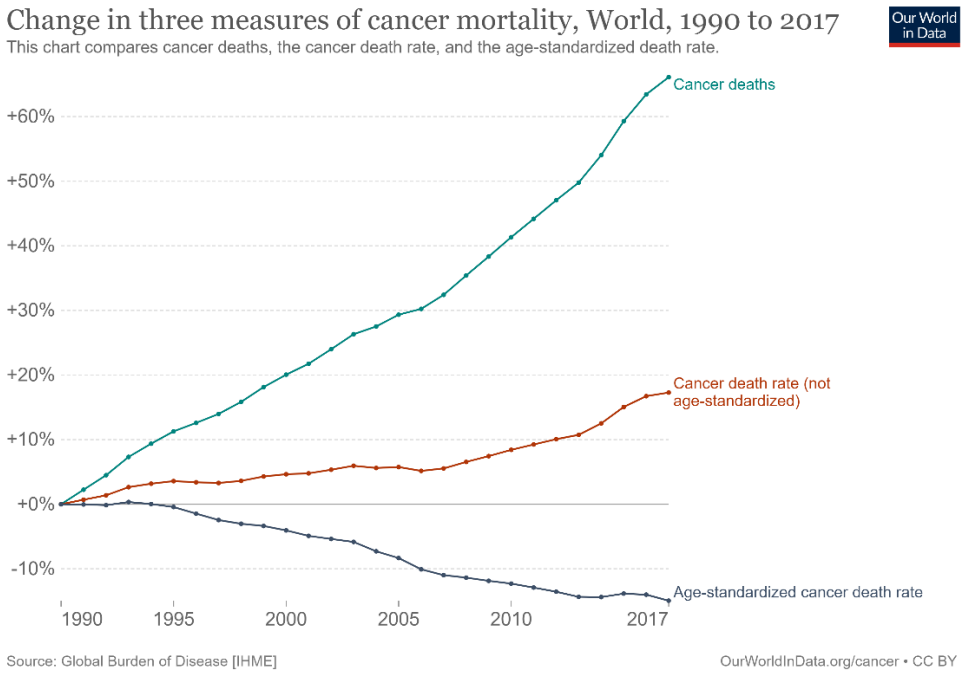

Figure 5. Chart comparing epidemiological measures of cancer mortality globally from 1990 to 2017. 'Cancer deaths' are the absolute number of deaths due to cancer per year. The 'cancer death rate' shows the number of cancer deaths per 100,000 people. These two measures reflect the rise in cancer-related deaths due to population growth and aging. When the cancer death rate is adjusted for these demographic changes (the 'age-standarized cancer death rate'), a relative decrease in cancer-caused deaths can be seen. This decrease can be largely attributed to changes in lifestyle, better diagnosis and treatment options. See more detailed information at https://ourworldindata.org/cancer.

Two seminal publications in the cancer biology field compiled all traits that define cancer cells and their surrounding microenvironment and coined them the ‘hallmarks of cancer’ [148, 149]. This valuable intellectual framework provides the opportunity to identify possible therapeutic avenues that, despite the heterogeneity of cancer, could selectively attack tumors over healthy tissue. Given the high selective pressure exerted on cells within a tumor, a challenge to developing successful treatments is the emergence of cells resistant to therapy after initial exposure to an intervention. Moreover, treating a tumor successfully often requires a combination of therapies that can target the heterogeneous cell population that forms the tumor, including cancer stem cells and the different cell types that infiltrate the tumor and are required for its growth. Hence, the study of potential new therapies and the development of novel chemotherapeutics is especially beneficial for the field of oncology to meet the high needs for treating this deadly disease.

2.5.1 Role of the UPS in cancer

Components of the UPS are often found to be directly mutated or dysregulated during the onset of cancer development. These can either result in increased degradation of tumor suppressor proteins or the stabilization of oncoproteins, molecular inhibitors and promoters of the malignant transformation, respectively [150]. The UPS also plays a supportive role where, despite not being directly responsible for oncogenesis, the UPS aids in the survival of malignant cells. Reflecting the hallmarks of cancer, this section will provide an overview of the dysregulated status of the UPS in cancer with a few key examples to illustrate its contribution to the disease.

2.5.1.1 *Uncontrolled cell proliferation*

Cell cycle progression is regulated by oscillation in the activity of cyclin-dependent kinases (CDK), which itself is dependent on the levels of CDK activators (cyclins A, B, D and E) and inhibitors, such as cyclin-dependent kinase inhibitor 1B (CDKN1B/p27) and cyclin-dependent kinase inhibitor 1A (CDKN1A/p21). The abundance of all such signaling molecules is controlled by the UPS, primarily through the activity of two RING ligases: the S-phase kinase-associated protein 1 (SKP1)-Cullin1-F-box protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C) [151].

A common target of driver cancer mutations in the UPS is the F-box and WD repeat domain containing 7 (*FBXW7*) gene, which encodes the F-box protein that mediates substrate recognition in the SCF complex. Loss of function of FBXW7 promotes stabilization of oncoproteins, such as cyclin E, MYC, c-JUN, NOTCH and MTOR, which can also result from mutations in the degrons of these substrate proteins [152]. This particular scenario is a characteristic of many cancers following mutation(s) in the degron of β-catenin, a prominent driver of oncogenesis [153]. In general, this pattern of mutating either the degron or the E3 ligase of a given oncoprotein is seen in several cancers and has been estimated to account for more than 10% of the somatic mutations that can drive cancer progression [154].

2.5.1.2 *Genomic instability and mutation*

Genome stability is frequently disrupted in cancer cells and given that many proteins involved in DNA repair and gene expression are ubiquitylated, the UPS therefore plays a key role in maintaining genomic integrity. Cervical cancers provide a clear example of how the UPS can support oncogenesis. The human papilloma virus (HPV), causative of cervical cancer, produces an E6 oncoprotein that binds to TP53 and promotes its degradation via

ubiquitylation by the E3 ligase UBE3A (also known as human papilloma virus E6-associated protein, E6-AP) [155], leading to genomic instability [156]. Another prominent E3 ligase associated with genomic integrity is MDM2, the E3 ligase mediating ubiquitin-dependent degradation of TP53. MDM2 is often overexpressed in cancers [157], and TP53 mutants with oncogenic gain-of-function (GOF) usually escape degradation by MDM2 [158]. Further indicating that UPS activity supports cancer progression, a study of these GOF properties found that mutated TP53 variants cooperate with NRF2 to activate transcription of proteasomal genes, and that increased proteasomal activity promoted the viability and migration of cultured cancer cells via the degradation of key tumor suppressors [159].

2.5.1.3 *Resistance to cell death*

Both uncontrolled cell division and genomic instability can quickly lead to cell death, thus, cancer cells often evolve mechanisms to cope with these vulnerabilities and promote cell survival. The transcription factor NF-κB is constitutively activated in many cancers [160], regulates the expression of genes related with inflammation, metastasis and angiogenesis, and confers pro-survival effects by increasing the levels of the inhibitor of apoptosis (IAP) protein family, among others [161]. Since the activity of NF-κB depends on the ubiquitylation and degradation of the inhibitory protein IκB, UPS activity is essential in this process [162].

2.5.1.4 Survival in conditions of proteotoxic stress

In physiological conditions, the capacity of the UPS is largely redundant. As already suggested by previous research, advanced electron cryotomography provided concrete evidence that only 20% of the proteasomes are engaged in processing substrates under non-stressed conditions, leaving room to respond to stress in the proteome [163]. However, this is not the case in cells with higher rates of protein synthesis, such as in aneuploid cells experiencing genomic instability. Aneuploid cells, an extremely common trait of solid tumors [164], suffer from constant proteotoxic stress, as mutations in some genes can result in unfolding and/or destabilization of the proteins they encode, thereby increasing the demand for functional chaperoning and proteolytic systems [165].

Further challenging these systems, cancer cells bear an increased burden of oxidized proteins due to elevated ROS levels [166]. Studies in budding yeast support a causal relationship between aneuploidy and an increased dependence on the UPS, with approximately one-third of single chromosomal aneuploidies rendering them hypersensitive to proteasome inhibitors [167]. Further supporting this theory, yeast cells that adapted to aneuploidy were found to

contain mutations in Ubp6, which enhance proteasomal activity [168]. Thus, the UPS in this context is operating close to its maximal capacity and is indispensable to sustain cancer progression. This concept of dependency and adaptation to stress phenotypes has been termed 'non-oncogenic addiction' [169], implying that drugs targeting these vulnerabilities will be more selective towards cancer cells, and therefore have acceptable therapeutic windows [169].

2.5.2 Role of autophagy in cancer

Analogous to the dysregulation of the UPS, mutations or loss of genes related to autophagy also occur during malignant cell transformation, although generally with a distinct pattern. These are, first, the downregulation of autophagic flux, linked to malignant transformation, followed by restoration of autophagic activity in cancer cells within established tumors to sustain their growth.

This section will provide a brief overview of the evidence linking autophagy to cancer. To emphasize the dichotomous impact of autophagy activity on cancer, key examples of autophagy dysregulation either early or late in the oncogenic process are presented.

2.5.2.1 Inhibition of autophagy promotes cancer initiation

Compromising autophagic flux, by reducing the expression of proteins implicated in autophagy initiation, results in the spontaneous formation of tumors in mice, shown first for the protein BECN1 [170, 171]. This tumor-promoting phenotype has been linked to the stabilization of oncoproteins, such as fusion protein BCR-ABL in leukemia [172] and GOF TP53 mutants [173]. Furthermore, oncogenic signaling through mutant TP53 can repress autophagy [173], indicating that inhibition of this pathway can promote cancer initiation [174].

Beyond a direct role in oncoprotein stabilization, autophagy also performs several other functions maintaining genomic integrity. These include autophagy supporting nuclear protein quality control degradation [175], limiting the impact of retrotransposon insertions in the genome [176] and promoting faithful chromosome segregation during mitosis [177]. In addition, selective degradation of damaged mitochondria can indirectly protect the genome from ROS-induced DNA damage [178], and the degradation of pathogens through xenophagy may provide a first-line defense from infections linked to carcinogenesis, such as those produced by HPV or hepatitis B/C viruses [179].

2.5.2.2 Autophagy supports tumor progression

Given the high metabolic needs of an expanding cell population, tumors suffer from oxygen deprivation (hypoxia) and scarcity of nutrients, with both conditions inducing metabolic stress. Such insults have been shown to stimulate autophagy in order to maintain a critical pool of metabolites required for sustained cell division and growth. This is exemplified well by pancreatic ductal adenocarcinoma, which displays a strong dependence on autophagy to maintain a high intracellular amino acid pool and sustain hyperactive protein synthesis [180]. Highlighting the adaptive role of autophagy, lung cancer cells deficient in autophagy have a normal metabolic profile under nutrient-rich conditions but show increased ROS and depletion of the nucleotide pool during starvation, leading to cell death [181].

Additionally, dormant cancer cells with the potential to resume growth and lead to metastasis are particularly sensitive to autophagy inhibitors [182]. Interestingly, knockdown of ATG7 but not BECN1 reduces metastatic potential, suggesting that these cells depend on non-canonical types of autophagy.

Overall, autophagy sustains cancer progression and survival through multiple mechanisms, such as proteomic stress resistance, tumor metabolism and drug resistance, although its specific involvement is likely dependent of the type of cancer, its genetic drivers, and its microenvironment. For instance, tumors driven by mutations in the oncogene KRAS show different responses to autophagy inhibitors depending on their TP53 status [183]. Therefore, while a growing body of evidence supports targeting autophagy as a cancer therapy strategy, further research is required to understand the underlying molecular signature of tumors that could benefit from autophagy-inhibiting interventions.

2.6 Inhibition of protein degradation for cancer treatment

Given the vital importance of proteostasis and other functions regulated by the UPS and autophagy in cancer cell survival, blocking protein degradation has been explored therapeutically. The following section outlines the emerging therapeutic targets and therapeutics in both proteolytic systems.

2.6.1 UPS inhibitors

2.6.1.1 Proteasome inhibitors

The development of proteasome inhibitors as research tools revealed their cytotoxic properties selective for cancer cells [184], an effect that was first attributed to the stabilization of pro-apoptotic proteins [185] and motivated the clinical trials of PS-341 (also known as bortezomib or Velcade). Encouraging results led to its approval for treating multiple myeloma (MM) resistant to refractory to chemotherapy and later, bortezomib was also approved as the first line therapy for patients with MM and mantle cell lymphoma [186]. These results motivated the exploration of new means to inhibit the UPS, either by novel proteasome inhibitors with improved clinical profiles, or through novel targets within the vast number of enzymes present in this pathway.

The arsenal of proteasome inhibitors can be divided depending on their 'warhead' into peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones (epoxomicin and eponomycin) and β-lactones (such as lactacystin) [187]. Of these, peptide boronates and epoxyketones were the first developed for clinical use, with bortezomib being the first peptide boronate employed. It targets the chymotrypsin-like (β5) activity of the 20S core and provides anti-tumor activity due to pleiotropic effects. These include the enhanced stabilization of tumor suppressors such as TP53 and CDKN1B/p27, the pro-apoptotic protein BAX and the NF-κB inhibitor IkB, as well as increasing proteotoxic stress by inducing the accumulation of misfolded proteins and unresolved unfolded protein response (UPR). Ultimately, this leads to cell cycle arrest, apoptosis and inhibition of tumor angiogenesis [188]. Unfortunately, bortezomib presents a narrow therapeutic window due to severe side effects, and its efficacy is hampered due to the emergence of resistance mechanisms [189]. In addition, none of the several clinical trials either as stand-alone therapy or in combination with other chemotherapeutics has shown efficacy in solid tumors [190].

To overcome these challenges, a second-generation of proteasome inhibitors with reduced cytotoxicity towards non-cancerous tissues, including the epoxyketone carfilzomib and the peptide boronic acid ixazomib, were developed [191]. Another two proteasome inhibitors,

oprozomib (structural analog of carfilzomib) and marizomib (β-lactone of the bacteria *Salinospora tropica*) are currently in clinical trials [192].

All compounds discussed above cannot be truly defined as proteasome inhibitors, but rather as 'β5-inhibitors', as they are selective for this catalytic site. Inhibiting other proteolytic activities on top of the β5 can enhance cytotoxicity [193]. In agreement, upregulation of β1- or β2-subunits presents a potential source of resistance to proteasome inhibitors, suggesting that compounds targeting several proteolytic sites could lead to greater potency and a lower chance of developing resistance [194].

Other compounds that also inhibit the proteasome but not via the 20S catalytic activities have started to emerge, such as RA190, an inhibitor of the proteasome ubiquitin receptor Rpn13 [195]. CRISPR/Cas9 knockout of Rpn13 demonstrated that Rpn13 is indeed needed for RA190's activity, and that the combination of RA190 with bortezomib promotes synergistic anti-MM activity [196].

2.6.1.2 Ubiquitylation inhibitors – E1 and E2 inhibitors

Downregulation of the E1 enzyme UBA1 results in selective cell death of MM cells [197], motivating the exploration of E1 inhibitors for cancer treatment. Inhibiting UBA1, such as with the recently developed TAK-243 (MLN7243) is effective in blocking protein clearance [198]. Located at the peak of the ubiquitylation cascade, inhibition of UBA1 leads to broad inhibition of all UPS substrates. On the contrary, inhibitors of the E1-enzyme for the ubiquitin-like protein NEDD8 (NEDD8-activating enzyme [NAE]) like MLN4924 are restricted to the subset of substrates regulated by the subfamily of CRLs, whose activity is regulated by NEDDylation and are of particular interest due to their role in regulating cell cycle progression [199].

Since all E1 and E1-like activities require ATP, inhibitors of these enzymes have similar structures and share a common mechanism coined 'substrate-assisted inhibition'. In this process, such inhibitors mimic ATP, enter the E1 active pocket and, aided by the catalytic cysteine of the enzyme, form covalent adducts with ubiquitin/UBL protein inside the active pocket that remain stable and block any further enzymatic activity [200]. Despite this similarity, inhibitors of UBA1 and NAE display selectivity towards their respective enzymes [198].

Inhibition of E2 enzymes can also be an attractive strategy to direct inhibition of degradation to a group of substrates. E2 acting in concert with the RING E3 ligases, which have no catalytic activity by themselves, is of particular interest. NSC697923 is a nitrofuran-

containing molecule found in a screening campaign for inhibitors of NF-kB and works by forming a covalent bond through the nucleophilic attack of the E2 cysteine, blocking formation of the E2~Ub conjugate [201]. This mechanism of action was also shared by the structurally related compound BAY 11-7082, previously identified as an NF-kB inhibitor [202]. Although the original study of NSC697923 reported selectivity towards the E2 UBE2N, this has since been refuted as both BAY 11-7082 and NSC697923 were shown to inhibit several E2s but also DUBs [202].

2.6.1.3 Ubiquitylation inhibitors - E3 inhibitors

Since E3 ligases dictate substrate selectivity, E3 inhibitors are anticipated to be more specific and less toxic than inhibitors of the proteasome. Given this advantage, many more E3 inhibitors have been developed. A summary of key examples follows.

Many research efforts are focused on the family of RING E3 ligases due to their prominent role in regulating the cell cycle. For instance, so-called compound 25 was identified as an inhibitor of SCF ligases that utilize the F-box protein SKP2 during *in silico* screening [203]. This compound blocks the interaction of SKP2 with the adaptor protein SKP1, inhibiting the ubiquitylation and degradation of SKP2-dependent substrates like the tumor suppressor CDKN1B/p27. This example is an encouraging proof-of-principle that specific protein-protein interactions can be blocked with small molecules.

Most of the E3 enzyme inhibitors that are not directed towards SCF ligases target the degradation of TP53. These include direct inhibitors of MDM22, an E3 ligase responsible for the ubiquitylation of TP53, like Nutlin-3a and its derivatives [204], and RITA, which binds to TP53 and disrupts its interaction with MDM2 [205]. These compounds lead to stabilization of TP53, resulting in TP53-dependent apoptosis. In addition to MDM2 inhibitors, the IAP family of E3 ligases has been targeted extensively for driving cancer cell death, typically using compounds inspired by the endogenous protein inhibiting IAPs, SMAC/DIABLO [206].

2.6.1.4 DUB inhibitors

Substrates destined for degradation at the proteasome lid need to be deubiquitylated in order to proceed to degradation; therefore, targeting proteasome-associated DUB activity could render the same outcome as general proteasome inhibition. b-AP15, inhibitor of both proteasome-associated DUBs UCHL5 and USP14, leads to the accumulation of polyubiquitylated substrates, cytotoxicity in cancer cell lines and inhibited tumor growth in four different solid tumors in mice, presenting proteasomal-DUB inhibition as a potential

new cancer therapy [207]. However, inhibition of USP14 alone with the selective compound IU1 enhances degradation of several ubiquitin-dependent substrates by the proteasome [208]. VLX1570 was identified in lead optimization efforts and is an analog of b-AP15 with good aqueous solubility and enhanced potency [209], yet a clinical trial for the compound in combination with a low dose of dexamethasone was terminated due to limiting toxicities [210]. Further studies later showed both b-AP15 and VXL1570 may form covalent adducts non-specifically with other DUBs and proteins, which may explain its limiting toxicity [211].

Inhibition of the proteasome's ubiquitin receptors has also been attempted. The inhibitor of Rpn11 capzimin shares common features with proteasome inhibition and, importantly, elicits cytotoxic responses in bortezomib-resistant cell lines [212].

Targeting non-proteasomal DUBs is a different approach to achieving selective inhibition of degradation for a discrete subset of proteins. Among these, USP7/HAUSP has a well-established link to carcinogenesis by stabilizing TP53, amongst other mechanisms [213] and as such, many USP7 inhibitors have been developed including P22077 and P5091 [214] [215]. These compounds cause TP53-dependent cell death and have shown promising effects in MM cell lines.

Since many DUBs depend on a catalytic cysteine residue, screening for selective DUB inhibitors is challenging, a limitation shared with inhibiting E1 and E2 enzymes, as potent compounds selected in screening campaigns are typically reactive species with potentially cross-inhibitory profiles.

2.6.1.5 VCP/p97 inhibitors

Inhibitors of VCP/p97 have been described to impair cancer cell growth [216], but most have not been developed further due to poor selectivity or limited potency. One apparent exception, DBeQ, was reported as a selective, reversible ATP-competitive VCP/p97 inhibitor [217] and its discovery led to more potent molecules like CB-5083, which underwent phase I studies in MM and metastatic solid tumors [47] that were terminated due to secondary off-target effects and interrupted its clinical development [218].

2.6.2 Autophagy inhibitors

Drugs like 3-methyladenine and wortmannin are ‘early-stage’ autophagy inhibitors that target PtIns3K complex upstream of the core autophagic machinery. Late-stage inhibitors that impair autophagosome-lysosome fusion, include bafilomycin A1 (BafA1), an inhibitor of lysosomal v-ATPase, and lysosomal proteases inhibitors like E64D and Pepstatin A [219]. However, these molecules are not suitable for clinical purposes due to poor solubility and toxicity limitations.

The only autophagy inhibitors currently used in clinical trials are chloroquine (CQ) and its derivative hydroxychloroquine (HCQ), lysosomotropic agents that increase lysosomal pH and autophagosome-lysosome fusion. However, these drugs present several challenges, such as the fact that CQ is less effective in inhibiting autophagy under acidic conditions, predominant in the tumor environment [220]. Moreover, the mechanism of action for either drug remains unclear. Recent findings suggest that CQ affects autophagy by impairing autophagosome-lysosome fusion without neutralizing lysosomal pH [221]. Additionally, several studies suggest that CQ exerts its antitumor and therapy-sensitizing effects via mechanisms independent of autophagy [222, 223].

More specific inhibitors of autophagy are under development and are expected to shed light into the potential of targeting autophagy in cancer [224], among those being inhibitors targeting PIK3C3 [225, 226], ULK1 [227], ATG4B [228, 229] and ATG7 [230].

2.7 Drug discovery

The work in this thesis seeks to identify novel compounds for impairing protein degradation in cancer cells within a general practice known as 'drug discovery', which comprises an extended and stepwise process to identify chemical compounds with desirable properties to potentially treat disease. A summary of the early steps in drug discovery follows with a view to outlining the scope of the work covered in this thesis.

2.7.1.1 High-throughput screening strategies

High-throughput screening involves the screening of a compound library either (i) directly against the drug target or (ii) in a more complex assay system, such as a cell-based assay. Whilst the latter example provides information on the impact of the compound on cell homeostasis, it also requires secondary assays to confirm the site of action of candidate molecules. Broadly, there are two main types of screening strategies: target-to-screen or screen-to-target.

In target-to-screen, a well-characterized biomolecular target is chosen and small molecules with modulatory potential are tested. Most commonly, an *in vitro* assay is developed in which only the target (most commonly a protein), or just a few factors, are tested in non-physiological conditions. These screens tend to be the preferred method for screening from a chemistry point of view, as they can provide precise correlations between the affinity of the compound for the target and the intended effect, usually an inhibitory interaction within a catalytic pocket, providing valuable information to guide compound optimization. However, this reductionist approach to disease fails to reproduce the complex biology within the cell, and therefore, may dismiss important components of the relevant biology.

In that respect, the screen-to-target approach is better suited to assess the outcome of the compound on a system that better recapitulates truthful biology (such as cell lines or *in vivo* models). With this strategy, living systems are exposed to a compound library and a subsequent activity readout helps identify candidate compounds, such as a molecular reporter of a signaling pathway or the detection of metabolite concentrations. This strategy also ensures that the compound fulfills minimum criteria for later stages of drug development (e.g, cell permeability) and without the need to select a target *a priori*. In these cases, however, identifying the mechanism of action represents the major bottleneck in the hit-to-lead pathway.

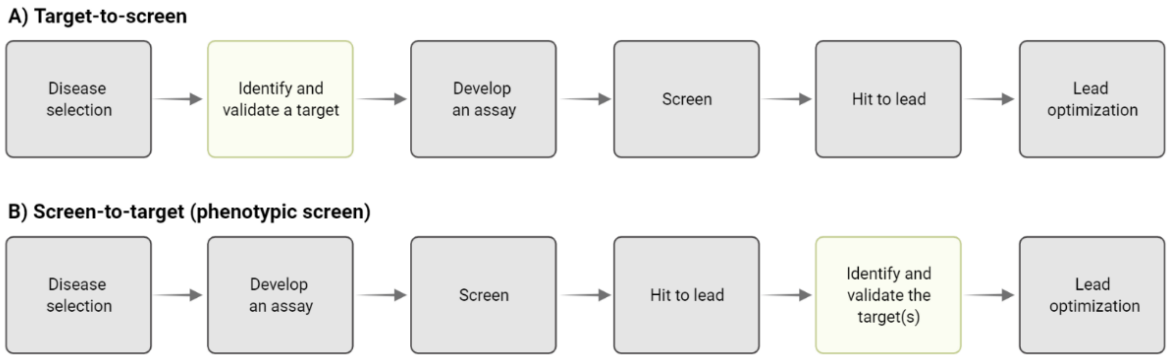

Figure 6. The two main compound screening strategies that can be performed and consequent steps in the early drug discovery process. A) The target-to-screen strategy first identifies an interesting target and then develops an assay to find compounds that modulate the activity of that target. B) The screen-to-target strategy involves assay development first without prior selection of a particular target. Figure created with BioRender.

2.7.1.2 Hit to lead

A 'hit' is defined as a compound that is found to be active in a compound screen and whose activity is confirmed upon retesting using alternative experimental techniques. Hits derived from a screening campaign are progressively narrowed down to promising candidates by a process called hit-to-lead or lead generation. Usually, two activities are initially undertaken: first, 'hit expansion', in which new compounds that were not present in the original screen but that resemble the screen hits in structure or biological activity are tested in the model to broaden the chemical space for hit selection. Once these compounds are tested, lead optimization is performed through structure-activity relationship (SAR) studies. In this process, successive modifications are performed in the compound's structure and their effect on the biological activity is assessed. One can then discern the structural components required for biological activity, which can inform changes to moieties that are not attractive for drug development, for example. SAR studies can also inform about positions in the molecule that can be exchanged without affecting the compound's activity, which can be used for the generation of probe compounds for subsequent target identification strategies.

Several computational methods can be applied at this stage to identify and remove potentially problematic compounds. These molecules include pan-assay interference compounds (PAINS), frequently hits found in unrelated compound screens due to unspecific effects, as well as compounds with unsuitable chemical profiles for further drug development [231, 232]. There are several means to identify the latter, including the failure to possess drug-like features mentioned in 'Lipinski's rule of five' [233]. However, concepts such as these are not definitive barriers to development and therefore a detailed assessment with medicinal chemists is required in order to decide which compounds are worth following up. This could

be complemented with *in silico* pharmacokinetic studies, which can predict areas of particular concern regarding the future efficacy of the compound in *in vivo* assays.

Given their roles in cancer, and the successful proof-of-concept of drugs targeting them, the UPS and autophagy remain valuable targets for drug development. These large and complex pathways have many potentially druggable enzymes, a feature that makes phenotypic screening attractive to survey a wide target landscape in a screening campaign. This may ultimately lead to identifying small molecules which can provide new mechanisms of action and even new targets to inform future target-to-screen campaigns.

3 RESEARCH AIMS

The work presented in this thesis is centered on the use of fluorescent proteins that allow monitoring of the UPS, with the two major aims of:

- **Identifying new small molecule inhibitors of the UPS and determine their mechanism of action and anti-cancer potential;**
- **Investigating modifiers of protein delivery to the proteasome with the overarching goal of identifying novel therapeutic targets.**

Specifically, the aims of the papers presented herein were:

- Papers I and II: define the mechanism of action of two novel inhibitors of the UPS;
- Paper III: to study the role of ubiquitylation of the ubiquitin-shuttle factor Rad23 and its effects on the delivery of substrates to the proteasome in *Saccharomyces cerevisiae*.

4 METHODOLOGICAL CONSIDERATIONS

4.1 Fluorescent UPS reporters

UPS reporter substrates are based on targeting an otherwise stable reporter protein (in this case, a fluorescent protein) for proteasomal degradation through the introduction of a degron [234]. Fluorescent proteins like GFP have long half-lives (>24 hours) but can be turned into short-lived proteins by various means:

- Protein fusions with ubiquitin in the N-terminus lead to deubiquitylation by endogenous DUBs and exposure of the N-terminal amino acid, which will determine the half-life of the engineered substrate following the N-degron pathway [235].
- Protein fusions with a mutated N-terminal ubiquitin, in which the final glycine of ubiquitin is changed to valine (G76V), preventing DUB cleavage. The uncleavable N-terminal ubiquitin is marked with ubiquitin chains that target it for proteasomal degradation by a different set of enzymes to the N-degron pathway, in a pathway called ubiquitin-fusion degradation (UFD) [236].
- C-terminal tagging of proteins with a short C-terminal linkage (CL) originally identified in a yeast screen (and coined CL1) results in a short-lived fusion protein that is degraded by the same battery of enzymes involved in the degradation of misfolded proteins from the ER. The CL1 linkage is rich in hydrophobic properties residues and therefore, unlike the N-end rule and UFD reporters, has a propensity to aggregate. This reporter, closely resembling a misfolded protein, is degraded in a ubiquitin-dependent manner [237].
- Proteins of natural origin have also been exploited as reporters for the UPS, but mainly outside of their usual context, such as the protein CD3δ. Usually expressed in T-lymphocytes, CD3δ is directly targeted for degradation by the ER-associated degradation (ERAD) pathway when ectopically expressed in cancer cells of other origins [238].
- The degron present in the ornithine decarboxylase (ODC) enzyme was found to be a versatile motif that can also target other proteins for degradation in a ubiquitin-independent manner. Hence, this reporter can be used to monitor this pathway in cells [239].

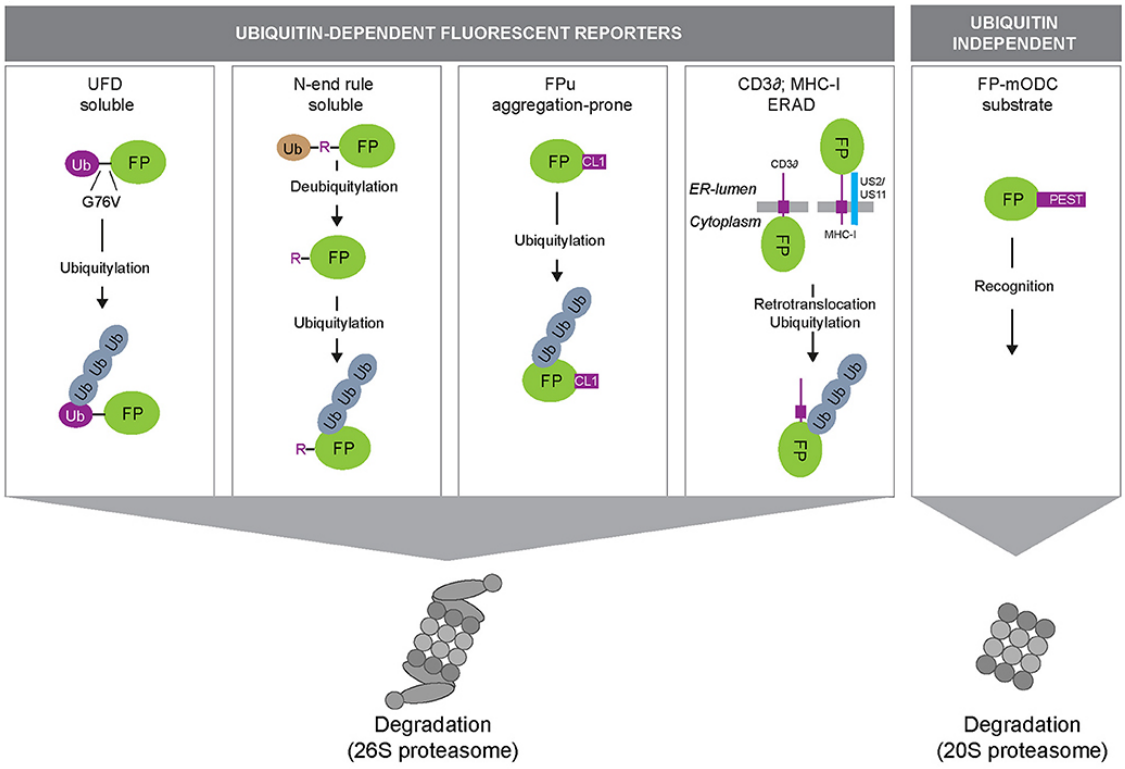

Figure 7. Fluorescent UPS reporters. Adapted from [70]. FP = fluorescent protein.

The main strength of using engineered fluorescent reporters to monitor the UPS is that, unlike endogenous substrates of the UPS, they lack biological activity and therefore do not possess unintended- nor confounding effects related to the many functions that endogenous UPS substrates perform (e.g, TP53 can accumulate following UPS impairment [240] but can also be functionally stabilized in response to genotoxic stress [241]). They are also readily detectable by an array of techniques, including fluorimetry, flow cytometry and fluorescence microscopy. Their use has been reported with a diverse range of cell lines and *in vivo* models, which means that the effectiveness of small molecules found in high-content screens can also be tested in more complex models of disease. Lastly, these reporters are holistic in nature and permit interrogation of the UPS in its entirety, in a target-agnostic approach that can lead to unanticipated targets and/or mechanisms of action that impair directly or indirectly the functionality of the pathway [70]. Limitations associated with the use of these reporters include their sensitivity to gene transcription and protein translation modulators. Introducing an internal, stable reference protein can help control for these eventualities [242], which presents the added advantage of enabling the discovery of UPS stimulators in genetic or compound screens.

Many reporter systems [234], active-site directed probes [243] and peptide-based model substrates are also available to monitor the activity of specific components of the UPS. Of specific interest for the work in this thesis are peptide-based substrates of the proteasome, as

well as activity-based probes for monitoring DUB activity. In brief, peptide-based proteasome model substrates are small (3-4 amino acids) peptide chains coupled to a fluorogenic group, such as 7-amino-4-methylcoumarin (AMC). The intrinsic fluorescence of AMC is quenched when attached to the peptides and recovered when released. The amino acids used for the peptide determine their specificity for β1, β2 or β5 activity [244]. Their small size allows them to bypass the need for ubiquitylation to enter the 20S proteasome core and, therefore, they can monitor 20S and 26S activity in cell lysates from compound-treated cells or be used *in vitro* with isolated proteasomes. To monitor overall DUB activity in lysates and *in vitro*, ubiquitin covalently linked to AMC (Ub-AMC) is also used [245].

Alternatively, active-site directed probes can also assess overall DUB activity with the added advantage of allowing monitoring of specific DUBs provided a specific antibody is available. These probes have epitope-tagged ubiquitin (HA-Ub) coupled to a thiol-reactive group, such as vinyl sulfone, that when recognized by DUBs *in vitro* act as 'suicide substrates' that remain attached to the DUB. Denaturing gel electrophoresis followed by western blot analysis with an anti-HA antibody then enables monitoring of all DUB activities, whilst probing for a specific DUB of interest results in a band shift (DUB+Ub) in the gel if the DUB is active [246].

4.2 Methods for monitoring autophagy

Use of the autophagosome marker LC3B

In paper II, we used a cancer cell line stably expressing GFP-tagged LC3B to assess whether CBK79 affected autophagy [247]. Compounds modulating autophagy can be easily identified by microscopy using this cell line by the formation of GFP-positive puncta in the cytoplasm. However, the induction of GFP-LC3B foci does not give information on the functionality of the pathway since an increase in puncta can correlate both with increased autophagic flux due to induction, or accumulation of autophagosomes due to inhibition at the level of the lysosome [248]. Hence, specific inhibitors of lysosome function, such as the v-ATPase inhibitor bafilomycin A1 (BafA1) should be used to discern between these possibilities.

Use of (un)specific autophagy inhibitors

BafA1 disrupts lysosome acidification and autophagosome-lysosome fusion [249]. Although these defects are usually linked to the inhibition of the v-ATPase, BafA1 has been shown to also inhibit the activity of the endoplasmic reticulum (ER) calcium pump Ca-P60A/SERCA [250], leading to increased calcium concentrations that can inhibit the fusion with the lysosome [251]. Chloroquine and its derivatives are also commonly used to neutralize lysosomal pH and lead to autophagy blockade. However, high concentrations can also induce

LC3 lipidation to membranes other than the autophagosome [106]. Overall, it is important to use several inhibitors when assessing autophagic flux and/or complement with genetic models to understand the mechanisms of action of novel autophagy modulators [94].

Assessing autophagic flux

To have a more direct readout of the functionality of the pathway, the tandem tagged version of LC3B (mRFP-GFP-LC3) can provide a direct measure of autophagic flux by microscopy or flow cytometry. While GFP is quenched in acidic compartments, mRFP is stable; hence, analysis of $GFP^+/mRFP^+$ versus $mRFP^+$-only puncta or the relative mRFP/GFP intensities, respectively, provide and assessment of autophagic flux [252]. This analysis can be complemented with the detection of the autophagy receptor SQSTM1/p62 by western blot, as it is degraded by autophagy together with its cargo [248].

Furthermore, the analysis of the turnover of long-lived proteins can provide a quantitative measurement of the efficiency of the flux. In this assay, radiolabelled amino acids are supplied to cells, followed by a 'chase' period with medium exchange including non-labelled amino acid, long enough to allow degradation of the labelled short-lived proteins [253]. After another medium exchange, a second 'chase' starts, in which is expected that only long-lived proteins in the cell remain labelled. After a few hours, protein degradation is assessed by precipitating the proteins with trichloroacetic acid and, after centrifugation, measuring the radioactivity present in the soluble fraction (free amino acids and small peptides resulting from degradation) and the insoluble fraction (undegraded proteins). The rates of degradation can be compared between vehicle-only and compound-treated cells, and the contribution of autophagy to the degradation of long-lived proteins revealed by treating cells with BafA1 [253].

Overall, only by combining several of the aforementioned methods a reliable assessment of the effect on autophagy of an experimental compound can be obtained.

4.3 Methods for target identification

Chemical proteomics: affinity-based protein profiling (ABPP)

The field of chemical proteomics research is an interdisciplinary area that integrates aspects of chemistry, mass spectrometry and cell biology to (i) enable the design of a probe compound and (ii) to isolate and identify the proteins interacting with the molecule in cells and/or cell lysates. We used an ABPP approach in which we introduced an alkyne into the compound to obtain a probe compound that allows covalent linkage of a tag-of-choice to facilitate biochemical isolation after treating the cells. This is an important feature: the

alkyne's small size and relatively low reactivity are desirable characteristics that are unlikely to modify the activity of the parental compound, although is important to verify this in a case-by-case basis by direct comparison to the parental compound in cell-based assays [254].

Lysates from cells treated with the probe can then be subjected to copper(I)-catalyzed azide alkyne cycloaddition (CuAAC, also known as click chemistry reaction) resulting in a covalent linkage with, in our case, a trifunctional linker containing the dye tetramethylrhodamine (TAMRA), followed by a linker and a biotin molecule. Streptavidin beads can then be used to isolate the probe and its interactors and given the strength of the streptavidin-biotin interactions, harsh conditions can be applied to enrich only covalent interactors of the molecule. The resulting enriched fractions can, ultimately, be subjected to trypsin digestion to generate small peptides, which will be separated by their physical properties using liquid chromatography (LC) and identified by mass spectrometry (MS). The abundance of the protein can either be inferred from the mass spectrometer signal (and obtain only a relative quantification between conditions) or be quantified if using SILAC (stable isotope labeling with amino acids in cell culture) [255]. Potential protein targets are then identified after benchmarking to the negative control, a sample processed in parallel in which vehicle is applied instead of probe compound. This is an essential negative control to exclude non-specific binders to the streptavidin beads [254].

Since this strategy is restricted to electrophilic compounds, which can have promiscuous binding inside the cell due to their reactive nature with nucleophiles present in several amino acids [256], several controls can be applied to understand which interactions are specific. For example, a competition assay in which excess of parental compound is pre- or co-applied with the probe can saturate specific binding pockets and therefore result in decreased interaction compared to the probe sample [257].

Thermal proteome profiling upon drug treatment

ABPP is limited by the need to introduce the tag into the parental compound, which can be a lengthy endeavor that requires expert chemistry knowledge and can ultimately fail if the structure-activity relationship studies reveal limited possibility to modify the compound without losing biological activity. Hence, other strategies have been developed to identify protein interactions with unmodified compounds.

The Cellular Thermal Shift Assay (CETSA) is a method to study compound-protein interactions based on protein stability. The technique is based on thermal stabilization of a protein when bound to a ligand, a concept that was used in classical techniques such as Drug

Affinity Responsive Target Stability (DARTS) [258]. CETSA allows monitoring of protein stability in living cells or tissues, an advantage that allows evaluation of protein interactions in a disease-relevant model. After compound treatment, cells are lysed and equal amounts of lysate are subjected to increasing temperatures. After separation of the aggregated protein fraction, proteins that remained soluble in the lysates can be used for SDS-PAGE followed by immunoblot with a specific antibody for the protein(s) of interest [259]. The melting curve for the protein can then be compared between compound-treated and a vehicle-only control to assess whether stabilization occurs, indicative of compound binding [259]. When combined with quantitative multiplexed proteomics, thermostability can be applied to interrogate the proteome in an unbiased manner through a technique called thermal proteome profiling (TPP) [255]. For quantitation, isobaric mass tags are introduced in the sample, which covalently bind the peptides present after trypsin digestion and allow absolute quantification of the peptides in the sample. These quantitative methods lie outside of the scope of this thesis, but are comprehensively reviewed in [260].

The main challenge of these proteomics-based approaches is the fact that membrane-bound proteins are usually lost during sample preparation. In addition, low abundant proteins are usually excluded or 'masked' by higher abundant ones [261]. Hence, these methods can miss important compound-protein interactions linked to biological activity.

4.4 Testing new compounds with potential anti-cancer properties

In paper I, we used a human tumor xenograft model to test the hypothesis that CBK77 effects can lead to reduced tumor growth *in vivo*. We used the NMRI mice homozygous for the nude spontaneous mutation (nu/nu), which results in athymic mice with limited cellular immunity (complete absence of T cells and partial defects in B cells) that allows the growth of cancer cell lines of human origin [262]. Cancer cells are injected under the skin to constitute a subcutaneous model of tumor growth amenable to test experimental compounds and their anti-cancer potential. This is a widely used model in cancer biology that, compared to testing compounds in cell lines grown *in vitro*, has the advantage of being a cost-effective way to translate cytotoxic phenotypes in a more relevant setting that recapitulates human tumors to a certain extent. However, the use of cell lines limits the reproducibility of findings, as these cells lose their initial characteristics during extended passages over time [263]. In that respect, results obtained with this xenograft model can be complemented with other disease models to gain confidence in the anti-cancer potential of a candidate compound. A good example of this pipeline includes the development of bortezomib [264].

Although mouse and human possess differences in aspects of their physiology, mouse models represent good pre-clinical models to address early pharmacokinetics, providing an insight into how tissues process the compound. Mouse models also enable study of pharmacodynamics (the effects of the compound on tissues) and limiting toxicities of experimental compounds when transitioning from the drug discovery to the development phase [265].

4.5 Ethical considerations

The work presented in this thesis uses three models to study the mechanisms of protein degradation and the mechanism(s) of action of novel compounds that inhibit the UPS: cancer cell lines of human origin, xenograft models in mice and *S. cerevisiae*. The ethical considerations regarding these models include the proper authorization from the human subjects who donated cells for the establishment of these cell lines and the application of proper welfare standards in animal studies. Besides HeLa cells, and to the best of our knowledge, there are no other ethical concerns regarding the remaining cell lines used in this work. The animal experiments presented in paper I were approved by the Stockholm ethics committee for animal research (no. N231/14), appointed and under the control of the Swedish Board of Agriculture and the Swedish Court.

5 RESULTS AND DISCUSSION

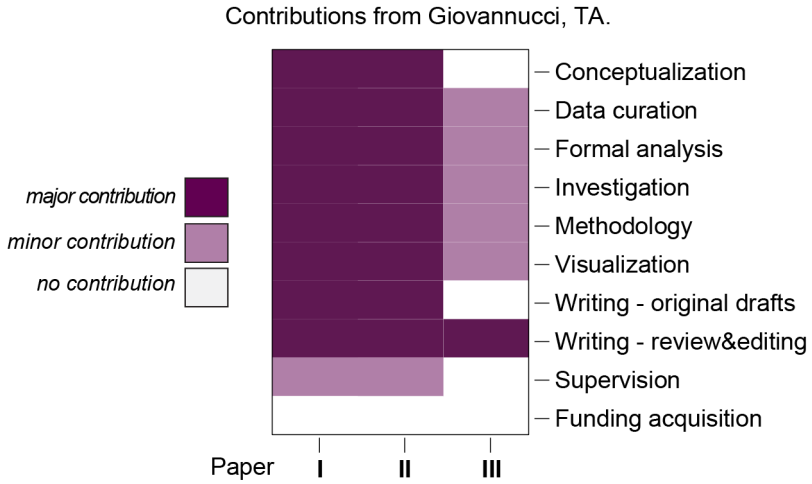

Figure 8. Matrix summarizing my contributions to each of the papers herein discussed following an adaptation of CRediT (contributor roles taxonomy). Supervision here refers to supervision of master level students' work.

5.1 Paper I: Inhibition of the UPS by an NQO1-activatable compound

The hyperactive state of the UPS in cancer cells makes it an attractive therapeutic target for drug development. Due to the extensive functional redundancy in the system, the shared functions of core proteins in the UPS with autophagy (e.g, VCP/p97, ubiquitin, and ubiquitin-binding proteins) and the array of cellular processes beyond proteolysis regulated by the UPS it is often difficult to predict, *a priori*, the net effect of targeting a specific component of the system. To overcome this limitation, we undertook an unbiased, phenotypic approach to screen small molecules using a fluorescent reporter protein that monitors ubiquitin-dependent degradation in cells. This reporter lacks biological activity and can be readily detected and quantified using high-content automated microscopy. The same strategy was also employed in paper II.

Summary of the results

We performed a phenotypic screen using a small but diverse library of 5720 compounds with drug-like properties and interrogated their capacity to inhibit protein degradation by monitoring the UPS fluorescent reporter ubiquitin^{G76V}-yellow fluorescent protein (Ub^{G76V}-YFP) reporter stably expressed in a melanoma cancer cell line [266]. Only one compound (CBK092352) induced accumulation of the reporter and showed signs of cellular toxicity. We found that the 5-nitrofuran ring in the molecule was required, and identified a position within the benzothiazole that accepted modification with several chemical groups. The latter finding allowed us to introduce an alkyne tag in the compound to create CBK77^{CLICK}, an activity-based probe for target identification. Whilst the presence of the nitrofuran ring raised

concerns due to its reactivity and potential for target promiscuity, the series of structural analogues to CBK092352 contained a molecule, CBK77, that was more potent compared to another analogue, CBK07, which closely resembled CBK77 at a structural level but had no biological activity. This suggested that the nitrofuran ring was required but not sufficient for UPS impairment and prompted us to study the mechanisms of action behind CBK77's effect, keeping CBK07 as an important negative control in our assays.

CBK77 had a dose-dependent and irreversible effect on the UPS and cell viability, with an IC_{50} in the micromolar range. We tested CBK77 in combination with either the caspase inhibitor Q-VD-OPh or the necroptosis inhibitor necrostatin, and found that only the caspase inhibitor could rescue cell viability, suggesting that CBK77 kills cells primarily by apoptosis.

The inhibitory effect on protein degradation affected several ubiquitin-dependent fluorescent reporters of a different nature (either soluble or aggregation-prone substrates with distinct enzymatic requirements for their degradation), as well as endogenous substrates such as TP53, whilst degradation of a ubiquitin-independent substrate was unaffected. The degradation of ubiquitylated substrates requires their transfer to the proteasome, where deubiquitylation and unfolding must take place to grant access to the proteolytic chamber of the proteasome. Hence, compounds inhibiting early steps, such as E1 inhibitors, or late steps in the pathway, such as the proteolytic activity of the proteasome or the proteasome-associated DUBs, would phenocopy the effects of CBK77. However, CBK77, but not CBK07, induced strong accumulation of polyubiquitin conjugates, arguing against an impairment at the level of the E1-E2-E3 enzymatic cascade. Proteasome activity assays and DUB assays with fluorogenic substrates also excluded inhibition of the β5-activity of the proteasome and a gross defect on DUB activity. To complement this finding, we used DUB activity probes, which also pointed to conserved overall DUB activity in cells and excluded the inhibition of the proteasome-associated DUBs USP14 and UCHL5.

We used a genome-wide cell viability CRISPR/Cas9 knockout screen to determine mechanisms of resistance to CBK77, which could provide information about possible mechanisms of action. We found a striking enrichment of guides targeting the gene NQO1 in CBK77-resistance cells, suggesting that NQO1 may be required for CBK77 activity. We validated these findings using different siRNAs targeting NQO1 and in MDA-MB-231 cells, naturally deficient in NQO1 due to a polymorphism that renders the protein unstable, which showed remarkable resistance to CBK77. In addition, using well validated inhibitors of NQO1, we found that its catalytic activity was required for CBK77's impairment of the UPS and subsequent cell death.

NQO1 is an oxidoreductase that can catalyze the reduction of quinones or nitroaromatic compounds [267] in a reaction mediated by a 2-electron transfer mechanism coupled to either of the two cofactors NADH or NAD(P)H [268]. *In vitro* assays with recombinant NQO1 showed that CBK77, but not CBK07, was metabolized by NQO1, suggesting that metabolites resulting from processing CBK77 are responsible for UPS impairment. In line with this assumption, we found by analytical HPLC-MS that CBK77 levels decreased in the presence of NQO1, whilst metabolites that matched to those expected by the 2-electron reductions of nitrofuran-containing compounds appeared. Conversely, CBK07 remained in the reaction and produced only trace amounts of some of these species.

To identify the direct targets of CBK77, we developed the probe compound CBK77^{CLICK}. Biochemical enrichment of proteins bound to CBK77^{CLICK} identified ubiquitin as one of the most abundant proteins in the interactome of CBK77. We could validate this interaction in ubiquitin-enriched samples from CBK77^{CLICK}-treated cells. Further, this interaction was abrogated by treatment with an NQO1 inhibitor, showing that CBK77 binding to ubiquitin also required bioactivation of the probe compound.

To discern if the compound was binding directly to ubiquitin or to a ubiquitylated substrate, we treated ubiquitin-enriched fractions with USP2, a promiscuous DUB that can cleave both polyubiquitin chains bound to substrates and free ubiquitin chains. We found a TAMRA-labelled band by SDS-PAGE that coincided with free ubiquitin detected by western blot, arguing in favor of direct binding to ubiquitin. When we tested the effects of such binding *in vitro*, we found that two unrelated DUBs from different classes were less efficient at cleaving ubiquitin chains that had been exposed to NQO1-activated CBK77.

Lastly, we generated a compound formulation suitable for *in vivo* assessment of CBK77 antitumor activity. We found a modest but significant reduction in tumor growth and weight following subcutaneous xenotransplantation of adenocarcinoma cells into mice without overt signs of toxicity.

Discussion of the results

Mechanisms underlying CBK77-induced cytotoxicity

Our study revealed that CBK77 does not interfere with global ubiquitylation, deubiquitylation or proteasome activity, differing from the conventional mode of action of proteasome inhibitors. We found that CBK77 inhibitory activity is confined to ubiquitin-dependent degradation of protein substrates, including the endogenous UPS substrate TP53, and demonstrated that their stabilization resulted from a delay in their clearance. However,

we cannot unequivocally exclude the possible contribution of functional stabilization of TP53 as well, as we show that CBK77 kills cancer cells by caspase-dependent mechanisms, and TP53 can be stabilized and induce apoptosis through caspases [269]. However, we did not detect an increase in the DNA-damage marker γH2AX (unpublished data), arguing against TP53 stabilization resulting from genomic damage. In addition, the immunodetection of TP53 by western blotting formed a characteristic ladder pattern that is consistent with ubiquitylated forms of TP53. This further argues against stabilization due to apoptotic mechanisms, for which deubiquitylation would be expected [270]. Our observations are more consistent with a model in which CBK77 induces global impairment of ubiquitin-dependent degradation, leading to accumulation of misfolded proteins that would in turn result in activation of the heat shock response [271]. In agreement, we observed a strong upregulation of the chaperone Hsp70 that mirrored the increase of ubiquitin conjugates.

Bioactivatable nature of CBK77

The CRISPR/Cas9 screen and following biochemical assays demonstrated that CBK77 activity strictly depends on bioactivation by NQO1. Given the nitrofuran ring present in CBK77, it is not surprising that reduction is required for its activation, as is reported for nitrofuran-containing compounds like nifurtimox in the treatment of Chagas disease [272]. In this particular instance, a type I nitroreductase (NTR) reduces- and therefore activates nifurtimox in disease-causing parasites. This enzyme utilizes FMN as a co-factor and, coupled to NAD(P)H, catalyzes the 2-electron reduction of the parental nitro group to generate nitroso and hydroxylamine derivatives [272]. The hydroxylamine can be further processed to generate the amine derivative or, alternatively, 'opening' of the furan ring may occur. This is true of nifurtimox, with the unsaturated open-chain nitril being the major peak detected upon NTR reduction by HPLC-MS [272] and the metabolite responsible for its cytotoxic effects.

Analogously, NQO1 utilizes FAD as co-factor and can also catalyze 2-electron reductions coupled to NAD(P)H or NADH. While quinones are its primary natural substrates, NQO1 can also reduce certain nitroaromatic compounds as we confirmed for CBK77, which was found to be reduced as efficiently as the classic NQO1 substrate quinone menadione [273]. However, unlike nifurtimox, our HPLC-MS analysis of CBK77 metabolites did not yield any detectable peak that would match to the open-chain nitril. Instead, the hydroxylamine derivative was detected, which has been suggested to form covalent adducts with proteins and nucleic acids *in vitro* [274]. The affinity-based protein profiling (ABPP) performed with $CBK77^{CLICK}$ identified several proteins that, given the denaturing conditions used, are expected to interact covalently. For a drug candidate, such polypharmacology may be an

advantage, as it would be more difficult to generate resistance and might be needed to reach the intended anti-cancer activity [275]. However, the extent to which those individual interactions influence the compound's activity, and their potential mechanisms of action, will require further investigation. An ABPP experiment including competition with parental compound may help to validate *bona fide* interactors of the compound and in evaluating their contribution to CBK77-induced protein degradation and cell death.

While CBK07 closely resembles CBK77, it did not induce the same cellular responses as CBK77, nor was an equally good substrate of NQO1. However, it was not completely inert: high concentrations and long incubations with CBK07 led to UPS impairment in some cells, and the NQO1 *in vitro* assay showed very limited but still detectable activity towards CBK07, which was abrogated by the NQO1 inhibitor dicoumarol. Analysis of the compound's effect on the thermal stability of NQO1 using the cellular thermal shift assay (CETSA) revealed that CBK07 stabilized NQO1, while CBK77 had little, if any effect, on NQO1's thermal stability (unpublished data). When analyzing known interactors of NQO1 by CETSA, we found that the inhibitor ES936 stabilized NQO1, in contrast to the substrate menadione, which did not have an effect NQO1's thermal stability (unpublished data). Thus, both the enzymatic and CETSA analysis depict substrate-like properties for CBK77, while CBK07 shared similarities with the effects observed with an NQO1 inhibitor. Together these data show that CBK77, in contrast to CBK07, is efficiently metabolized by NQO1, giving rise to unstable, reactive metabolite(s) that could covalently bind to intracellular targets. Further, it would be interesting to study if CBK07 could impair NQO1's enzymatic activity, either directly or through the formation of reactive metabolite(s). Mass spectrometry analysis of covalent adduct formation with recombinant NQO1 incubated with CBK07 could provide some insights into this possibility.

CBK77 mechanism of action

One potential explanation for why CBK77 only affects ubiquitin-dependent degradation is that it may target a central protein for this process. In the ABPP experiment, ubiquitin appeared as a prominent interactor with CBK77. Furthermore, CBK77 hindered the cleavage of ubiquitin chains *in vitro* by DUBs of different families without impairing DUB or proteasomal activity.

UPS impairment by ubiquitin-interacting compounds is not unprecedented, as was shown for the ubiquitin-interacting molecules ubistatins [276]. These are a group of large molecules

that non-covalently interact with the hydrophobic conserved patch of ubiquitin. Ubistatins' symmetrical structure endows them with the capacity to simultaneously bind two ubiquitin monomers present in polyubiquitin chains, acting as a ubiquitin-chain intercalator impairing binding to diverse ubiquitin-binding domains, which serves to block the interaction of ubiquitin with DUBs, shuttle factors and other UPS components [277]. While ubistatins display profound inhibitory activity *in vitro*, their chemical structure is less suitable for drug development and CBK77's characteristics might render it a better candidate. First, its low molecular weight provides greater cell permeability. Second, the need for enzymatic bioactivation could restrict CBK77 effects to cancer cells expressing elevated levels of NQO1.

The irreversible collapse of the UPS in the absence of global inhibition of either ubiquitylation, DUB or proteasome activity all support a model in which impaired or inefficient disassembly of polyubiquitin chains leads to an accumulation of UPS substrates that ultimately causes cell death. However, we cannot conclusively discard the possibility that other target(s) are involved. The lack of overall DUB inhibition does not exclude specific DUBs possessing key roles in the UPS that may be modified by CBK77. For example, while we did not observe inhibition of USP14 or UCHL5, we did not study Rpn11/POH1, the JAMM metalloprotease located in the lid of the 26S proteasome. Further work addressing the CBK77-ubiquitin interaction, including the identification of the binding site(s), could help to elucidate the mechanisms behind CBK77-induced UPS impairment.

CBK77 as a chemotherapeutic agent

To the best of our knowledge, CBK77 is the first UPS inhibitor that relies on activation by an endogenous enzyme for its inhibitory activity. While the first non-peptidic proteasome inhibitor, the natural product lactacystin, also behaves as a prodrug, its active component is generated by a spontaneous chemical reaction without involvement of cellular enzymes [278]. The use of prodrugs that are activated in the altered intracellular environment of cancer cells is an attractive strategy for enhancing tumor specificity. It has been previously proposed that NQO1 can be exploited for this purpose since it is upregulated in many solid tumors as part of the antioxidant response governed by the transcription factor NRF2 [279]. The elevated levels of this oxidoreductase can thereby restrict the effect of redox-activated prodrugs primarily to cancer cells.

The xenotransplant model presented shows limited but significant inhibition of tumor growth in treated mice, without acute signs of toxicity. We have yet to explore target engagement within the tumor, which could start by studying biomarkers of UPS impairment in tumor sections and, if possible, identifying CBK77 metabolites in tumor samples by mass spectrometry. This preliminary *in vivo* assessment supports but does not conclusively show selective targeting of cancer cells compared to non-transformed cells, which could be further assessed *in vitro* by treating primary non-cancer cell lines. Additional experiments to understand the concentration of drug reached in the model compared to the concentrations used *in vitro* to obtained UPS inhibition are also necessary to understand CBK77 pharmacodynamics *in vivo*.

5.2 PAPER II: Identification of a novel compound that simultaneously impairs the UPS and autophagy

The work presented in this manuscript was performed in parallel to paper I and represents a characterization of another hit identified whilst seeking novel anticancer small molecules disturbing proteostasis. The motivation to perform a second screen came from the availability of a larger chemical library at the Chemical Biology Consortium Umeå (CBCU), and the possibility to access a streamlined and automated pipeline of cell seeding, liquid handling and high-content imaging as opposed to the semi-automated workflow performed in paper I.

Summary of the results

We performed a phenotypic screen using a library consisting of 17,500 chemically-diverse compounds with drug-like properties, and interrogated their capacity to inhibit protein degradation by monitoring the fluorescent Ub^{G76V}-YFP reporter stably expressed on a cancer cell line. One molecule, CBK267272 (or ChemBridge 7869981) induced accumulation of the Ub^{G76V}-YFP substrate in a dose-dependent manner, correlating with a decrease in cell viability. The molecule contained an aminothiazole and two pyridine rings and accepted a few chemical modifications to its structure without losing activity. One such accepted modification, the addition of a methyl group on position 3 on the left-side pyridine ring, resulted in a compound with markedly increased potency, coined CBK79, which we selected for further study.

To characterize its effects on the UPS, we treated several cancer cell lines expressing fluorescent reporters of the UPS with CBK79 and found that it induced a global UPS impairment that affected both ubiquitin-dependent and -independent substrates, as well as endogenous substrates such as TP53 and HIF1A. This impairment was not due to inhibition of the proteasome β5 activity, which was largely maintained. Simultaneously, CBK79 also induced accumulation of GFP-tagged LC3B, a classical marker for autophagosomes, which appeared mostly restricted to the perinuclear area. We confirmed that the accumulation of LC3B puncta reflected lipidated LC3B embedded in vesicles, as opposed to aggregated LC3B, by observing that this accumulation was absent in cells devoid of the E3 ligase required for lipidation, ATG16L1. This was further confirmed by western blot analysis with an LC3B-specific antibody that showed the appearance of a faster migrating band attributable to lipidated LC3B (LC3B-II). U2OS cells stably expressing the tandem reporter mRFP-GFP-LC3B showed a profound decrease in mRFP/GFP ratio when analyzed by flow cytometry, suggesting that the increase of LC3B-II upon CBK79 administration was due to a blockade of the autophagic flux at the level of the lysosome. This observation was confirmed by co-treatment with the autophagy inducer Torin-1, which further increased the amount of LC3B-

II compared to treatment with CBK79 alone. The impairment of the flux correlated with a decrease in degradation of long-lived proteins, a common readout to assess functionality of the pathway, demonstrating that CBK79 blocks the autophagic flux under basal conditions.

Interestingly, co-treatment of CBK79 with bafilomycin A1 (BafA1), inhibitor of the lysosomal v-ATPase and therefore a broad autophagy inhibitor, abolished LC3B lipidation. Conversely, co-treatment with chloroquine, another commonly used autophagy inhibitor with the capacity to block lysosomal degradation, did not. We additionally found that LC3B lipidation mediated by CBK79 was insensitive to 3-MA, inhibitor of the initiation PtdIns3K complex, and still occurred in cells devoid of ATG13, an essential component of the ULK1 complex that is also needed for canonical initiation of autophagy. This indicated that, alongside the impairment on the known canonical autophagy, CBK79 also induced an unconventional type of LC3B lipidation. We validated this further by checking CBK79 effects on LC3B in a cell line that can only execute canonical autophagy (ATG16L1[1-249] lacking the WD40 domain) and found only a minimal increase in LC3B-II on western blot, suggesting that the majority of LC3B-II upon CBK79 treatment can be attributed to a noncanonical form of LC3B lipidation.

After CBK79 treatment, we observed activation of several stress responses commonly upregulated upon proteotoxic stress, including aggresome formation, nuclear enrichment of heat shock factor 1 (HSF1) together with induction of Hsp70, and a decrease in protein synthesis. In addition, CBK79 induced the formation of cytosolic granules positive for the RNA binding protein and stress granule marker G3BP1 in puromycin-sensitized cells, as demonstrated by other compounds that induce proteotoxic stress [280]. In agreement, exposing cells to a transient temperature rise prior to administration of CBK79 increased the levels of Hsp70 and prevented the accumulation of the UPS reporter and polyubiquitin conjugates, whilst the levels of LC3B-II remained unchanged. This preconditioning of cells strongly argued that CBK79 mediates its biological activity through induction of proteotoxic stress and further indicated that the distinct effects on autophagy and the UPS are uncoupled.

Discussion

Simultaneous global impairment of the UPS and autophagy inhibition following CBK79 treatment

Owing to their essential homeostatic functions in all cells, the UPS and autophagy have both been explored as therapeutics targets for cancer treatment. This has led to the development of clinically approved proteasome inhibitors for the treatment of multiple myeloma and mantle cell lymphoma, and to the exploration of chloroquine (CQ) and hydroxychloroquine

(HCQ), two FDA-approved drugs that target lysosomal degradation, in various clinical trials [281]. Our data for the novel compound CBK79, identified in a screen for UPS inhibitors, revealed that this active molecule inhibits both the ubiquitin-dependent and independent degradation of short-lived proteins by the UPS, as well as degradation of long-lived proteins by autophagy.

The UPS and autophagy share many components and points of molecular crosstalk that might explain why inhibiting one of these two processes could lead to modulation of the other as well. In many instances, this leads to compensatory mechanisms that can hamper the efficacy of anticancer agents targeting these pathways. Inhibition of UPS-mediated degradation has been reported in a model demonstrating that long-term inhibition of autophagy results in the inhibition of ubiquitin-dependent degradation through the progressive, SQSTM1/p62-mediated sequestration of UPS substrates [126]. Since we detected simultaneous- rather than sequential inhibition of these pathways, it is, however, unlikely that CBK79-induced impairment of both pathways is mediated by this type of mechanism. Instead, it likely places these effects downstream of CBK79's potential target(s).

Given that chaperones operate upstream of the degradative functions of these pathways, one possibility is that CBK79 affects the chaperoning capacity of the cell by directly inhibiting specific components of the chaperone pathway. VCP/p97 is a crucial molecular chaperone shared by both degradative pathways, which has well-established roles in modulating ubiquitin-dependent degradation by the proteasome [42], as well as in early and late stages of autophagosome formation [44, 45]. Inhibition of VCP/p97 has profound detrimental effects on proteostasis that lead to the accumulation of ubiquitylated proteins and cancer cell death. The observation that CBK79 also impairs the degradation of the ubiquitin-independent substrate ZsGreen-ODC may argue against a role of VCP/p97, which is largely restricted to the homeostasis of ubiquitylated material [282]. However, the defect in degrading this particular substrate was only evident after prolonged treatments, once inhibition of ubiquitin-dependent substrates was impaired. Hence, we cannot conclusively discard a possible role of VCP/p97 since the overload of the UPS with ubiquitylated substrates could cause a global impairment of proteolysis by the proteasome [119].

Alternatively, CBK79 could cause damage and/or unfolding of proteins (e.g., by inducing oxidative stress or by targeting a component critical for homeostasis of newly-synthesized proteins), leading to a sharp increase in misfolded proteins that can overload the chaperoning machinery. We found that inducing thermotolerance by applying mild heat shock prior to CBK79 treatment prevented UPS impairment but not LC3B lipidation, suggesting that the

effects on the UPS could be explained by this mechanism and that CBK79-induced impairment of autophagy is a phenomenon disconnected from its inhibitory effect on the UPS.

To elucidate the molecular target(s) behind these effects, we are using ABPP, as we did in paper I. The chemical structure of CBK79, unlike CBK77, is devoid of electrophiles and it is therefore not expected to readily form covalent adducts with proteins. Hence, a photocrosslinker moiety has been introduced to preserve non-covalent interactions.

CBK79 induces cellular stress responses

We observed that treatment with CBK79 >8h reduced protein synthesis significantly. The unfolded protein response (UPR) is a prominent cellular response to stress that can lead to such a reduction [128], which would be in line with the proteotoxic stress inflicted by CBK79. One of the hallmarks of UPR activation is the splicing of the XBP1 transcript, which upregulates the transcription of components of the ERAD pathway to respond to decreased protein folding rates in the ER [129]. We detected only a transient increase in this transcript upon CBK79 treatment that was minimal compared to other ER stressors, such as tunicamycin (unpublished data), rendering it unlikely to be a downstream effect of IRE1α activation. It remains unclear if other branches of the UPR could be mediating this effect. Combining CBK79 with inhibitors of PERK and/or eIF2α, alongside the detection of alternative UPR sensor ATF6 by western blot, could provide clues to whether the UPR drives this stress response.

CBK79 induces noncanonical autophagy

Noncanonical autophagy is an umbrella term employed to classify all types of LC3 lipidation-related processes that do not follow the classical recruitment or action of the core autophagy proteins described in the canonical pathway of autophagosome formation [83]. BafA1-sensitive LC3B lipidation has been reported on single membrane vesicles of the endocytic system (opposed to the double membrane of the autophagosome) in processes such as LC3-associated phagocytosis (LAP) of pathogens and in ‘entosis’, the engulfment of living cells by epithelial cancer cells [102].

Recently, induction of noncanonical LC3B lipidation has also been described in response to a group of ionophores or lysosomotropic agents that are established canonical autophagy inhibitors. Ionophores are compounds that can reversibly bind to ions [283], while lysosomotropic agents, such as CQ and HQC, are weak bases that are selectively taken up into lysosomes and accumulate in their protonated forms, neutralizing lysosomal pH and

inducing non-selective impairment of autophagy [281]. This compound-induced and unconventional type of LC3 lipidation is dependent on a specific residue on the WD40 C-terminal domain in ATG16L1 (K490) [284] and occurs in single membrane endosomes/lysosomes that are 'perturbed' by the ionic or osmotic imbalance driven by these agents. The proposed mechanism for this alternative mechanism of LC3 lipidation states that neutralization of the pH in endolysosomal membranes leads to association of the v-ATPase subunits V0-V1 to increase the functional pool of v-ATPase, which in turn specifies the site of LC3 lipidation by recruitment of ATG16L1 through the aforementioned C-terminal domain [284].

CBK79-induced LC3B lipidation mirrored these characteristics and demonstrated an equivalent phenotype to lysosomotropic agents. Specifically, we observed BafA1-sensitive LC3B lipidation, an effect independent of canonical ATG proteins, and a dependence on the WD40 domain of ATG16L1, therefore raising the possibility that CBK79 perturbs intracellular vesicles as well. We are yet to identify what types of vesicles are decorated by LC3B under CBK79 treatment (single or double layered). Correlative light and electron microscopy (CLEM) would allow multi-color labeling to detect markers of endolysosomal membranes (including pH sensitive dyes such as Lysotracker), whilst also obtaining important ultrastructural information that could help to clarify the observed effects on LC3B mediated by CBK79.

The noncanonical lipidation following exposure to autophagy inhibitors, such as CQ, typically occurs in parallel to their inhibitory effects on autophagy [285], yet it is presently unclear if there is a relationship between the two. Given that both routes share the same pool of LC3, it seems plausible that LC3 could become rate-limiting under the increased demands of one of these routes, as occurs with the shared free ubiquitin pool in ubiquitin-dependent processes [3] and resulting in inhibition of the other. However, there are six hATG8 proteins, as opposed to a single ubiquitin. Further characterization of the possible roles that other hATG8 proteins may have on these noncanonical LC3-related processes could shed light on this question. In addition, one could study the flux through the canonical autophagy pathway in the 'canonical-only' ATG16L1[1-249] cell line upon CBK79 treatment. If the pathway is impaired in that cell line, it would be unlikely to be due to this 'competition' model.

Overall, our results indicate that CBK79 adds to the growing list of compounds that can induce noncanonical LC3 lipidation in parallel to an inhibition of canonical autophagy. Collectively, ours and other reports highlight the importance of using several means to study the mechanism of action following the use of GFP-LC3 as a readout for autophagy, which is

very common in high-content screens. They also underline the importance of contrasting findings made from treatment with compounds with those obtained after modulating autophagy using genetic tools, considering the broad effects of the commonly used autophagy modulators. A deeper investigation of CBK79's effects could ultimately give new insights into the interplay between canonical and noncanonical forms of autophagy.

Simultaneous inhibition of the UPS and autophagy for anticancer therapy

Separate from its molecular mechanistic underpinnings, the dual effect of CBK79 on both pathways can be an interesting starting point for therapeutics targeting proteostasis. Proteasome inhibition by bortezomib results in the accumulation of misfolded proteins and induces compensatory autophagy in cancer cells. Under these circumstances, autophagic activity upregulated via IRE1α [286] has been interpreted as a protective mechanism that renders cancer cells more resistant to bortezomib-induced cell death. Indeed, inhibition of autophagy concomitantly to proteasome inhibition increased polyubiquitylated material, induced aggregates, and restored sensitivity to bortezomib-induced cell death [287]. These dual autophagy-UPS targeting approaches have also been attempted in clinical trials combining bortezomib with HCQ, which seemed to be well tolerated and did not induce limiting toxicities [288]. Developing more specific drugs that target autophagy, or single molecules targeting both processes, could shed light into the anti-cancer potential of such interventions.

5.3 PAPER III: The deubiquitylating enzyme Ubp12 regulates Rad23-dependent proteasomal degradation

VCP/p97 regulates the degradation of a myriad of ubiquitylated substrates in collaboration with the proteasome, a task of crucial importance for PQC of ER-resident proteins (in ERAD), newly-synthesized proteins (in ribosome-associated degradation) and nuclear proteins, amongst others. The proteasome can recognize a broad variety of ubiquitin chains, but in order to be efficiently degraded by the proteasome, the substrate also needs an unstructured initiation site to catalyze unfolding of the globular structure [289, 290]. VCP/p97 possesses an ATP-dependent unfoldase activity that is required for many but not all proteasomal substrates [282], and therefore ATP-competitive inhibition of its activity is of particular interest to overcome resistance to proteasome inhibitors [47].

To deliver its partially unfolded proteins to the proteasome, VCP/p97 recruits proteins that contain UBA-UBL domains, allowing recognition of the ubiquitin chain by their UBA domain and facilitating docking at the proteasome via the UBL domain. Hence, these factors, including HHR23A/B, UBQLN proteins and DDI1/2, can participate in as many processes as VCP/p97, including autophagy [291] and the endoplasmic reticulum (ER)-associated protein degradation pathway (ERAD) [292]. Interestingly, there is evidence suggesting that they can regulate distinct pools of substrates [53], which might add a layer of specificity to the UPS downstream of the ubiquitylation machinery. Therefore, the activity of these shuttle factors might provide an attractive point of intervention for targeting proteostasis in cancer cells.

Despite their role in PQC, the underlying requirements for a substrate to be delivered to the proteasome via these factors remain largely unknown and it is unclear if these requirements are rooted at the level of the substrate (e.g, by different ubiquitin chain topologies) or at the level of the shuttle factor. There are a few examples of modulation of shuttle factor activity by PTMs, namely the phosphorylation of the shuttle factor Rad23 to regulate its interaction with the proteasome [293], while the ubiquitylation of the related shuttle factor Dsk2 reduces its binding to ubiquitin chains [294]. This opens up the possibility of several regulatory mechanisms underlying ubiquitin shuttle factors' capacity to deliver substrates to the UPS. In paper III, we study this possibility in *S. cerevisiae* and characterize a novel PTM in the yeast homologue of HHR23A/B, Rad23, that modulates substrate delivery to the proteasome.

Rad23 contains an N-terminal ubiquitin-like (UbL) domain, followed by its first ubiquitin-associated (UBA) domain (UBA1), an XPC/Rad4 interacting domain and, finally, a second UBA domain (UBA2) [295]. The XPC/Rad4 binding domain determines the function of Rad23 in nucleotide excision repair (NER), while the UBA domains bind mono- and poly-

ubiquitin chains [296]. The UBA domains can also interact with the UbL to form a closed conformation [297], as well as interacting with the UBA domains in other UbL-UBA containing proteins, such as Dsk2 and Ddi1 [298]. Moreover, the UBA2 has been shown to stabilize the protein, making Rad23 a long-lived protein resistant to proteasomal degradation [299, 300].

The UbL domain interacts with Rpn1 at the proteasome to facilitate the delivery of ubiquitylated substrates close to the 20S gate [297]. The UbL domain is required for Rad23 function in the UPS, as UbL deletion ($Rad23^{UbL\Delta}$) strains accumulate ubiquitylated proteins [293]. In addition, this domain can be phosphorylated at two serine residues (Ser47, Ser73), subsequently preventing Rad23 from interacting with the proteasome and mediating substrate delivery [293].

In this study, we found that Rad23 was ubiquitylated within the UbL domain and further characterized the DUB Ubp12 as a new protein interactor of the segregase Cdc48 that modulates the degradation of Rad23-dependent substrates.

Summary of the results

We identified the DUB enzyme Ubp12 as a new interactor of Cdc48 (yeast homologue of VCP/p97) and showed that Ubp12 overexpression impaired the degradation of two fluorescent reporters of the UPS, but not the degradation of the model substrate DEG1-GFP. While degradation of these substrates is dependent on Cdc48 activity, they differ in their requirement for Rad23, with DEG1-GFP being a Rad23-independent substrate. This difference prompted us to investigate whether Ubp12 plays a role in Rad23-dependent degradation, given that Rad23 acts downstream of Cdc48 in the escort pathway [43]. We showed that Ubp12 and Rad23 directly interact and mapped this interaction to the UbL domain of Rad23. By performing denaturing pulldowns, we confirmed that Rad23 was ubiquitylated and genetic removal of all lysine residues, leading to the the $Rad23^{UbLK0}$ mutant, was required to abolish ubiquitylation. Overexpressing Ubp12 led to a decrease in ubiquitylated Rad23, while deletion of Ubp12 increased it, demonstrating that Rad23 is a substrate of Ubp12.

Further, by overexpressing a ubiquitin variant that lacked all lysines (UbK0), we demonstrated that Rad23 was carrying ubiquitin chains as opposed to several mono-ubiquitylations. The predominant forms of ubiquitylated Rad23 appeared to be short ubiquitin chains that were too short to be a good degradation signal [17]. To directly test if ubiquitylation of the UbL domain could mediate degradation of Rad23, we employed constructs expressing Rad23 containing a point mutation in the UBA2 domain (L392A) that

abrogates the stabilizing influence of this domain [54]. These showed that Rad23 L392A and Rad23^{UbLK0,L392A} were degraded at a very similar rate, suggesting that ubiquitylation of the UbL domain in Rad23 did not influence its turnover.

The UbL domain in Rad23 plays an essential role in the shuttling function of Rad23 in the UPS, raising the question of how ubiquitylation on this domain impacts protein degradation. Rad23-dependent substrates were less efficiently degraded in the absence of ubiquitylation, as seen by either Rad23^{UbLK0} expression or Ubp12 overexpression. We excluded a general defect in protein degradation because Rad23^{UbLK0} expression did not increase the general levels of ubiquitin, nor did it increase the amount of ubiquitylated substrates bound to Dsk2.

We also observed an increase of ubiquitylated proteins bound to Rad23^{UbLK0} and showed that most of the ubiquitylated proteins were bound to the UBA1 domain, but that UBA2 also had a small contribution. This finding suggested that ubiquitylation of Rad23 might be needed for efficient substrate delivery to the proteasome. Since wildtype Rad23 and Rad23^{UbLK0} interacted equally well with the proteasome, we reasoned that ubiquitylation of Rad23 most likely facilitates the transfer of substrates to the proteasome.

Rad23 is also important for DNA repair upon UV radiation through its interaction with Rad4 to promote nucleotide excision repair (NER). Ubiquitylation of Rad23 is dispensable for this function, as Rad23^{UbLK0} was proficient in DNA repair. In contrast, the functional significance of this PTM in proteostasis was evident given that yeast that either overexpressed Ubp12 or Rad23^{UbLK0} were more sensitive to diverse proteotoxic stress insults.

Discussion

Role of Ubp12 in the Cdc48-Rad23 axis

In this paper, we conclusively show that Ubp12 is a DUB that regulates the ubiquitylation status of Rad23, and that this modification is important for cell resilience in situations of proteotoxic stress. Unexpectedly, abolishing Rad23's ubiquitylation by mutating all lysine residues does not lead to stabilization of Rad23, which would be expected if the ubiquitin chains were signaling for proteasomal degradation. Hence, ubiquitylation of Rad23 is likely a regulatory event that modulates Rad23 function in the UPS.

The mechanism by which ubiquitylated Rad23 modulates protein degradation is not presently clear, but the data presented opens up the possibility that ubiquitylation of Rad23 operates at the level of the proteasome to facilitate substrate transfer. This goes in line with the reporting of multiple ubiquitin ligase and deubiquitylase activities associated with the proteasome regulating substrate delivery [301]. It is tempting to speculate that ubiquitin chains in Rad23

increase the retention time of Rad23 to facilitate transfer of the substrate. Alternatively, the retention time might remain the same, but ubiquitylation of Rad23 may ensure that Rad23 persists in an open conformation that facilitates substrate binding/delivery by impeding intramolecular interactions between UBA-UBL domains. In either of these two scenarios, Ubp12 might be required to terminate the function of Rad23 at the proteasome, either by shortening the time Rad23 'docks' there, or by promoting the return to a closed conformation that liberates the substrate. Identifying the E3 ligase mediating ubiquitylation of Rad23, and whether it operates at the proteasome, is an important avenue of future research to further elucidate the mechanisms governing substrate delivery by Rad23 to the proteasome.

Situations of UPS overload due to protein misfolding, through conditions like acute oxidative stress or heat shock, can limit the quantity of free ubiquitin available to regulate processes [118]. Speculatively, in such situations, ubiquitylation of Rad23 might be compromised, reducing the number of substrates at the proteasome potentially as a protective response. Indeed, proteasome inhibitor-induced apoptosis can be abrogated following knockdown of the Rad23 human homologue HHR23B [302].

We currently lack direct evidence in support of Rad23 and Ubp12 acting on the same pathway. We argue that the stabilizing effect of Ubp12 overexpression and of lysine substitutions in the UbL domain of Rad23 on UPS reporter substrates are similar and suggest that they are mechanistically linked. Since Ubp12 is a DUB, however, it could generally act on ubiquitylated substrates independently of Rad23. We attempted to address this limitation genetically by driving overexpression of Ubp12 in conjunction with Rad23^{UbLK0}, which, if operating sequentially in the same pathway, should not result in additive effects on cell growth compared to the single overexpressions. However, we found that combined expression of wildtype Rad23 and overexpression of Ubp12 reduces yeast growth quite dramatically. We reason that overexpression of Rad23 without a concomitant increase in E3 levels will lead to overexpression of unmodified Rad23, which has been demonstrated to inhibit protein degradation [303]. Given this limitation, we cannot exclude the possibility that the growth defect in Ubp12-overexpressing yeast is (partly) due to Rad23-independent functions.

Distinct regulatory mechanisms between the different shuttle factors

As mentioned earlier, the UBA domains can drive intra- but also inter-molecular interactions, leading to homo- or heterodimers [51]. Dsk2, another shuttle factor, has been shown to cooperate with Rad23 in common substrates [304] and to also be ubiquitylated in its UbL domain [294]. However, ubiquitylation of Dsk2, unlike Rad23, regulated its turnover. We

presently do not know the E3 ligase responsible for- nor the topology of the ubiquitin chains on Rad23 and Dsk2. It is possible that different E3 ligases and/or different ubiquitin chains are responsible for the different effects of these ubiquitylation events. Further, these data suggest that ubiquitylation fine-tunes their roles in their common pool of substrates.

A role for Cdc48 in the Rad23-Ubp12 model

While we cannot discard that the action of Ubp12 on Rad23 is independent of their individual capacities to bind to Cdc48, we believe that a role of Ubp12 downstream of Cdc48 would be consistent with previous research and would provide a straightforward explanation for our data. We found Ubp12 as an interactor of Cdc48, a protein possessing a central role in proteostasis by way of its crucial function in mediating ubiquitin-dependent degradation both through its own ATP-dependent unfoldase activity and its large network of ubiquitin-binding co-factors [48]. The balance between ubiquitin ligases and DUBs binding to Cdc48 has been shown to regulate the fate of ubiquitylated substrates [305]. It is possible that Cdc48-associated DUBs, such as Ubp12, could also regulate substrate fate indirectly by modulating the activity of associated shuttle factors that are responsible for their delivery to the proteasome.

Interestingly, a recent study uncovered a new substrate of Ubp12, Fzo1, although Ubp12 does not stabilize Fzo1, as we also observe for Rad23. Ubp12 has affinity for short di-ubiquitin K48 chains on Fzo1 and authors propose a model in which Ubp12's affinity for short ubiquitin chains likely render its substrates to be poor proteasomal substrates [306]. This is the case for the substrates currently known for Ubp12, Gpa1 [307] and Ubp2 [306]. The same study identifying Fzo1 also observed binding of both the substrate and the DUB to Cdc48 and demonstrate that the ATPase activity of Cdc48 is required to regulate the process. Altogether, these and our findings would support a general regulatory role of Ubp12 in concert with Cdc48.

Rad23 in cancer

The human homologues of Rad23, HHR23A and HHR23B have overlapping but also distinct functions. Both are implicated in NER and the UPS, albeit to different extents depending on the context. For example, HHR23B is acetylated in cells and required for HDAC inhibitor-induced apoptosis, unlike HHR23A [302]. A proteomic study defining their interactors found proteasome subunits and stress-response proteins enriched with both proteins, while HHR23B formed unique interactions with BRCA1, VCP/p97 and vimentin, and HHR23A interacted with BRCA2 [308].

HHR23A/B may sustain tumor growth either by stabilizing oncoproteins or by degrading tumor suppressors. While their roles in cancer have not been thoroughly investigated, some current evidence links HHR23A/B with breast cancer progression, such as HHR23A binding to- and stabilizing RNF115, E3 ligase involved in breast cancer progression [309]. Moreover, high cytoplasmic levels of HHR23B correlate with invasive breast cancer in tumor samples, attributed to the role of HHR23B in ERAD [310].

The evidence for targeting HHR23A/B proteins in cancer is still emerging. Further efforts to characterize the substrates that are Rad23 -dependent and -independent may help in pinpointing if there are, and which type, of cancers would benefit from targeting these shuttles. It is presently difficult to predict if blocking these shuttle factors would result in a broad or a restricted inhibitory effect of the UPS. While evidence so far supports that there might be a distinct pool of substrates regulated by HHR23A/B, one outstanding question concerns how the docking of several shuttle factors is regulated at the proteasome lid and subsequently complicates predicting whether blocking a shuttle factor could affect the delivery of other substrates by others as well. Regardless, the Cdc48-Rad23 pathway is a major route to the proteasome in yeast [311], and HHR23B has been shown to be specific for K48-linked chains [312], strengthening the notion that targeting this shuttle factor would lead to profound proteotoxic stress.

Their lack of enzymatic activity makes targeting HHR23 proteins as a therapeutic intervention more challenging than other components of UPS, but not impossible. Indeed, this would represent a similar scenario to targeting E3 ligases of the RING family, which also lack a catalytic pocket but have still been studied extensively for treating cancer. Targeting SCF^{SKP2} with compound 25 is a good illustration of the anticancer potential of non-enzymatic proteins of the UPS [313]. Likewise, once identified, our results suggest that the E3 ligase mediating Rad23 ubiquitylation would be an interesting target to explore. To screen for inhibitors of protein-protein interactions, assays monitoring the thermal stability of the protein upon compound binding have been successfully applied [314].

Overall, our results shed new light on the poorly studied mechanisms regulating the activity of protein shuttle factors. Regulating HHR23A/B activity might be a novel avenue to modulate proteostasis in certain cancers.

6 CONCLUDING REMARKS

The work presented in this thesis shows that high content screening with fluorescent UPS reporters can identify small molecules with UPS inhibitory properties of a different chemical nature and with different mechanisms of action. Moreover, the study of the turnover of such fluorescent reporters can identify novel regulatory mechanisms in the UPS with the potential to pinpoint novel avenues for future research.

Specifically, the conclusions of the papers presented herein were:

- Paper I:
    - CBK77 is a first-in-class UPS inhibitor that is bioactivated by the enzyme NQO1 within the cancer cell environment. The elevated levels of NQO1 in tumours may provide a valuable link to the UPS, conferring greater specificity of CBK77 in targeting cancer cells.
    - Accordingly, CBK77 reduces the growth of NQO1-positive tumors in a xenotransplant model.
    - NQO1-activated CBK77 binds to ubiquitin, impairing deubiquitylating enzyme activity on ubiquitin chains. This mechanism of action may be explored further to understand CBK77 potential as a chemical probe for research and/or development of UPS-targeting drugs.
- Paper II:
    - CBK79 is a novel small molecule offering dual potential as a proteostasis-targeting inhibitor. As an aminothiazole simultaneously disturbing protein degradation both by autophagy and the UPS in cancer cells, CBK79 induces profound proteotoxic stress and cell death.
    - In parallel to its inhibitory effect on autophagy, CBK79 induces noncanonical lipidation of LC3B. Our work warrants further attention to this phenomenon and its link to autophagy impairment. It also calls for due care whilst interpretating assays monitoring autophagy with the commonly used marker of autophagosomes GFP-LC3, given its many roles beyond (macro)autophagy.
    - Elucidating the upstream mechanism(s) leading to the inhibition of protein degradation by CBK79 is key to defining its potential as an anti-cancer agent that can block common cellular compensatory mechanisms of- and provide greater efficacy than- inhibitors acting only on the UPS.

- Paper III:
    - Ubiquitylation of ubiquitin shuttle factor Rad23 is regulated by the deubiquitylating enzyme Ubp12. Preventing ubiquitylation of Rad23 impairs protein delivery to the proteasome and their eventual delivery to the proteasome, revealing a novel mechanism to block protein degradation.
    - This process applies to a subset of Cdc48-dependent substrates, suggesting that targeting this regulatory step can modulate the turnover of a specific pool of UPS substrates.
    - These results warrant further research focussed on identifying the E3 required for Rad23 ubiquitylation, as this may represent a potential drug target for blocking the UPS.
    - Further study should also aim to define the exact mechanism by which this ubiquitylation operates, and the precise endogenous substrates regulated by this process.

7 POINTS OF PERSPECTIVE

The fields of research studying the UPS and autophagy are expanding enormously. Yet, as is often the case, recent findings have only opened up more questions, as novel actors and mechanisms are discovered. This also applies to the work presented in this thesis, and below I outline future avenues of research to build on our observations.

In paper I, we showed CBK77 being bioactivated by the oxidoreductase NQO1 andthat cellular sensitivity to CBK77 was largely dependent on NQO1 activity. It would therefore be interesting to determine whether sensitivity to CBK77 amongst cancer cell lines of different origins corresponds to their varying levels of NQO1 expression [279]. Underscoring its potential value to chemotherapy, it has been shown that carfilzomib-resistant cell lines exhibit a compensatory upregulation of the transcription factor *NFE2L2* with a concomitant increase in its target genes, including NQO1 [315]. It could also be worth exploring whether CBK77 are able to impair proteostasis in proteasome inhibitor-resistant cells. Lastly, we found that CBK77 binds to ubiquitin and hinders deubiquitylating activity. Since ubiquitin has many other roles in the cell besides proteolysis, it would be valuable to test the effect of CBK77 on other ubiquitin-dependent processes, such as DNA repair.

The presence of a large group of enzymes whose activity depends on reactive thiols (E1, E2, and DUBs of the USP family) is a general challenge for screening for compounds that inhibit the UPS as hit compounds from such screening campaigns can be biased towards non-specific compounds that target cysteines in general, precluding their development due to unwanted side effects due to unspecific protein binding. This caveat could be potentially mitigated with (i) a good compound library design, (ii) attempting to identify possible secondary targets of the compounds by methods like CETSA or TPP, and (iii) by assessing potential toxicities early *in vivo*.

In paper II, we presented CBK79, a novel compound that inhibits both autophagy and the UPS and provokes proteotoxic stress. Besides impairing autophagic flux, CBK79 also induced noncanonical lipidation of the ATG8 protein LC3B, a feature recently identified also for commonly used autophagy inhibitors. What is the fate of these noncanonically labelled LC3B-positive vesicles? Emerging evidence may suggest a link between organelle damage, noncanonical lipidation and organelle homeostasis, as it has been reported to occur upon mitochondrial damage inflicted by CCCP [285] and upon osmotic damage to lysosomes [316]. Further studies are bound to address the functional relevance of these forms of noncanonical lipidation, and to what extent they are linked to proteostasis. Indeed, given that ATG8 proteins are ubiquitin-like proteins, it is tempting to speculate that an involvement of

this protein family in many processes, similar to the ubiquitin code, will start to emerge. There is emerging evidence of ATG8 conjugation to other substrates beyond phosphatidylethanolamine, such as phosphatidylserine [105], and conjugation of LC3 and GABARAP to other proteins, in a process resembling that of ubiquitin and therefore coined 'ATG8ylation' [89, 317, 318]. It will be exciting to see how these concepts evolve to help us understand the full potential of the ATG8 proteins in health and disease.

In paper III, we found and explored the functional significance of ubiquitylation of the ubiquitin shuttle factor Rad23, a novel post-translational modification regulated by the deubiquitylating enzyme Ubp12 that influences the fate of Rad23-dependent substrates. Many proteins bind and mediate proteasomal degradation of ubiquitylated proteins, and given that yeast strains lacking all known ubiquitin receptors are apparently still viable in basal conditions [319], the question remains as to whether there are yet-unknown ubiquitin receptors or unrecognized mechanisms to counteract such deficiencies.

To conclude, it is encouraging to see how the UPS has moved from being 'undruggable' to a prime target for developing novel cancer therapeutics. Whilst in this thesis the focus of discussion has been inhibition of the UPS, harnessing its proteolytic activity for degrading oncoproteins is also a very active field of research. Proteolysis-targeting chimaeras (PROTACs) and molecular glue degraders are molecules that bring an E3 ligase in close proximity to a substrate protein to induce its degradation [320]. NX-2127, developed to target a prominent protein driver in B-cell cancers, is the first drug of this class that has demonstrated targeted degradation in patients (NCT04830137). This proof-of-concept, together with novel strategies to screen for both inhibitors [321] and 'hijackers' [322] of E3 ligases may mean that no endogenous protein will ultimately remain 'undruggable'.

8 ACKNOWLEDGEMENTS

I wanted this section to be short (*she said while decreasing the paragraph spacing*) but as it seems obvious by the size of this book, I am not able to skimp on words for what feels important to me. It's been a long journey and every person that has helped me on the way, directly or indirectly, deserves a space here. Yet, it is impossible to name you all! Here goes my probably insufficient attempt to transmit how truly grateful I feel:

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'So few grains of happiness
measured against all the dark
and still the scales balance.

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