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Characterization of proteasome tissue expression and the role of AKIR-1 in regulating proteasome subcellular function

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Tiivistelmä

Eliöiden solunsisäisten proteiinien määrän ja laadun säätelyyn osallistuu monia tekijöitä ja mekanismeja, jotka yhdessä ylläpitävät solun toiminnan kannalta oikeanlaista proteiinitasapainotilaa eli proteostaasia. Proteostaasia ylläpidetään säätelemällä tarkasti proteiinien synteesiä, laskostumista ja oikea-aikaista hävitystä. Ihmisen ja muiden aitotumaisten eliöiden solunsisäisten proteiinien hävityksestä huolehtii kaksi hajotusjärjestelmää: autofagi-lysosomijärjestelmä ja ubikitiini-proteasomijärjestelmä. Ubikitiini-proteasomijärjestelmä vastaa useimpien solulimassa ja tumassa olevien proteiinien hävityksestä. Hävitettäväksi tarkoitetut solun proteiinit merkitään ensin ubikitiini-proteiinilla, hajotussignaalilla, jonka jälkeen ubikitinoidut proteiinit ohjataan pilkottaviksi proteasomiin. Proteasomi, tarkemmin sanottuna 26S proteasomi, on useasta alayksiköstä koostuva tynnyrimäinen proteiinikompleksi, jonka sisällä olevat entsyymit pilkkovat useista kymmenistä tai sadoista aminohapoista koostuvat polypeptidiketjut pieniksi, noin kahdesta tai muutamasta kymmenestä aminohaposta koostuviksi oligopeptideiksi. Ubikitiini-proteasomijärjestelmä vastaa väärinlaskostuneiden ja viallisten proteiinien hävityksen lisäksi useiden lyhytikäisten säätelyproteiinien hajotuksesta ja osallistuu näin ollen monien solun toimintojen, esimerkiksi geenien ilmentymisen ja solujen jakautumisen säätelyyn. Häiriöt ubikitiinivälitteisessä proteiinien hajotuksessa liittyvät muun muassa hermorappeumasairauksiin, kuten Alzheimerin tautiin, Parkinsonin tautiin ja Huntingtonin tautiin, sekä immuunijärjestelmän sairauksiin ja myös erilaisiin syöpiin. Huolimatta ubikitiini-proteasomijärjestelmän tärkeydestä tiedämme edelleen hyvin vähän mekanismeista, jotka säätelevät järjestelmän eri osien toimintaa monisoluisessa eliössä. Väitöskirjatyöni pyrki selvittämään, kuinka ubikitiini-proteasomijärjestelmän toimintaa säädellään monisoluisessa eliössä käyttäen tutkimusmallina *Caenorhabditis elegans (C. elegans)* -sukkulamatoa.

Ensimmäisessä osatyössäni tutkin sisäsyntyisesti tuotettujen eli endogeenisten proteasomien ilmentymistä sukkulamadon kudoksissa pystyttämällä immunohistokemiallisen tutkimusmenetelmän sukkulamato leikkeille. Osoitin, että proteasomia ilmennetään useissa sukkulamadon soluissa ja kudoksissa, ja että proteasomi-immunopositiivisuuden voimakkuus vaihteli solunsisäisten osien välillä: kaikkien tutkittujen solujen tumissa esiintyi solulimaan verrattuna voimakkaampi proteasomi-immunopositiivisuus. Lisäksi osoitin, että proteasomikompleksin alayksikön hiljennys johti kudoskohtaisiin vasteisiin proteasomin ilmentymisessä ja että suolen solun solulimassa havaittu proteasomi-immunopositiivisuuden vahvistuminen oli riippuvainen SKN-1-transkritiotekijästä.

Tunnistaaksemme uusia tekijöitä, jotka säätelevät proteasomin toimintaa, ryhmämme on aiemmin toteuttanut genominlaajuisen seulonnan, jossa selvitettiin yksittäisten geenien

hiljennysten vaikutusta proteasomissa hajotettavien proteiinien määrään suolen soluissa. Toinen osatyöni näytti, että yksi seulonnassa tunnistettu tekijä AKIR-1, evolutiivisesti konservoitunut tumaproteiini, säätelee proteasomin solunsisäistä sijoittumista ja tuloksemme vahvistavat, että Akirin/AKIR-1-välitteinen proteasomin tumasijoittumista ohjaava säätelymekanismi on evolutiivisesti konservoitunut. Näytimme myös, että AKIR-1-proteiini vaikuttaa proteasomin entsymaattiseen aktiivisuuteen. Lisäksi tunnistimme importiini-proteiineja, jotka säätelevät suolen soluissa ubikitinoitujen proteiinien solunsisäistä sijoittumista.

Tässä väitöskirjassa kuvataan ensimäistä kertaa endogeenisten proteasomikompleksien ilmentymistä monisoluisen *C. elegans* -sukkulamadon kudoksissa. Lisäksi tämä väitöskirja kuvaa ensimmäistä kertaa, että AKIR-1-proteiini ohjaa proteasomin tumasijoittumista ja entsymaattista aktiivisuutta monisoluisessa eliössä. Väitöskirjani laajentaa tietämystämme proteasomin toiminnan säätelystä, ja antaa näin ollen tärkeää tietoa solujen normaalista toiminnasta mutta tarjoaa myös paremmat mahdollisuudet kehittää tulevaisuudessa uusia ja parempia lääkinnällisiä hoitoja sairauksiin, jotka syntyvät proteasomin toiminnan häiriöistä.

Abstract

Protein homeostasis, proteostasis, refers to the complex network of cellular factors and mechanisms maintaining a cellular proteome, an optimized pool of cellular proteins required for the normal function of the cell and the viability of an organism under different conditions. Proteostasis is achieved by the coherent action of factors regulating protein synthesis and folding, and timely coordinated protein destruction. In eukaryotic cells, protein degradation is manifested by two pathways, the autophagy-lysosome pathway and ubiquitin-proteasome system (UPS). The UPS is responsible for the degradation of most cytosolic and nuclear proteins. Proteins selected for destruction are marked with a ubiquitin-degron prior to being delivered to the 26S proteasome, a multi-subunit protease complex cleaving long polypeptides into short oligopeptides. The UPS regulates the degradation of misfolded and damaged cellular proteins, and additionally, contributes to the regulation of several cellular processes such as transcription and cell cycle by adjusting, through the degradation, the levels of many short-lived regulatory proteins. Dysfunctions in the UPS are linked to the pathogenesis of several human disorders, for instance neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, as well as disorders of the immune system, and many cancers. However, the mechanisms regulating UPS function in a multicellular organism are still not fully understood. Thus, the purpose of this thesis was to study the proteasome function and regulation in a multicellular organism utilizing an animal model, *Caenorhabditis elegans* (*C. elegans*) -nematode.

In the first part of this study, I developed an immunohistochemical method on *C. elegans* sections and revealed a positive expression of endogenous proteasomes in several tissues and cell types in *C. elegans*. Moreover, we showed that the level of positive proteasome immunoreactivity varied within cellular compartments. Interestingly, in all studied tissues the nuclear proteasome immunoreactivity was higher compared to the immunoreactivity in the cytoplasm. In addition, we found that systemic proteasome impairment by proteasome subunit knockdown resulted in tissue-specific changes in proteasome immunoreactivity. We showed that the increase in proteasome immunoreactivity in the cytoplasm of the intestinal cells upon proteasome impairment was dependent on a transcription factor SKN-1.

To find new factors modifying proteasome function in a multicellular organism, our group has performed a genome-wide RNAi screen in *C. elegans* to identify genes changing the level of proteasomal substrates and thus potentially being proteasome regulators. In the second part of the study, I examined one of the identified hit gene, which encodes the nuclear protein AKIR-1. We demonstrated that *C. elegans* AKIR-1 regulates the nuclear transport of proteasomes and revealed that AKIR-1 also affects the proteolytic activity of

the proteasome. Our study supports the evolutionarily conserved role for the Akirin/AKIR-1-proteins in mediating nuclear translocation of the proteasome. In addition, we revealed a role for some *C. elegans* importins in regulating the subcellular distribution of ubiquitinated proteasomal substrates.

This study demonstrated for the first time the expression of endogenous proteasomes at tissue-level resolution in a multicellular animal model, *C. elegans*. Moreover, it revealed the role of AKIR-1 in regulating proteasome nuclear localization and activity in a multicellular organism. My thesis provides new information concerning proteasome function and regulation in a multicellular organism and broadens our knowledge on this basic cellular process. Deeper understanding of the mechanisms regulating proteasome in a multicellular organism could help to develop ways to prevent and treat disorders related to dysfunctional proteasomes.

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Elisa Mikkonen
Helsinki, 2025

List of abbreviations

AD	Alzheimer's disease
APF	ATP-dependent Protelysis factor
APP	Amyloid Precursor Protein
ATP	Adenosine triphosphate
Aβ	Amyloid β
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
CMA	Chaperone-mediated autophagy
CP	Core particle
Crm	Chromosomal region maintenance
CZI	Carl Zeiss Image
DNA	Deoxyribonucleic acid
DUB	Deubiquitinating enzyme
E1-E3	Polyubiquitinating enzyme 1-3
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation pathway
ERAD	Endoplasmic reticulum associated degradation
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
FOXO	Forkhead box protein O
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
hESC	Human embryonic stem cell
HRP	Horseradish peroxidase
IGF	Insulin/insulin-like growth factor
INF-γ	Interferon-gamma
iPSC	Induced pluripotent stem cell
IPTG	Isopropyl β-D-1-thiogalactopyranoside

JUNQ	Juxtanuclear compartment
kDa	Kilodalton
LLPS	Liquid-liquid phase separation
LLVY	Leu-Leu-Val-Tyr
Lys	Lysine
MDa	Megadalton
Met	Methionine
MHC	Major histocompatibility complex
MM	Multiple myeloma
mm	Millimeter
mRNA	Messenger RNA
MTOC	Microtubule organizing center
mTORC	Mechanistic target of rapamycin complex
NEB	Nuclear envelope budding
NEM	N-ethylmaleimide
NF-Y	Nuclear transcription factor Y
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
nm	Nanometer
NPC	Nuclear pore complex
Nrf	Nuclear factor erythroid 2-related factor
NuRD	Nucleosome remodeling and deacetylase
ODC	Ornithine decarboxylase
PA200	Proteasome activator 200-kDa
PA700	Proteasome activator 700,000 dalton
PAC	Proteasome assembling chaperone
PACE	Proteasome-associated control element
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Parkinson's disease
PKA	Protein kinase A
POMP	Proteasome Maturation Protein
POST	Polyubiquitinated substrate transporter
PQC	Protein quality control
PSG	Proteasome storage granules
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RAC	Regulatory particle assembly chaperones
Ran	Ras-related Nuclear protein
REG	11S regulator
RNA	Ribonucleic acid

RNAi	RNA interference
RP	Regulatory particle
RPN	Regulatory Particle Non-ATPases
RPT	Regulatory Particle ATPases
SDS	Sodium dodecyl-sulphate
SIPAN	Starvation-induced proteasome assemblies in the nucleus
SQSTM	Sequestosome
STAT	Signal transducer and activator of transcription
Suc-LLVY-AMC	Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcourmarin
TIFF	Tagged image file format
TNF	Tumor necrosis factor
UBA	Ubiquitin-associated
UBE4B	Ubiquitination factor E4B
UBH-4	Ubiquitin carboxyl-terminal hydrolase 4
UBL	Ubiquitin-like
UCHL5	Ubiquitin carboxyl-terminal hydrolase L5
UIM	Ubiquitin-interacting motif
UPS	Ubiquitin-proteasome system
USP14	Ubiquitin-specific protease 14
UTR	Untranslated region
UTR	Ultraviolet light
XPO	Exportin
ZFAND5	Zinc finger AN1-type domain family

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List of original publications

This thesis is based on the following publications:

I Mikkonen, E., Haglund, C. & Holmberg, C. I. Immunohistochemical analysis reveals variations in proteasome tissue expression in *C. elegans*. *PLoS One* **12**, e0183403 (2017). https://doi.org/10.1371/journal.pone.0183403

II Pispa J*., Mikkonen E*., Arpalahti L., Jin C., Martínez-Fernández C., Cerón J., and Holmberg C. I. AKIR-1 regulates proteasome subcellular function in *Caenorhabditis elegans*. iScience. 2023 Oct 20;26(10):107886, https://doi.org/10.1016/j.isci.2023.107886

* These authors contributed equally.

The publications are referred to in the text by their roman numerals (I and II).

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1 Introduction

Protein synthesis, folding, and degradation are regulated processes maintaining cellular protein homeostasis and organismal fitness. There are two proteolytic pathways, namely, the autophagy-lysosome pathway and the ubiquitin-proteasome system (UPS) coordinating the degradation of cellular proteins in eukaryotic cells. The UPS is mostly responsible for the clearance of misfolded and damaged soluble proteins, as well as for the timely-mannered degradation of short-lived regulatory proteins, whereas autophagy primarily deals with long-lived proteins, large insoluble protein aggregates, cytosolic structures such as protein complexes, macromolecular compounds, organelles, or pathogens. Dysfunctions in these degradation systems are related to the pathogenesis of several, often late-onset diseases such as neurodegenerative diseases as well as different types of cancers, and disorders of the immune system. Despite the fundamental role of proteasome, it is still uncovered how proteasome function by finetuning its expression, localization, and/or proteolytic activity is manifested in animals to maintain cellular proteome under changing conditions. The aim of my thesis was to study the tissue-level expression of endogenous proteasomes in a multicellular organism by developing an immunohistochemical method on *C. elegans*. Moreover, the purpose was to examine how our recently identified putative UPS modulator affects proteasome function in a multicellular organism. The literature review will first present a short history regarding the research of intracellular protein degradation followed by discussions on the components and regulation of the UPS, and the role of UPS in proteostasis and disease onset. Lastly, it will present some techniques and the animal model *C. elegans* used in this thesis to study UPS function. The results and discussion section will present the tissue-level expression of endogenous proteasomes in *C. elegans* and the role of AKIR-1 in regulating proteasomes' function and subcellular localization and contrasts the obtained results with prior research.

2 Review of the literature

2.1 Short history of intracellular protein degradation

For a long time, structural and functional cellular proteins, so-called body proteins, in living systems were believed to be highly stable and static molecules. In addition, dietary proteins were considered to function solely as an energy source and being independent from the cellular proteins (reviewed in (Ciechanover 2017)). The idea that body proteins are instead dynamic, started to evolve in the late 1930s, when Rudolf Schoenheimer and colleagues challenged the contemporary concept that proteins are static molecules. They utilized nitrogen isotope (15N)-labelled amino acids, which enabled them to follow proteins' dynamic *in vivo* (Schoenheimer et al. 1939, Ratner et al. 1940). Their work demonstrated for the first time that dietary proteins were incorporated in proteins in the tissues and that proteins are in a dynamic state (Schoenheimer et al. 1939, Ratner et al. 1940). This view was strengthened in the mid-1950s, when the lysosome, an intracellular protein degradation system, was discovered by Christian de Duve (De Duve et al. 1953, Gianetto and De Duve 1955). Unfortunately, after its discovery, lysosomes were considered to be the only existing machinery degrading cellular proteins and the whole process concerning intracellular protein degradation were regarded as "solved" and little effort was put on resolving the details of protein degradation (reviewed in (Ciechanover 2005)).

The function of the lysosome, in which "random batch" of cytosolic proteins were segregated within a membrane-bound vesicles, which then fused with the lysosome to mediate the degradation of their content, could not, however, explain some known features in the degradation of intracellular proteins. Firstly, individual proteins had variable half-lives, ranging from few minutes to hours and days. Secondly, different proteins responded differently to lysosomal inhibitors and changes in physiological conditions. Lastly, the observation that the degradation of cellular proteins, and more importantly the function of the proteolytic machinery itself is dependent on metabolic energy was in contrast with the known exergonic action of the lysosome (reviewed in (Ciechanover 2017)). Therefore, it was strongly suggested that cells contained also a non-lysosomal

degradation system, and clear evidence on that was revealed by Rabinovitz and Fisher. They found that rabbit reticulocytes, which are terminally differentiated red blood cell lacking lysosomes, were able to degrade abnormal hemoglobin (Rabinovitz and Fisher 1964). Further support came from the experiments performed by Brian Poole and his colleagues. They followed the degradation of differently labelled exogenous and endogenous proteins in macrophages upon lysosomal inhibition and showed that the inhibition of lysosomal activity specifically prevented the degradation of extracellular proteins, but did not influence the degradation of intracellular proteins (Poole et al. 1977). They summarized their results as follows: *"The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins are broken down during protein turnover"* (Poole et al. 1978). Finally, a non-lysosomal ATP-dependent proteolytic system, now known as the ubiquitin-proteasome system (UPS), was isolated and characterized from cell-free extracts prepared from reticulocytes by Etlinger and Goldberg (Etlinger and Goldberg 1977). About the same time, Hershko and colleagues also isolated and characterized a similar system (Hershko et al. 1978). Their further studies led to the purification and characterization of the enzymatic components mediating the protein degradation in these extracts. They identified an ATP-dependent Proteolysis factor 1 (APF-1), which was later identified as a ubiquitin (Wilkinson et al. 1980), and the enzymes mediating the ubiquitin conjugation to proteolytic substrates (Ciechanover et al. 1982, Hershko et al. 1983). The protease degrading ubiquitinated substrates, the proteasome, was later discover by Hough and colleagues (Hough et al. 1986). Avram Hershko, Aaron Ciechanover, and Irwin Rose were jointly awarded the Nobel Prize in Chemistry 2004 for their discovery of ubiquitin-mediated protein degradation.

Currently, degradation of intracellular proteins is known to be a complex and tightly regulated cellular process mediated by two degradation systems: the autophagy-lysosome pathway and the ubiquitin-proteasome system. Autophagy can be divided into three different forms, namely, the chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. In the CMA, individual cytosolic proteins can be delivered for lysosomal degradation by chaperone proteins. In microautophagy, cytosolic material in microautophagic bodies is directly engulfed by the lysosome through lysosome membrane invagination. The details of this process however are mostly unknown. The last form of autophagy, macroautophagy, which is the most well-known, is responsible for the large-scale turnover of intracellular components like proteins, lipids, nuclei acids, and organelles. In the first step of macroautophagy, cytoplasmic materials are enclosed into the double-membraned autophagosomes, which later fuses with the lysosomes to form autolysosomes. In these autolysosomes the internal contents

are degraded by the lysosomal hydrolases and degradation products are released into the cytosol and recycled. (reviewed in (Yim and Mizushima 2020).

This thesis focuses on the UPS, which regulates almost all cellular processes, such as cell cycle, development, differentiation, transcription, antigen presentation, signal transduction, and receptor-mediated endocytosis by mediating the degradation of short-lived regulatory proteins in a timely and spatial manner (reviewed by (Ciechanover 2017, Mao 2021)). In addition, it has a crucial role in maintaining cellular protein quality control (PQC) by degrading misfolded and damaged proteins. Dysfunctions in the UPS have been linked to the pathogenesis of several human disorders, such as neurodegenerative diseases (*e.g.* Alzheimer's disease, Parkinson's disease, and Huntington's disease) as well as disorders of the immune and inflammatory system and different types of cancers. In the following chapters, I will discuss in more detail the components of the UPS.

2.2 The ubiquitin-proteasome system

The UPS is a selective proteolytic pathway, in which ubiquitinated substrates are degraded by the proteasome. In eukaryotic cells, the UPS mediates the degradation of most nuclear and cytosolic proteins, including misfolded and denatured proteins, as well as short-lived regulatory proteins, but also some long-lived cellular proteins (Rock et al. 1994, Zhao et al. 2015). The ubiquitin-proteasome system relays on two main components: a small signal molecule, the ubiquitin, and a large multi-catalytic protease complex, the 26S proteasome. In addition, a large group of various enzymes facilitating the ubiquitination and the deubiquitination are needed.

2.2.1 Ubiquitination

Ubiquitin is a small peptide of 76-amino acids that is highly conserved in all eukaryotes (Goldstein et al., 1975; Ciehanover et al., 1978; Wilkinson et al., 1980). Ubiquitination, the covalent attachment of the ubiquitin molecule to the substrate, is a multistep process catalyzed by three types of enzymes acting cooperatively: a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Haas et al. 1982, Hershko et al. 1983) (**Figure 1**). Prior to attachment of ubiquitin to the substrate, ubiquitin monomers are processed from immature precursor proteins by specific proteases (Kimura and Tanaka 2010). Ubiquitin monomers are then activated by the E1 enzyme in an adenosine triphosphate (ATP) -dependent manner. E1 enzyme catalyzes the formation of a thioester bond between the carboxyl-terminus of the ubiquitin and the active cysteine of the E1. Then the E1-bound, active ubiquitin is attached with a thioester

bond to an E2 enzyme, also known as ubiquitin-transferring enzyme. Finally, E3 enzyme binds E2 and catalyzes the covalent attachment of the ubiquitin from the E2 to the substrate by forming an isopeptide bond between the carboxyl-terminus glycine of the ubiquitin and the epsilon-amino group of any lysine side chain exposed within the surface of the target protein (Goldknopf and Busch 1977, Hershko et al. 1983). In addition to the lysine side chain, ubiquitin can be conjugated to non-lysine residues such as serine, threonine, or cysteine (reviewed in (Kravtsova-Ivantsiv and Ciechanover 2012)). Additional ubiquitin moieties are conjugated to the substrate by E3s or in some cases by E4 ligases, which are ubiquitin-chain elongating factors that catalyze the assembly of a long multiubiquitin chain on a substrate already having one or a few conjugated ubiquitin(s) (Koegl et al. 1999, Hoppe 2005).

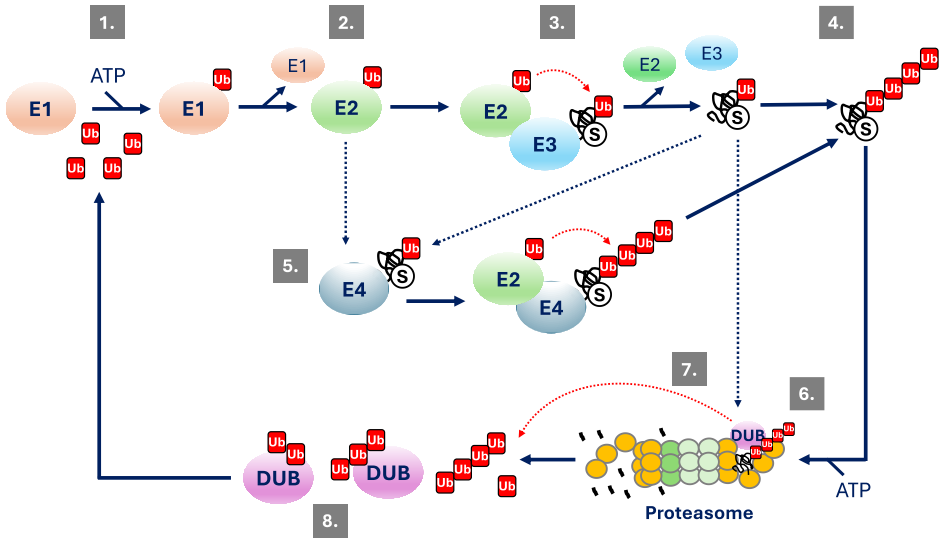

Figure 1. Substrate ubiquitination and deubiquitination. Ubiquitination, the covalent attachment of a ubiquitin (Ub) to a substrate (S) is a multi-step process mediated by different enzymes. **1.** A ubiquitin activating enzyme (E1) activates the ubiquitin in the presence of ATP. **2.** The E1-bound, active ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2). **3.** A ubiquitin ligase (E3) binds both the ubiquitin-holding E2 and the substrate and catalyzes the ligation of the ubiquitin to the substrate. **4.** Repeated cycles of ubiquitination results in polyubiquitin chain formation. **5.** The ligation of ubiquitin from E2 to monoubiquitinated or polyubiquitinated substrates can be additionally catalyzed by a specific ubiquitin-chain elongating enzyme (E4). **6.** Polyubiquitinated and monoubiquitinated substrates are delivered to the proteasome for ATP-dependent proteolysis, in which substrates are cleaved into short peptides, which are then released from the proteasome. **7.** Proteasome-associated deubiquitinating enzymes (DUBs) remove the conjugated ubiquitin(s) from the substrate prior to proteolysis. **8.** The released polyubiquitin chains are further hydrolyzed into ubiquitin monomers by the free DUBs in the cytosol. Of note, the figure is not to scale.

Like many other post-translational modifications, the ubiquitination is a reversible reaction. Conjugated monoubiquitin or polyubiquitin chains can be removed by certain proteases, deubiquitinating enzymes (DUBs), which catalyze the hydrolysis of the peptide or isopeptide bond between ubiquitin and the substrate or between two ubiquitin moieties. There are about 100 genes in human genome and 45 genes in *C. elegans* genome encoding DUBs (Nijman et al. 2005, Komander et al. 2009, Papaevgeniou and Chondrogianni 2014).

The number of enzymes facilitating the different steps of ubiquitination varies. In humans, there are two E1s (UBA1 and UBA6/UBE1L2), and in *C. elegans* there is only one E1 enzyme (UBA-1) (Chiu et al. 2007, Jin et al. 2007, Pelzer et al. 2007, Kulkarni and Smith 2008). The number of enzymes (E2s and E3s) in the last two steps increases drastically: the human genome encodes about 40 E2s facilitating the transfer of ubiquitin or ubiquitin-like proteins (Stewart et al. 2016) and more than 600 different E3 enzymes (Yang et al. 2021). In *C. elegans* there are 22 E2s and more than two hundred E3s (Reviewed in (Papaevgeniou and Chondrogianni 2014)). Both human and *C. elegans* have one E4 enzyme: ubiquitination factor E4B (UBE4B) in human and UFD-2 in *C. elegans* (Koegl et al. 1999). The specificity of the ubiquitination is mediated by the great number of different E3 ligases, which selectively ubiquitinate certain target proteins by recognizing specific structures and motifs on the substrates. In general, a substrate to be targeted for proteasomal degradation, must have a degradation signal, a degron, which is recognized by the E3 ligases. Inherent degrons are specific amino acids such as short destruction motifs, or amino- and carboxyl-terminal amino acid sequences (N-degrons and C-degrons, respectively). Inherent degrons usually contain a hydrophobic sequence that is buried in the protein core of native protein or protein complexes. In addition to these inherent degrons, a transient post-translational modification, such as phosphorylation, can function as an acquired degron and trigger ubiquitination (reviewed in (Ella et al. 2019).

2.2.2 Ubiquitin code

Once one ubiquitin molecule is covalently bound to a substrate (monoubiquitination), another ubiquitin can be conjugated to any of the seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63), or the amino-terminus methionine (Met1) of the previous ubiquitin, or any lysine residues on the substrate (multi-monoubiquitination) (reviewed by (Kwon and Ciechanover 2017). In animals, Lys48-linkage (K48), which targets protein substrates for proteasomal degradation, is the most abundant linkage type (Chau et al. 1989, Hershko and Ciechanover 1998). The Lys63-linkage (K63) generates a degron for autophagy-lysosome pathway, but is also involved in other non-

proteolytic pathways, such as the repair of deoxyribonucleic acid (DNA) (Liu et al. 2018). Compared to these canonical K48- and K63-linkages, the biological roles of the atypical, less-abundant linkage types (Lys6-, Lys11-, Lys27-, Lys29-, and Lys33-linkages) are fairly unknow and especially their role in proteasomal degradation remain still controversial. There are some evidences that K11-linkages found in a mixed or branched chain with K48- or K63-linkages can promote proteasomal degradation (Bremm et al. 2014, Locke et al. 2014, Qin et al. 2014).

Substrate polyubiquitination, more precisely the attachment of a polyubiquitin chain of at least four ubiquitin moieties conjugated via K48-linkages, has been considered as the minimum requirement targeting most eukaryotic proteins for degradation (Thrower 2000). However, there are several studies showing that monoubiquitination or multi-monoubiquitination alone can function as a proteolytic signal for the proteasome, although monoubiquitination has been considered as a non-proteolytic signal regulating protein interactions and activity. One study demonstrated that proteins shorter that 150 residues can be degraded following monoubiquitination (Shabek et al. 2012), and another one identified a myriad of proteins in both yeast (*Saccharomyces cerevisiae*; of note yeast hereafter refers to *S. cerevisiae* unless otherwise stated) and in human cells being degraded by the proteasome upon monoubiquitination and multi-monoubiquitination (Braten et al. 2016). Some proteins, such as the enzyme facilitating the biosynthesis of polyamines, the ornithine decarboxylase (ODC), can be degraded by the proteasome even without any added ubiquitin moieties in a ubiquitin-independent manner (Murakami et al. 1992). In addition, a recent study identified thousands of human peptides and several full-length proteins undergoing ubiquitin-independent proteasomal degradation (Makaros et al. 2023). Moreover, another recent study revealed a so-called midnolin-proteasome pathway in which midnolin, a largely uncharacterized protein, promotes the proteasomal degradation of many nuclear protein in a ubiquitin-independent manner (Gu et al. 2023). Substrates targeted for degradation should also contain an unstructured initiation region, as lack of an unfolded initiation site might even protect the protein from proteasomal degradation (Prakash et al. 2004). Altogether, the next step, the poteolysis is manifested by the proteasome.

2.2.3 Structure and function of the proteasome

The 26S proteasome is a large, 2,5-megadalton (MDa), ATP-dependent multi-catalytic protease complex. The 26S holoenzyme composes of two separately stable and functionally distinct sub-complexes, the core particle (CP) and the regulatory particle (RP) capping either or both ends of the core particle (**Figure 2**) (reviewed in (Tomko and Hochstrasser 2013)). More specifically, the CP capped at one end is referred to as 26S proteasome and the CP capped at both ends is referred to as 30S. In this thesis, the terms "proteasome" and "26S proteasome" are used interchangeably, and are referring to both 26S and 30S complexes. The catalytic CP is ubiquitously found in prokaryotes and eukaryotes (Coux et al. 1996), but the regulatory particle exists only in eukaryotic cells. The atomic structure of archaeal, yeast, and human CP has been solved revealing its highly stable structure (Löwe et al. 1995, Groll et al. 1997, Harshbarger et al. 2015, Schrader et al. 2016). On the contrary, the structure of the RP is highly dynamic. Recent advances in single-particle cryo-electron microscopy and cryo-electron tomography have enabled atomic resolution modelling of the human and yeast 26S holocomplexes and provided molecular resolution images of the holocomplexes in their natural environments, respectively (De La Peña et al., 2018; Dong et al., 2019).

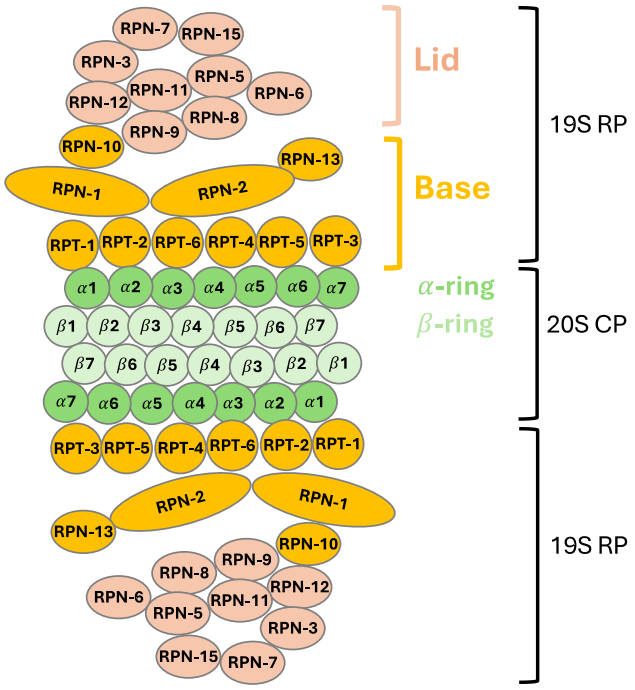

Figure 2. The simplified architecture of proteasome subunits and subcomplexes. 20S CP, core particle, consists of 14 α- and 14 β-subunits arranged into two seven subunit α-rings and two seven subunit β-rings. 19S RP, regulatory particle, consists of the base and lid subcomplexes, each composing of several subunits; RPN, RP non-ATPase; RPT, RP ATPase.

2.2.3.1 Core particle (CP)

The structure of the core particle, also known as 20S proteasome or 20S CP, has been studied comprehensively by X-ray crystallography in several species and is known to be highly conserved (Löwe et al. 1995, Seemüller et al. 1995, Groll et al. 1997, Unno et al. 2002). Eukaryotic and archaeal 20S proteasome as well as the proteolytic units of bacterial proteases, all display a similar cylinder-like structure composed of multi-subunit rings stacked on top of each other (Löwe et al. 1995, Bochtler et al. 1997, Groll et al. 1997, Wang et al. 1997*b*).

The archaeal and eukaryotic 20S proteasome is a 700-kilodalton (kDa) complex composing of four axially stacked seven-subunit rings, two outer α-rings and two inner β-rings, forming a cylinder-shaped barrel (Löwe et al. 1995, Groll et al. 1997). In eukaryotes, the two inner β-rings are each made of seven distinct β-subunits, and three of the subunits, namely, β1, β2, and β5, contain active sites with caspase-like, trypsin-like, and chymotrypsin-like activity, respectively (Arendt and Hochstrasser 1997, Heinemeyer et al. 1997). Therefore, each mature eukaryotic 20S proteasome houses six proteolytically active threonine residues sequestered inside the catalytic barrel that mediate degradation of protein substrates into small peptides. The number and diversity of the active sites within the core particle allow a fast and efficient degradation of a variety of cellular proteins that have entered the catalytic chamber. Similarly to the β-rings, the outer α-rings are each composing of seven similar yet distinct α-subunits. The heptameric α-rings, positioned on both sides of the two β-rings, regulates substrate entry into the core particle by controlling the opening of the central axial pore, the CP gate. The amino-terminal loops of three α-type subunits, α2, α3, and α4, block the CP gate, particularly the amino-terminal loop of α3-subunit occupies most of the space at the CP gate (Groll et al. 2000). Opening of the CP gate is allosterically controlled by the interactions between the CP and the regulatory particle. On the other hand, there are several studies showing that 20S CP can meditate protein degradation without the regulatory particle(s), but the mechanism opening the entrance into the free CP are still unknown (reviewed by (Kumar Deshmukh et al. 2019)). In yeast, the opening of the CP gate and the degradation of oxidized and unstructured proteins were induced when cysteines of the α5-subunit of the CP were S-glutathionylated (Silva et al. 2012).

In addition to the constitutive CP, in mammalian cells, there are other CP subtypes displaying altered catalytic properties and thus fulfilling better the tissue-specific requirements in protein degradation. Immunoproteasomes, in which the three constitutive active site-containing β-subunits are substituted with the immunosubunits, β1i, β2i, and β5i, are found in lymphoid tissues and cells subjected to proinflammatory cytokines, such as interferon-gamma (INF-γ) and

tumor necrosis factor (TNF) (Wright et al. 1995, Groettrup et al. 1996, Hisamatsu et al. 1996, Nandi et al. 1996). INF-γ induces the synthesis of β1i-, β2i-, and β5i-immunosubunits, which are incorporated into a newly formed CP in place of their constitutive counterparts. The altered peptide cleavage properties of the immunoproteasomes enable production of more favorable peptides for major histocompatibility complex (MHC) class I antigen-presenting receptors. Another CP subtype is the thymoproteasome, which is expressed only in the cortical epithelial cells of the thymus and regulates the development of cytotoxic T cells (Murata et al. 2007). Thymoproteasome displays a specific β5t-subunit, which is replacing the β5- or β5i-subunit (Murata et al. 2007). Immunoproteasomes and thymoproteasomes do not exist in *C. elegans*. Phylogenetic studies have addressed that these CP subtypes occur only in jawed vertebrates excluding birds, which for unknown reasons have lost both immunoproteasomes and thymoproteasomes (reviewed in (Murata et al. 2018)). In mammals, testis-specific CPs, so called spermatoproteasomes contain an α4s-subunit replacing the α4-subunit of the constitutive CP (Qian et al. 2013, Uechi et al. 2014).

2.2.3.2 Regulatory particle (RP)

The regulatory particle, also known as 19S or PA700 (proteasome activator 700,000 daltons), is composed of two multi-subunit subcomplexes named the lid and the base (Glickman et al. 1998). The base subcomplex directly contacts the CP and consists of four Regulatory Particle Non-ATPases (RPN1, RPN2, RPN10, and RPN13) and six distinct Regulatory Particle ATPases (RPT1 to RPT6) forming a heterohexameric ATPase ring, which acts as a mechanical motor mediating the conformational changes of the 26S proteasome during substrate processing (Matyskiela et al. 2013, Unverdorben et al. 2014). In addition, the RPT subunits (except RPT4) allosterically control the opening of the CP gate by inserting their carboxyl-termini into the α-pockets between the α-subunits of the α-ring (Smith et al. 2007, Rabl et al. 2008). The lid subcomplex of the 19S RP comprises of RPN subunits (RPN3, RPN5, RPN6, RPN7, RPN8, RPN9, RPN11, RPN12, and RPN15) (Lander et al. 2012). The base subunit RPN10 indirectly stabilizes the whole regulatory particle by interacting with the lid subunit RPN9 (Lander et al. 2012, Lasker et al. 2012). Besides the carboxyl-terminal motifs of the RPT subunits, the base subunit RPN6 helps anchoring the RP onto the CP (Pathare et al. 2012).

The RP is responsible for recruiting, deubiquitinating, unfolding, and translocating of protein substrates into the CP chamber for degradation in an ATP-dependent manner (reviewed in (Hanna and Finley 2007, Finley 2009)). The ubiquitin receptors of the base subcomplex, RPN1 (Shi et al. 2016), RPN10 (Deveraux et al. 1994, Nocker et al. 1996), and RPN13 (Husnjak et al. 2008,

Schreiner et al. 2008) recognize substrates targeted for degradation through the ubiquitin chain. Besides the ubiquitin receptors intrinsically residing in the proteasome, ubiquitinated substrates can also be recruited and delivered to the proteasome by extrinsic ubiquitin receptors. These proteins consist of a ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains, and includes proteins such as the nucleotide excision repair protein RAD23A/B (Rad23 in yeast) (Elsasser et al. 2004), ubiquitin domain-containing protein UBQLN1/2/3/4 (Dsk2 in yeast) (Zhang et al. 2009), and the ubiquitin-dependent protease DDI1 (Ddi1 in yeast) (Kaplun et al. 2005). These proteins interact with the intrinsic ubiquitin-binding sites of the proteasome via their UBL domain and function as shuttle factors delivering proteins marked for destruction to the proteasome. Rad-23 and Dsk2 can bind to all three ubiquitin receptors of the proteasome, whereas Dd1 seems to only bind to the ubiquitin receptor Rpn1 (Gomez et al. 2011). Once recognized by the lid ubiquitin receptors (**Figure 3**), the flexible initiation region of the substrate reaches into the ATPase ring, which converts the chemical energy of ATP hydrolysis to mechanical force to unfold and translocate the ubiquitin receptor - bound substrate into the CP. The ubiquitin chains need to be removed from the substrates before they enter the proteolytic core of the proteasome. The proteasome-associated DUB, RPN11 (a subunit of the lid subcomplex) catalyzes co-translocationally the release of ubiquitin moieties from the substrate before its entry into the ATPase motor (De La Peña et al. 2018). Removed ubiquitin molecules can eventually be recycled and attached to another target molecule.

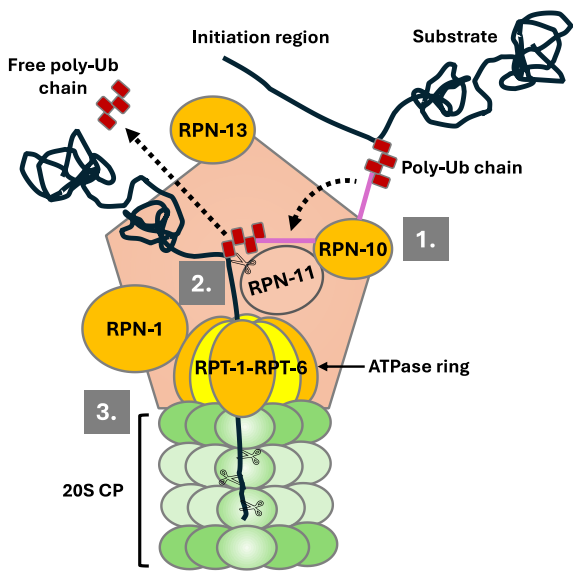

Figure 3. The 26S proteasome-mediated proteolysis of ubiquitinated substrates. 1. The ubiquitin receptors of the proteasome e.g. RPN-10 binds the substrate through the ubiquitin. **2.** The flexible initiation region of the substrate enters the ATPase ring composing of six different RPT-subunits and causes a conformational change enabling the proteasome-associated DUB, RPN-11 to co-translocationally cleave the polyubiquitin chain from the substrate. **3.** The deubiquitinated substrate is unfolded and translocated into the 20S CP for proteolysis by the ATPase ring with the energy released from ATP hydrolysis.

2.2.3.3 Alternate CP activators

In eukaryotes, besides the 19S regulatory particle another similar ATPase ring-containing protein complex, Cdc48 (also known as VCP or p97) has been shown to bind the core particle and forming an alternative ATP-powered Cdc48-20S proteasome complex (Barthelme and Sauer 2012, Esaki et al. 2018). There are also two other CP activator families, 11S regulator (also known as PA28 or REG) and PA200 (proteasome activator 200-kDa (Dubiel et al. 1992, Li and Rechsteiner 2001, Schmidt et al. 2005). Both families function in an ATP-independent manner and do not display unfoldase activity. In vertebrates, PA28 is formed from the three highly homologous subunits, α, β, γ. The biological roles of the two forms of the PA28 regulator, PA28α,β and PA28γ are still fairly unknown, however PA28α,β seems to be primarily located in the cytoplasm and PA28γ is constitutively expressed in the nucleus, and both forms show increased expression after acute oxidative stress in cells, suggesting a role in degradation of oxidized proteins (Baldin et al. 2008, Pickering and Davies 2012). The mammalian proteasome activator PA200 or its yeast ortholog Blm10 promotes the acetylation-dependent degradation of the core histones during transcription, DNA repair, and spermatogenesis, and PA200 is suggested to play an important role in preventing cellular senescence, the permanent cell cycle arrest (Ustrell 2002, Wen et al. 2024). Both PA28 and PA200 can participate in the assembly of hybrid proteasomes, in which different types of CP activators bind to opposite sides of the same CP.

2.3 Regulation of proteasome function

Proteasomes are highly regulated as their coordinated function and adaptability in variable conditions play a key role in maintaining cellular protein homeostasis. The function of the proteasome is regulated in several ways affecting its biogenesis and assembly, subcellular localization, and finally the coordinated degradation of individual subunits or the proteasome as a whole. In addition, there are several cellular factors that regulate the action of proteasomes by transiently interacting with the complex or modulating the complex through post-translational modifications.

2.3.1 Biogenesis of proteasomes

Several signaling pathways and transcription factors regulate the expression of the proteasome subunits and/or assembly chaperones, and thus, maintain cellular proteasome homeostasis. In yeast, the zinc-finger transcription factor Rpn4 regulates the gene expression of proteasome subunits and assembly chaperones. Rpn4 upregulates the gene expression by binding to a conserved proteasome-associated control element (PACE) found in the promoter region of all proteasomal subunit genes and in some assembly chaperone genes (Mannhaupt et al. 1999, Jelinsky et al. 2000, Shirozu et al. 2015). Under normal growth conditions, Rpn4 is rapidly degraded by the proteasome, through both ubiquitin-dependent and ubiquitin-independent pathways, making it a very short-lived protein with about a two-minutes half-life (Xie and Varshavsky 2001, Ju and Xie 2004). When proteasome function is compromised, Rpn4 accumulates and promotes the expression of genes encoding proteasome subunits.

Mammalian cells lack an ortholog for yeast Rpn4, but a similar negative feedback loop exists, by which an inhibition of proteasome function is compensated by transcriptional activation of all 26S proteasome subunit genes leading to *de novo* proteasome synthesis (Meiners et al. 2003). In mammals, the activation of proteasomal gene expression in response to proteasome inhibition is mediated by the transcription factor NRF1 (nuclear factor erythroid 2-related factor 1) (Radhakrishnan et al. 2010). Under standard condition, NRF1 is integrated into the endoplasmic reticulum (ER) membrane, and degraded by the proteasome through the ER-associated protein degradation pathway (ERAD) (Steffen et al. 2010). However, when proteolytic activity is inhibited, NRF1 translocates to the nucleus and upregulates proteasomal genes by binding to the regulatory regions of all 33 proteasome subunit genes (Steffen et al. 2010). In addition, the 20S assembly chaperone, Proteasome Maturation Protein (POMP), and PA200 are found to be upregulated upon proteasome inhibition in an NRF1-dependent manner (Steffen et al. 2010). This negative feedback loop mediated by the NRF1 is called the proteasome recovery pathway or the proteasome 'bounce-back' response. The expression of NRF1 itself is for instance in mouse and human cells mediated by an active mTORC1 (mechanistic target of rapamycin complex 1), the growth-controlling kinase pathway responding to growth factors or increased nutrient level by increasing protein synthesis and NRF1-mediated increase in proteasome levels and activity (Zhang et al. 2014).

In addition to NRF1, another member of the protein family, NRF2, has been shown to regulate proteasome expression. NRF2 upregulates proteasome expression and activity in colon cancer cells (Arlt et al. 2009) and it has been shown to mediate the proteasome inhibition-induced enhancement in proteasome

activity in human cells (Kraft et al. 2006). A third member of the NRF-family, the NRF3 has been shown to enhance 26S proteasome assembly by inducing expression of the POMP gene in cancer cells (Waku et al. 2020). In *C. elegans*, the NRF1/2/3 ortholog SKN-1 contributes to the expression of many proteasome subunit genes under normal conditions and mediates the proteasome bounce-back response by controlling *in vivo* the compensatory upregulation of proteasome genes upon proteasome inhibition (Li et al. 2011).

In mammalian cells, several transcription factors involved in regulating the expression of a subset of proteasome subunits have been identified. In rat neuronal cell line, the expression of β1i and β5i is induced by the transcription factor Zif268 (James et al. 2006). In addition, in human cells, the NF-Y (nuclear transcription factor Y) regulates basal expression of some proteasome core particle subunits (α2, α5, α7, β3, β4, and β6), five RP subunits (RPT1, RPT5, RPT6, RPN10, and RPN11), and one RP assembly chaperone (p28) (Xu et al. 2012). In human embryonic stem cells (hESCs) and in induced pluripotent stem cells (iPSCs), the FOXO4 (forkhead box protein O4) transcription factor regulates the expression of the 19S lid subunit RPN6 (PSMD11 in humans) and maintains the proteasome activity at a higher level in human embryonic stem cells (Vilchez et al. 2012*a*). Moreover, the role of FOXO in regulating the expression of 19S subunit is conserved, as the *C. elegans* ortholog of FOXO, DAF-16, was shown to regulate the expression of rpn-6.1, a *C. elegans* ortholog of human PSMD11, in long-lived *glp-1* mutants (Vilchez et al. 2012*b*). The induction of epidermal growth factor (EGF) by STAT3 (signal transducer and activator of transcription 3). upregulates β5-subunit (Vangala et al. 2014). In addition, there are studies suggesting that overexpression of a single proteasome subunit, β5, may upregulate the level of other subunits, as well as proteasome assembly and activity both in human cell lines and at organismal level in *C. elegans* (Chondrogianni et al. 2005, 2015).

2.3.2 Assembly of proteasomes

Assembly of the 26S holoenzyme requires the formation of mature 20S CP, the independent assembly of the two RP subcomplexes (the base and the lid) to form 19S regulatory particle, and finally the association of the 19S regulatory particle to the mature 20S core particle. The assembly of the eukaryotic core particle is a complex multi-step process mediated by a set of extrinsic and intrinsic chaperones (Budenholzer et al. 2017). The CP assembly can be divided into three separate steps: the formation of the α-ring, β-ring, and the half-CP dimerization followed by proteasome maturation (reviewed by (Rousseau and Bertolotti 2018)). In humans there are four proteasome assembling chaperones (PAC1-PAC4) and a proteasome maturation protein (Witt et al. 2000, Murata et al. 2009). Two PAC-

heterodimers, the PAC1-PAC2 and the PAC3-PAC4 guide the α-ring assembly (Hirano et al. 2005, 2006, Le Tallec et al. 2007). The PAC3-PAC4 heterodimers disassociates from the α-ring already during the incorporation of the β-ring subunit, but the PAC1-PAC2 heterodimer stays attached to the α-ring until being degraded by the newly assembled CP after dimerization of the half-CP. The α-ring serves as a template for the incorporation of the β-subunits to form the β-ring. The POMP, which regulates the joining of two half-CP assemblies together, associates with the α-ring at the same time as the first β-subunit, β2, is incorporated and prevents half-CP dimerization until all β-subunits are properly incorporated (Hirano et al. 2006, 2008, Li et al. 2007). Most of the β-subunits, β1, β2, β5, β6, and β7, are synthesized as proproteins and their amino-terminal propeptides need to be cleaved to activate the CP (Chen and Hochstrasser 1996, Schmidtke et al. 1996, Seemüller et al. 1996).

In mammalian cells, the assembly of the base subcomplex of the 19S RP is regulated by four RP assembly chaperones (RACs), which each binds the carboxy-terminal domain of a distinct RPT subunits forming three intermediate complexes (reviewed in (Tomko and Hochstrasser 2013)). The pathway of lid assembly in mammalian cells in not well known and no RACs have yet been identified to be assisting the lid assembly *in vivo*. In yeast, the lid assembly is mediated by the formation of two intermediate complexes, which associate and are joined together by the Rpn12 subunit to form the mature lid, which can bind to the base (Tomko and Hochstrasser 2011, 2013, Tomko et al. 2015, Dambacher et al. 2016). There are two possible assembly pathways mediating the last RP-CP association step in formation of the holoenzyme. In mammalian cells, it seems that that the lid and base assemble into a free RP intermediate, which then assembles with the mature CP (Thompson et al. 2009, Lu et al. 2017). This is in contrast to the pathway dominating in yeast cells, in which the CP templates the assembly of the base and the CP-base intermediate guides the lid assembly to form the holoenzyme (Park et al. 2009, 2013*a*).

2.3.3 Subcellular localization of proteasomes

In eukaryotes, 26S proteasomes are present both in the nucleus and the cytoplasm. Studies in yeast and mammalian cells have showed that changes in cellular conditions coordinate the localization of the 26S proteasomes, for example glucose exhaustion prior to quiescence in yeast cells and amino acids starvation in mammalian cells affect the subcellular localization of the 26S proteasomes (Laporte et al. 2008, Livneh et al. 2023). In proliferating yeast cells, 26S proteasomes are primarily localized to the nuclei (Laporte et al., 2008; Pack et al., 2014; Russell et al., 1999; Wilkinson, 1998). In addition, in yeast cells the nuclear

fraction of proteasomes seems to be crucial for survival, as nuclear proteasome-depleted cells exhibit a lethal phenotype (Tsuchiya et al. 2013). Recent studies with mammalian cells have also shown a similar strong nuclear localization for the 26S proteasome under basal condition (Tomita et al. 2019, de Almeida et al. 2021, Livneh et al. 2023, Park et al. 2024). Interestingly, even though 26S proteasomes seem to predominantly be localized in the nucleus, it is not yet understood, why cells need to display relatively high proteasome levels in the nucleus compared to the cytosol. It might be related to the role of proteasomes regulating nuclear functions, such as transcription (Salghetti et al. 2000) and DNA replication (Roseaulin et al. 2013) in the nucleus. On the contrary, a study performed with cultured human cells and yeast cells claims that the nuclei would even lack peptidase activity (Dang et al. 2016). In addition, there are also studies demonstrating that some nuclear proteins are exported from the nucleus to the cytoplasm for degradation (Chen and Madura 2014, Hirayama et al. 2018). Thus, further studies are required to fully understand the subcellular localization and function of the 26S proteasome as well as the role of subcellular compartmentalization of the proteasomal substrates.

2.3.3.1 Nuclear transport

Nuclear transport, import and export, are conserved processes in which proteins are delivered from the cytoplasm into the nucleus or the other way round through the nuclear pore complex (NPC), a large protein structure crossing the double-membraned nuclear envelop, which surrounds the nucleus in eukaryotic cells (reviewed by (Macara 2001)). Small, water-soluble molecules and ions can passively diffuse through the net of intrinsically disordered proteins, FG-Nups (phenylalanine-glycine-rich nucleoporins) filling the nuclear pores (reviewed in (Matsuda et al. 2024)). Larger molecules (>40 KDa), for instance proteasomes, require an active process dependent on the Ran-GTP (Ras-related Nuclear protein-Guanosine-5'-triphosphate) hydrolases and soluble transport receptors, such as karyopherins. Karyopherins are a large family of proteins, including importins, exportins, and biportins (reviewed by (Wing et al. 2022)). The importins are divided into an α- and β-subgroup.

The canonical nuclear import of cytoplasmic proteins (**Figure 4**) is mediated by a heterodimer consisting of an importin-α that recognizes and binds the nuclear localization signal (NLS) of the cargo protein and an importin-β that facilitates an interaction with the NPC. The heterodimer/cargo-complex diffuses into the nucleus and once inside, the complex interacts with the Ran-GTP, which disassociates the importin-α/cargo complex from the importin-β/Ran-GTP complex. The disassociation of the importin-α/cargo complex leads to the release

of the cargo from the importin-α. The importins are then returned to the cytoplasm as bound to Ran-GTPs. In the cytoplasm, the Ran-GTPs bound to importin-α or -β is hydrolyzed into Ran-guanosine diphosphates (GDPs) releasing the importins. Besides the canonical import system, importin-α can translocate into the nucleus in an importin-β- and a Ran-independent manner (reviewed by (Miyamoto 2002)). The other karyopherin proteins, the exportins, mediate the transport of nuclear proteins into the cytoplasm. The exportin binds the cargo through a nuclear export signal (NES) and associates with the Ran-GTP. The complex diffuses through the nuclear pore to the cytoplasm, where the Ran-GTP hydrolysis into Ran-GDP releases the cargo and the exportin in the cytosol.

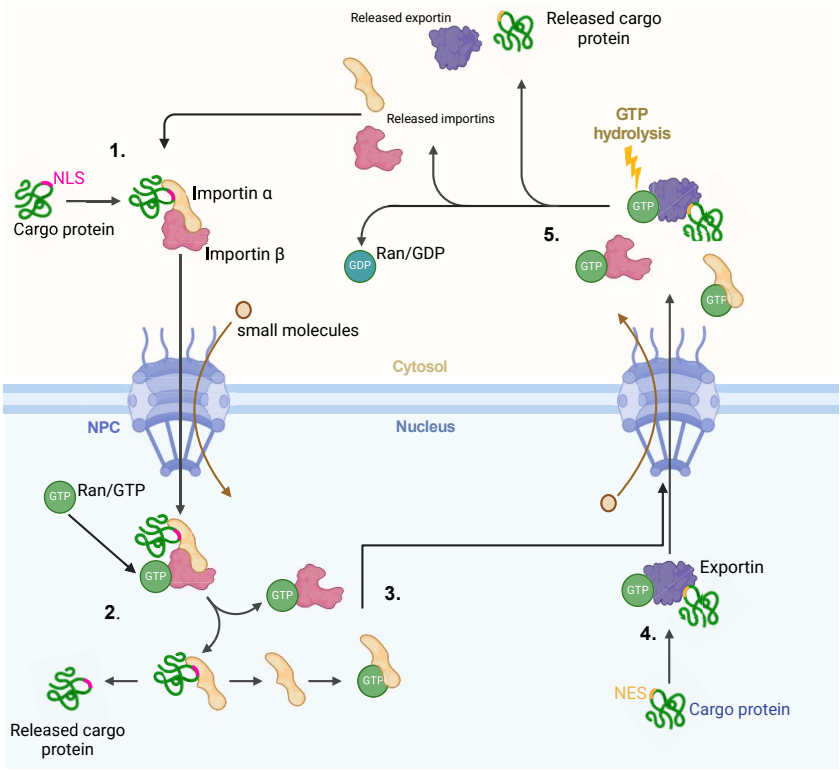

Figure 4. Karyopherin-mediate nuclear transport. Small molecules passively diffuse through the nuclear pore complexes (NPC). Large proteins are actively transported through the NPC with transport receptors mediating the nuclear import (on the left side) and nuclear export (on the right side). **1.** The importin-α of the importin α/β heterodimer recognizes and binds a nuclear localization signal (NLS)-containing cytosolic cargo protein and the importin-β mediates the interaction with the NPC. **2.** In the nucleus, the binding of the Ran-GTP disassociates the importin-α/cargo complex from importin-β resulting in cargo release. **3.** Importins are exported to the cytosol by Ran-GTPs. **4.** The exportin recognizes and binds a nuclear export signal (NES)-containing cargo protein, interacts with Ran-GTP, whereafter, the complex diffuses through the NPC to the cytosol. **5.** In the cytosol, Ran-GTP is hydrolyzed into Ran-GDP releasing the cargo protein and exportin, as well as importins in the cytosol. Figure adapted from Biorender.com (2023) using the template "Simplified Overview of Transport Through Nuclear Pore Complexes". Retrieved from https://app.biorender.com/biorender-templates.

2.3.3.2 Nuclear import of proteasomes

It has been revealed that several proteasome subunits contain canonical NLSs (cNLSs): the α1, α2, α3, and α4 subunits of the 20S CP (Nederlof et al. 1995, Wang et al. 1997*a*) and the RPT2 and RPN2 subunits of the 19S RP, of which the NLS in RPN2 is primarily mediating the nuclear import of the 19S base complex (Wendler et al. 2004). However, the role of these cNLSs in mediating the nuclear import of the 26S proteasome holocomplex is unclear. Moreover, it is still not clear whether proteasomes are imported into the nucleus as subcomplexes and assembled into holocomplexes inside the nucleus, as shown in some studies (Lehmann et al. 2002, Wendler et al. 2004, Isono et al. 2007), or as holocomplexes, as shown in other studies (Reits 1997, Chen et al. 2011, Savulescu et al. 2011, Pack et al. 2014, Budenholzer et al. 2020). It has been demonstrated that the nuclear pore complex can expand to facilitate the transport of cargos with a diameter up to 39 nanometers (nm) (Panté and Kann 2002), therefore in theory, the dimensions of the 26S proteasome holocomplex (~20 nm x 45 nm) could allow it to be transported along its longitudinal axis through the NCP channel (Pack et al. 2014).

A recent study in yeast, demonstrated that the nuclear import of the fully assembled 26S proteasome and some lid assembly intermediates through the NPC is mediated by an NLS-containing adaptor protein, Sts1 (Budenholzer et al. 2020). In fission yeast (*Schizosaccharomyces pombe*), proteasome nuclear localization is mediated by a proteasome nuclear anchor protein, Cut8, which bears a strong sequence similarity with the Sts1 (Tatebe and Yanagida 2000, Takeda and Yanagida 2005). Sts1 displays a weak interaction with Rpn11, a subunit of the lid subcomplex of the 19S RP (Tabb et al. 2000, Chen et al. 2011, Ha et al. 2014). Sts1 also contains an amino-terminus bipartite NLS sequence sufficient for binding the Srp1, the yeast importin-α (Budenholzer et al. 2020). In addition, Sts1 is a short-lived protein, which degradation is mediated by the proteasome in a ubiquitin-independent manner (Budenholzer et al. 2020). A recent study suggested the similar role for human AKIRIN2 to function as a proteasome import adaptor in cancer cell lines by interacting with fully assembled proteasomes and importin 9 (IPO9) (de Almeida et al. 2021). In *Drosophila*, importin 9 (Ipo9), has been previously shown to facilitate proteasome trafficking into the germ cell nuclei during spermiogenesis (Palacios et al. 2021). In addition, importin 5 (IPO5) mediates the post mitotic import of 20S proteasomes into the nuclei in human melanoma cells (Spits et al. 2019). In yeast, the continuation of cell proliferation after stress-induced quiescence requires reimport of proteasomes from cytosolic granules (Laporte et al. 2008). The proteasome activator Blm10 has been shown to participate in this nuclear relocalization by mediating the import of CPs after quiescence (Weberruss et al. 2013). Sts1 does not localize to those cytosolic granules during carbon starvation and does not participate in the nuclear reimport

of proteasomes upon glucose refeeding (Breckel et al. 2024), suggesting that in yeast cells the nuclear import of proteasomes is mediated by different factors and mechanisms under different conditions.

2.3.3.3 Nuclear export of proteasomes

A recent study demonstrated that in cultured human cells proteasomes are actively shuttled between the nucleus and the cytoplasm under normal growth condition (Livneh et al. 2023), and that upon amino acids starvation or hypoxia proteasomes are translocated from their large nuclear pool to the cytosol in HeLa cells and in various *in vivo* animal tumor models (Livneh et al. 2023, 2024). Similar nuclear export of proteasomes has been shown to occur also in yeast cells, in which during quiescence or carbon starvation, nuclear proteasomes are exported to the cytosol and concentrated in membraneless structures called proteasome storage granules (PSG) (Laporte et al. 2008). Interestingly, the starvation-induced translocation of proteasomes in mammalian cells seems to differ from formation of proteasome storage granules in yeast cells (Livneh et al. 2023, 2024). In cultured cancer cell lines, it has been reported that proteasome translocation to the cytosol stimulates protein degradation, thereby supplying starved cells with recycled amino acids required for the synthesis of essential proteins (Vabulas and Hartl 2005). Inhibition of the proteasome translocation to the cytoplasm, which leads to proteasome sequestration in the nucleus, resulted in amino acid deprivation and cell death (Livneh et al. 2023). In addition, the inhibition of proteasome translocation causes a tumor growth arrest in animal tumor models (Livneh et al. 2024). The mechanism regulating the nuclear export of proteasomes are not well understood. In human cells, the starvation-induced translocation of the proteasome as well as the active shuttling of proteasomes between the nucleus and the cytoplasm under normal metabolic conditions seems to be dependent on polyubiquitin binding protein p62/SQSTM1 (sequestosome 1) and its interacting partner, NBR1 (neighbor of BRCA1 gene 1) (Livneh et al. 2023). The translocation of the proteasome was almost completely inhibited when both p62 and NBR1 were silenced (Livneh et al. 2023). In yeast, the function of the canonical exportin 1 is required for proteasome export under nitrogen starvation (Nemec et al. 2017).

2.3.3.4 Subcellular localization of proteasomal substrates

One well-known cellular process trafficking proteasomal substrates within the cell is the endoplasmic reticulum associated degradation (ERAD). In the ERAD misfolded or misassembled proteins in the ER are recognized by molecular chaperones and translocated from the ER into the cytosol for UPS mediated degradation (reviewed in (Ruggiano et al. 2014)). In addition, there are studies

showing that proteasomal substrates are translocated both from the nucleus to the cytoplasm and from cytoplasm to the nucleus. In mammalian cells, polyubiquitinated substrates are exported from the nucleus to the cytoplasm by the UBIN-POST system as a response to proteasome inhibition (Hirayama et al. 2018). Hirayama and colleagues showed that the UBIN (also known as Ubiquilin 4), a UBL and a UBA domain containing protein, binds various types of ubiquitin chains via its UBA domain. In addition, they showed that the export of UBIN-bound polyubiquitinated substrates were mediated by POST (polyubiquitinated substrate transporter), a UBIN interacting protein containing a NES. The POST mediated the export of the complex from the nucleus to the cytosol by interacting with the Crm1/Xpo1- (Chromosomal region maintenance 1/Exportin 1) karyopherin, which mediates the export of NES containing cargo proteins (Hirayama et al. 2018). They further showed that the UBIN/POST system could mediate the export of ubiquitinated proteins also in *C. elegans* (Hirayama et al. 2018). In yeast, Rad23 shuttles nuclear substrates to the cytoplasm for proteasomal degradation (Okeke et al. 2020). There is also a study showing that in yeast a nuclear envelope budding (NEB), a method to transport nuclear material to the cytoplasm, which is too large to be transported through the NPCs, is enhanced upon proteasome inhibition and under induction of protein misfolding (Panagaki et al. 2021). They also showed that NEB event is more conserved than previously thought and is occurring for instance also in human cells and in *C. elegans* (Panagaki et al. 2021). On the opposite, certain cytoplasmic substrates are imported into the nucleus for proteasomal degradation (Park et al., 2013; Prasad et al., 2010). Interestingly, a recent study demonstrated that human cells seem to respond to autophagy deficiency-mediated cytoplasmic accumulation of autophagy substrates by increasing their trafficking from the cytoplasm into the nucleus via nuclear pores to be degraded by the nuclear proteasome (Park et al., 2024).

In addition, it has been shown that both in mammalian cells and in yeast cells, proteasomal substrates and proteasomes are compartmentalized into separate structures called proteasome foci under different stress conditions. One example, occurring in the nuclear envelope of both yeast cells and mammalian cells, is the proteasome inhibition-induced formation of a juxtanuclear compartment (JUNQ) containing polyubiquitinated substrates and proteasomes. These compartments are disassembled and dissolved quickly upon proteasome reactivation (Kaganovich et al. 2008). In mammalian cells there are several types of proteasome foci formulated upon different stress factors, such as the formation of transient proteasome foci in the nucleus upon high glucose induced hyperosmotic stress (Yasuda et al. 2020), formation of polyubiquitin binding protein p62 foci in the nucleus under different stressors and inhibition of nuclear export (Fu et al.

2021), and formation of a starvation-induced proteasome assemblies in the nucleus of mammalian cells (SIPAN) due to amino acid deprivation (Uriarte et al. 2021). A common feature of those proteasome foci formed in the nucleus of mammalian cells is that they behave like liquid-liquid phase separation (LLPS) organelles, which allows soluble proteins to condensate in aqueous environment without being enclosed by lipid membranes. These proteasome foci usually contain proteasomes and polyubiquitinated proteins, but some of them also contain ubiquitin shuttling receptors (RAD23B) and/or ribosomal or proteasomal orphan proteins or enzymes related to ubiquitination. In mammalian cells, when the production of misfolded proteins exceeds the degradation capacity of the proteasome, aggregation-prone proteins aggregate and form aggresomes located in the cytoplasm around the microtubule organizing center (MTOC) (Johnston et al. 1998).

2.3.4 Degradation of proteasomes

Proteasomes are very stable complexes (Pack et al. 2014). The half-life of proteasomes is about 16 hours in mouse embryonic fibroblasts and 12-15 days in rat liver cells (Tanaka and Ichihara 1989, Tomita et al. 2019). Different pathways regulate the degradation of single subunits of the 26S proteasome complex and the turnover of whole complex. In human cells, following induction of apoptosis, the subunits of the 19S regulatory particle, RPT5, RPN10, and RPN2 are cleaved by the caspase-3 protease. The cleavage of the subunits inhibits both the ubiquitin-dependent and ubiquitin-independent proteasome-mediated degradation of cellular substrates and advances the programmed cell death (Sun et al. 2004). In yeast cells, dysfunctional proteasome subunits are degraded by the UPS itself to prevent them being assembled into the proteasome holoenzyme (Peters et al. 2015). In addition, in yeast cells under normal condition during the proteasome assembly, any excess Rpt subunits of the ATPase ring in the 19S RP base complex are degraded by the proteasome (Nahar et al. 2019). In mammalian cells, amino acid starvation leads to proteasome ubiquitination in a subunit- and site-specific manner, which targets the proteasome for degradation by the autophagy-lysosome system (Cohen-Kaplan et al. 2016). The detection and delivery of the ubiquitinated proteasomes to the autophagy-lysosomes is mediated by the polyubiquitin binding protein p62/SQSTM1 (Cohen-Kaplan et al. 2016).

2.3.5 Proteasome-associated proteins and post-translational modifications

There are several cellular factors called proteasome-associated proteins that regulate the function of the proteasome by transiently or reversible associating

with the proteasome, as well as factors altering proteasome function through post-translational modifications. Several of these reversible proteasome-associated proteins are in some way related to ubiquitin, for instance some E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases, the extrinsic ubiquitin receptors, and the proteasome-associated DUBs, such as USP14 (Ubiquitin specific peptidase 14) and UCHL5 (Ubiquitin C-terminal hydrolase L5) are known to transiently bind proteasome (reviewed in (Mao 2021)). The non-ubiquitin related proteasome-associated proteins include for example the protein called Ecm29 (Extracellular mutants 29), which in yeast induces proteasome holocomplex disassembly in response to oxidative stress (Wang et al. 2010, Park et al. 2011) and a member of the zinc finger AN1-type domain family, ZFAND5/ZNF216, which binds to 26S proteasomes and increases cellular protein breakdown by enhancing activities of 26S proteasomes (Lee et al. 2018).

The 26S proteasome function, its abundance, assembly, localization, and activity are also affected by many post-translational modifications (PTMs), which permit the 26S proteasome to fine-tune its function according to cellular conditions. The PTMs found in the 26S proteasome can be categorized into the following modification types: proteolytic truncation, N-acetylation, N-methylation, N-myristylation, poly(ADP-ribosyl)ation, O-glycosylation, oxidation, sumoylation, succinylation, ubiquitination, and phosphorylation. As an example, the cyclic adenosine monophosphate (cAMP) activated kinase, protein kinase A (PKA), phosphorylates the 19S regulatory particle lid-subcomplex subunit RPN6 on serine 14. This PKA-mediated phosphorylation enhances degradation of short-lived regulatory and misfolded proteins by increasing the peptidase activity hydrolysis by the CP and ATP hydrolysis by the RP (Lokireddy et al. 2015). In addition, the above mentioned Ecm29 interaction with the proteasome is mediated by phosphorylation of the 20S subunit α7 (Wani et al. 2016). Besides these few mentioned modifications, there are in total over 300 post-translational modifications found occurring in the 26S proteasome, but the exact function and mechanisms are still unknown for the majority of these PTMs.

2.4 Roles of the UPS in proteostasis and diseases

In eukaryotic cells, the UPS is a vital protein quality control (PQC) process, which maintains cellular protein homeostasis, proteostasis, together with coordinated protein synthesis and chaperones-mediated protein folding. Normal cellular processes and the health of an organism relies on functional cellular proteins, which are folded into their proper, native conformation (reviewed by (Balchin et al. 2016)). However, several factors, such as gene mutations, mistakes in protein translation, stressors (toxins, oxidative and heat stress), as well as aging, and

proteins' inherently dynamic nature are constantly challenging the native conformation of proteins (Lee et al. 2006, Liu et al. 2008, Jacobson et al. 2012, Meller and Shalgi 2021). Therefore, protein misfolding and the formation of non-native protein conformations are common events in cells, which are, to certain extent, handled by the PQC processes (reviewed in (Kandel et al. 2024)).

In cells, damaged proteins (misfolded and/or unfolded proteins) tend to interact and form larger protein aggregates (soluble oligomeric aggregates) due to their hydrophobic regions being exposed. Protein aggregates can further form various structures, such as soluble oligomers, amyloid fibrils (amyloids), and insoluble protein aggregates (reviewed in (Kandel et al. 2024)). In general, damaged and aggregated proteins cause proteotoxicity and/or cellular damage, and protein aggregation is linked to the pathogenesis of several human diseases, particularly neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases, but also some cardiomyopathies (reviewed in (Ross and Poirier 2004, Henning and Brundel 2017)). In Alzheimer's disease (AD), extracellular amyloid β (Aβ) protein deposits are present in the brain. The Aβ and its precursor protein, APP (Amyloid Precursor Protein), seems to have a role in neuronal processes, but their normal physiological functions are not completely understood. Proteasome function is reduced in the brains of AD patients and in AD mouse models, and the common Aβ isoform, Aβ42, causes proteasome inhibition *in vitro* (Gregori et al. 1995, Keller et al. 2000, Keck et al. 2003, Almeida et al. 2006). In addition, genetically or pharmacologically increased proteasome activity protects against cell death in mouse, fly, and cell culture Aβ models of AD (Chocron et al. 2022). In *C. elegans*, proteasome activation in the nervous system prevented Aβ-mediated proteotoxicity in the muscle through the enhanced activity of the proteasome in the muscle cells (Panagiotidou et al. 2023). In Alzheimer's disease, another protein, Tau, which normally is involved in microtubule assembly, forms tau aggregates (fibrils). In healthy cells, Tau is a UPS substrate, unlike the extracellular Aβ, and accumulation of Tau seems to directly inhibit proteasome function, and the accumulation of ubiquitinated tau can be reduced by activating the proteasome (Myeku et al. 2016, Schaler and Myeku 2018). Parkinson's disease (PD) is characterized by aggregated protein formations known as Lewy Bodies containing α-synuclein and ubiquitin in the substantia nigra. As with the Aβ and APP, the normal function of α-synuclein is not really known. Most PD cases are sporadic with no clear genetic cause, but some mutations in the UPS, such as E3 ligase Parkin, have been found to be associated with familial PD (Kitada et al. 1998). A mouse model has suggested that dysfunctional 26S proteasome can lead to a PD-like neurodegenerative disease (Bedford et al. 2008). Thus, increasing UPS capacity could be harnessed to treat or even prevent diseases related to the accumulation of protein aggregates. For several disease-causing proteins, the

oligomers have been shown to impair proteasome function and bind directly to proteasome in *in vitro* experiments (Thibaudeau et al. 2018). Therefore, it is important to understand how UPS is affected during the early stages of neurodegenerative diseases in animal models.

Changes in proteasome function have also been implicated in the tumorigenesis of many types of cancers. In general, excessively proliferating tumor cells rely on highly functional proteasomes, which maintain "cellular homeostasis" by preventing the accumulation of unwanted and misfolded protein and thus protecting tumors from proteotoxic stress. In addition, proteasomes are an important regulator of many so-called tumor signaling pathways, which regulate for instance cell cycle, cell growth, and apoptosis. Cyclins and cyclin-dependent kinases (CDKs) control the progression of the cell cycle, which namely relies on the coordinated fluctuations in the expression and UPS-mediated degradation of different cyclins. Dysfunctions in degradation of cyclins can lead to tumor formation (King et al. 1996, Wang et al. 2015). In addition, low levels of p21 family members (p21, p27, and p57), which regulate the progression of cell cycle by inhibiting the activity of the CDK complex, are associated with poor prognosis in several types of cancer, thus, in tumor cells up-regulation of these proteins by proteasome inhibition could lead to apoptosis (Chiarle et al. 2000). Moreover, proteasome is an important regulator of the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, a tumor signaling pathway regulating immune responses and cell survival. Dysregulation of this pathway has been implicated in the development and progression of many types of cancer and inflammation-related diseases (Yu et al. 2020). Proteasome inhibitors could inhibit NF-κB transcription factor activity by preventing the degradation of a NF-κB pathway inhibitor. The inhibition of NF-κB activity in cancer cells in turn leads to defects in cell survival, tumor growth, and angiogenesis, as well as the induction of apoptosis (Sunwoo et al. 2001). Another crucial proteasome regulated tumor-related factor is the p53 tumor suppressor, which regulates cell cycle progression, apoptosis, and senescence (Vogelstein et al. 2000). Most human cancers are associated with inactivated, mutated form of p53 (Hernandez-Boussard et al. 1999). Proteasome inhibition—induced p53-dependent apoptosis in murine mammary epithelial cells (MacLaren et al. 2001). The first proteasome inhibitor, bortezomib, an inhibitor of the chymotrypsin-like site of the β5-subunit, was approved by the United States Food and Drug Administration (FDA) in 2003 for the treatment of multiple myeloma (MM), a type of bone marrow cancer. The second proteasome inhibitor, carfilzomib, also a β5-subunit specific inhibitor, was approved by the FDA in 2012 to treat relapsed and refractory multiple myeloma, and the third, Ixazomib, the first orally taken proteasome inhibitor, was approved by the FDA in 2015 for multiple myeloma treatment to be used in combination

with other drugs used for MM after at least one prior therapy. However, there are still several challenges in the use of proteasome inhibitors in the treatment of cancers, such as the development of drug resistance, the drug toxicity, and their low efficiency for solid tumors (reviewed in (Manasanch and Orlowski 2017)), and thus more research is required to overcome the current challenges.

2.5 Methods to study UPS function

In this thesis, I utilized several *in vitro* and *in vivo* methods to study the UPS in a multicellular animal model, *C. elegans*. The total level of the endogenous 20S α-subunits can be examined from whole animal lysates by Western blotting method. In Western blotting, denatured proteins are separated by their length with the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred onto a membrane prior to the detection with an anti-20S antibody recognizing six out of the seven α-subunits (α1,2,3,5,6,7) of the 20S proteasome. In addition to the Western blotting, changes in the level of different proteasome complexes: core particles, core particles associated with one regulatory particle, and core particles associated with two regulatory particles, can be examined by blotting non-denatured proteasome complexes separated using native PAGE gels (Elsasser et al. 2005). Total enzymatic activity of proteasome complexes and individual complexes separated with native PAGE can be determined by using an in-gel activity assay, in which the hydrolysis capacity of the 20's chymotryptic-like site of each complex is determined by measuring the release of a fluorescent compound AMC from the Suc-LLVY-AMC (Suc-Leu-Leu-Val-Tyr-AMC) substrate subjected on the gel (Elsasser et al. 2005). The function of the 26S proteasome can, in addition, be studied indirectly by determining the total amount of endogenous polyubiquitinated proteins, proteasomal substrates, from whole animal lysates analyzed by Western blotting with an antibody against polyubiquitinated proteins, which recognizes poly-ubiquitinated proteins but not mono-ubiquitinated proteins or free ubiquitin.

In addition to those whole animal lysate-based *in vitro* methods, which exclude tissue-specificity, our group has created transgenic *C. elegans* strains to study the UPS function in different tissues *in vivo* (**Figure 5**). Our group have generated UPS-reporter strains that provide a readout for *in vivo* UPS-mediated protein degradation (Hamer et al. 2010, Li et al. 2011, Matilainen et al. 2013). These reporter strains express under cell type-specific promoters a photoconvertible fluorescent protein Dendra2, which is fused to a mutated, non-hydrolysable form of ubiquitin (UbG76V) (**Figure 5A**). The mutation in the ubiquitin leads to the polyubiquitination of the UbG76V-Dendra2 reporter by E3 ligases forming a short-lived substrate targeted for proteasomal degradation (Hamer et al. 2010).

To monitor the degradation rate, UbG76V-Dendra2 is irreversible photoconverted with 405 nanometer (nm) light from a green to a red fluorescent form and the change in the intensity of the red fluorescent proteins is followed as a function of time (Hamer et al. 2010). The monitoring of the photoconverted red fluorescent pool of the reporter allows us to separate reporter degradation from the newly translated green fluorescent, non-photoconverted pool. In this thesis, I utilized transgenic strains expressing the UPS reporter in body-wall muscle cells and intestinal cells (Hamer et al. 2010, Li et al. 2011). Moreover, our lab has generated transgenic *C. elegans* strains expressing a fluorescent polyubiquitin-binding reporter in the intestinal cells and body-wall muscle cells (Matilainen et al. 2013, Jha and Holmberg 2020, Pispa et al. 2020). The polyubiquitin-binding reporter (**Figure 5B**) consists of the ZsProSensor protein fused with ubiquitin-interacting motifs (UIMs) derived from the ubiquitin receptor, RPN-10 (Matilainen et al. 2013). The ZsProSensor protein comprises of a fluorescent ZsGreen-green protein tagged with the MODC (mouse ornithine decarboxylase) domain, which mediates the ubiquitin-independent degradation of the reporter. Accumulation of endogenous polyubiquitinated proteins enhances the fluorescence signal by stabilizing the reporter, which recognizes K48-linked polyubiquitin chains on the proteasome substrates (Matilainen et al. 2013).

To study the proteasome subcellular localization in *C. elegans*, we used a transgenic *C. elegans* strain expressing green fluorescent protein (GFP) –tagged UBH-4 (ubiquitin carboxyl-terminal hydrolase 4), a proteasome-associated DUB (Martinez-Fernandez et al. 2023), and a strain expressing GFP-tagged RPT-5, a subunit of the 19S regulatory particle (see the publication II). The proteasome subcellular localization was also examined by immunostaining of dissection-extruded gonads and intestine with the anti-20S antibody. Dissection of the animals improves the antibody access to the tissue but excludes some tissues and cell types from the analysis. Moreover, an immunohistochemical method on *C. elegans* sections was developed in this thesis (see the publication I) to study proteasome expression in a cell- and tissue-level resolution.

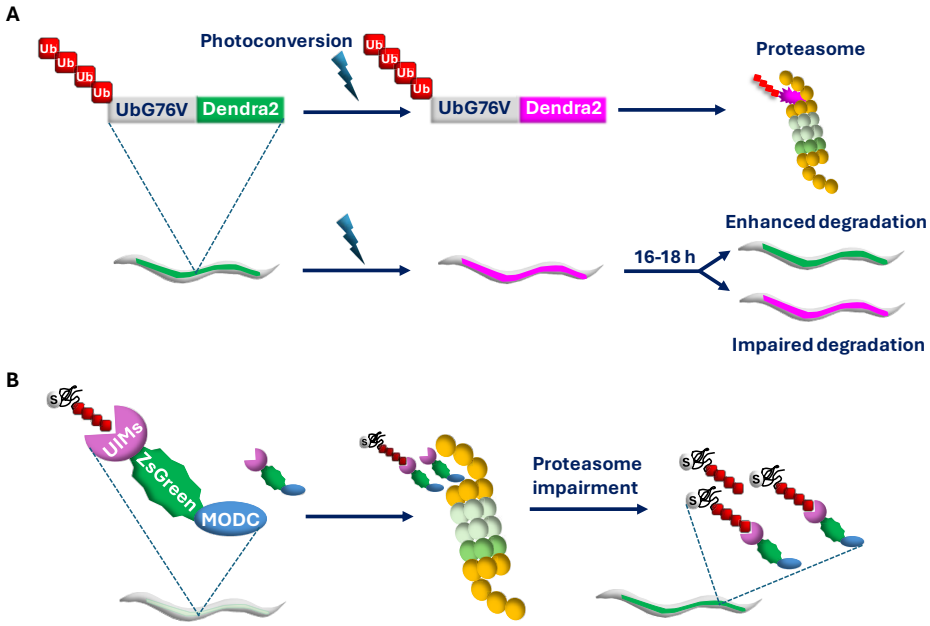

Figure 5. Fluorescent in vivo reporter systems to study UPS-mediated proteolysis in *C. elegans*. A) UPS reporter consists of fluorescent photoconvertible Dendra2 protein tagged with the uncleavable ubiquitin molecule (UbG76V), which mediates reporter ubiquitination and proteasomal degradation. Dendra2 is irreversible photoconverted from a green to a red state (purple Dendra2) to measure proteasome-mediated degradation of the photoconverted reporter. Below is a schematic illustration of a transgenic *C. elegans* expressing UbG76V-Dendra2 reporter in intestinal cells to measure tissue-specific protein degradation rate. The level of photoconverted reporter proteins is measured 16-18 hours after the photoconversion; low levels indicate enhanced degradation, and high levels indicate impaired degradation. **B)** Polyubiquitin-binding reporter consists of the fluorescent ZsGreen protein, the ubiquitin-interacting motifs (UIMs), and the mouse ornithine decarboxylase (MODC) domain. MODC mediates ubiquitin-independent degradation of the reporter. Proteasome impairment leads to accumulation of polyubiquitinated proteins stabilizing the reporter and enhancing fluorescence signal. Underneath is a schematic presentation of a transgenic *C. elegans* strain expressing polyubiquitin-binding reporter in intestinal cells in normal condition and upon proteasome impairment.

2.6 *Caenorhabditis elegans* as a model organism in biomedical research

The experimental model *Caenorhabditis elegans* utilized throughout this thesis, is a well-established multicellular organism used in thousands of laboratories worldwide. *C. elegans* has been used to uncover mechanisms and factors regulating basic cellular functions, but also to model molecular events underlying the pathogenesis of human diseases. Several groundbreaking findings have been made by using this small nematode, and in total four different Nobel prizes have been awarded to scientist working with *C. elegans*. The latest one in 2024, when the Nobel Prize in Physiology or Medicine was jointly awarded to Professor Victor Ambros and Professor Gary Ruvkun for their discovery of microRNA, a class of RNA molecules that plays a crucial role in post-transcriptional gene regulation, a mechanism, that they originally identified using *C. elegans*. Previous Nobel Prizes have been awarded for the discovery of the genetic pathway of programmed cell death and *C. elegans* cell lineage, the discovery of RNA interference – gene silencing by double-stranded RNA, and for the discovery and development of the GFP.

C. elegans is non-hazardous, non-infectious, non-pathogenic, and non-parasitic free-living nematode found worldwide in soil, especially in rotting vegetation, where it feeds microbes such as bacteria. *C. elegans* is an exceptional research model for several reasons, including its small size, short life cycle, anatomical simplicity, transparency, and well-annotated small genome. Newly hatched L1 larvae are about 0.25 millimeters (mm) long and even adults are only 1 mm long, which makes them suitable for laboratory cultivation, as thousands of animals can be grown on a Petri dish containing agar, which is seeded with a lawn of the bacterium *Escherichia coli* as a food source. Wild-type *C. elegans* has two sexes, self-fertilizing hermaphrodites and males. A single hermaphrodite can produce 300-350 self-progeny that are fertilized by the sperm stored in the spermatheca. Most of the self-fertilized progeny are hermaphrodites, and the incidence of males is only 0.1-0.2%, as males arise only after a spontaneous sex-chromosome non-disjunction (Hodgkin and Doniach 1997). Both sexes are diploid for the five autosomal chromosomes, and hermaphrodites have two X chromosomes (XX) whereas males have a single X chromosome (XO). The life cycle of *C. elegans* from an egg to an egg-laying adult is about 3.5 days at optimal temperature (20°) and the whole lifespan is just 2-3 weeks under standard laboratory conditions. After hatching, *C. elegans* larvae proceed through four larval stages, L1 to L4, before becoming fertile adults. Adult hermaphrodites can be self-fertile for about 3-4 days.

C. elegans has an invariant number of somatic cells: the number of somatic cells in hermaphrodites is 959 and 1031 in males, and out of these 302 are neurons. *C. elegans* is the only multicellular organism with a complete cell lineage; the development of an adult hermaphrodite or male from the single-celled zygote through the cell divisions is known (Sulston et al. 1975, 1980, 1983, Kimble and Hirsh 1979). Despite its simplicity, *C. elegans* has defined tissues, epidermis (also called hypodermis), muscles, intestine, nervous system, and tissue related to reproduction. Due to its transparency, single cells and their subcellular details can be easily visualized using Nomarski microscopy. In addition, different fluorescent proteins can be used to tag proteins and subcellular compartments to study their function *in vivo*.

C. elegans was the first multicellular eukaryotic organism to have its genome sequenced (The C. elegans Sequencing Consortium* 1998). The entire *C. elegans* genome has 19.983 protein-coding genes (Wormbase release WS293, Oct. 2024). It has been estimated that about 38% or more of the *C. elegans* protein-coding genes have predicted human orthologs (Shaye and Greenwald 2011) and *C. elegans* orthologs have been identified for about 60-80% of human genes (Sonnhammer and Durbin 1997, Lai et al. 2000). Moreover, 40-50% of known human disease genes have an ortholog in *C. elegans* (Ahringer 1997, Culetto 2000). Thus, *C. elegans* suits excellently to model general metazoan biology due to the high similarity in the cellular and molecular processes occurring in *C. elegans* and other animals such as humans. In addition, the genetic manipulation of *C. elegans* for instance through RNA interference to reduce gene activity is effortless making it a convenient model to examine the function of a gene or a protein.

3 AIMS OF THE STUDY

There is vast knowledge about proteasome assembly and structure as well as its catalytic function, however, rather little about its dynamic expression and localization within a cell, especially in a multicellular organism. In the first part of my thesis, I examined proteasome expression in *C. elegans* at a cell- and tissue-level resolution. The second part was based on a genome-wide RNAi screen conducted by our group to reveal new genes regulating proteasome function in *C. elegans*. Here, my thesis focused on the nuclear protein AKIR-1, which is an orthologue of human AKIRIN2, and which was identified as a putative proteasome regulator in the genome-wide RNAi screen.

More specifically, the aims were:

1. to study the tissue-specific expression of the proteasome during basal condition, under proteasome impairment, as well as in a long-lived, stress-resistant mutant background by developing an immunohistochemical analysis on *C. elegans* sections and

2. to study how lack of AKIR-1 affects the subcellular distribution of both proteasomal substrates and the proteasome itself, as well as proteasome activity in *C. elegans*.

4 MATERIALS AND METHODS

List of experimental methods used in this thesis are indicated in **Table 1**. The detailed description of the methods can be found in the associated publications, however, a brief description of the main steps in each method are provided in the following section.

Table 1 Methods used in this thesis.

Method	Used in publication(s)
C. elegans strains maintenance	I and II
Generation of a transgenic line	II
C. elegans RNA interference (RNAi)	I and II
C. elegans in vivo imaging	I and II
C. elegans Hoechst nuclear staining	II
Lifespan assay	II
Progeny count	II
Immunohistochemical analysis	I
Immunofluorescence with dissected *C. elegans*	II
Quantitative real-time polymerase chain reaction (qPCR)	II
Western blot analysis	I and II
Native gel analysis	I and II
Fluorescence signal quantification	II
Statistical analysis	I and II

4.1 Strains and *in vivo* methods

4.1.1 *C. elegans* strains and growth conditions

C. elegans strains used in this thesis and their sources or the original publication describing the generation of the strain are indicated in **Table 2**. Animals were cultured under standard conditions as previously described (Brenner 1974) at 20°C and fed with *Escherichia coli* (OP50 strain). Unless otherwise stated, bleach solution (0.25 M NaOH and 0.54% NaOCl) was used for age-synchronization and

animals were cultured on bacteria seeded plates from the first stage (L1) larvae (day 1) until first day of adulthood (day 4). Age-synchronized *akir-1* mutant L1 larvae were placed on bacteria-seeded plates 10–17 hours before the age-synchronized N2 L1 larvae as *akir-1* mutants displayed slower growth compared to wild-type N2 animals. Animals from both strains were collected at first day of adulthood (day 4 with N2 and day 5 with *akir-1* mutant animals). Animals used for *in vitro* assays were collected in M9 buffer (22 mM KH_2PO_4, 41 mM Na_2HPO_4, 8.5 mM NaCl, and 19 mM NH_4Cl) and animal pellets were stored at −80°C prior to lysis.

Table 2 *C. elegans* strains used in this thesis and their original sources.

CGC: Caenorhabditis Genetics Center.

Strain	Used in publication(s)	Original reference/ source
N2 (wild-type, Bristol isolate)	I and II	CGC
CB1370 *daf-2(e1370)III*	I	CGC
NL2099 [*rrf-3(pk1426)II*]	II	CGC
VC1056 [*akir-1(gk528)I*]	II	CGC
GR1702 *Is*[*sur-5::GFP*]	I and II	From Gary Ruvkun lab
YD3 [*xzEx3[unc-54p::UbG76V::Dendra2]]*	II	(Hamer et al. 2010)
YD27 [*xzEx27[vha-6p::UbG76V::Dendra2]*]	II	(Li et al. 2011)
YD90 [*xzIs1[vha-6p::UIM2::ZsProSensor]*]	II	(Matilainen et al. 2013)
YD114 [*xzIs2[unc-54p::UIM2::ZsProSensor]*]	II	(Pispa et al. 2020)
YD116 [*rrf-3(pk1426);xzIs2[unc-54p::UIM2::ZsProSensor]*]	II	(Jha and Holmberg 2020)
YD25 [*xzEx25[vha-6p::Dendra2]*]	II	(Li et al. 2011)
YD117 [*xzEx113[rpt-5p::GFP::RPT-5]*]	II	Publication II
CER395 [*ubh-4(cer68[ubh-4::egfp])II*]	II	(Martinez-Fernandez et al. 2023)

4.1.2 Generation of a transgenic line

To make a reporter strain expressing GFP tagged RPT-5 under its endogenous *rpt-5* promoter (YD117), GFP::RPT-5 translational fusion construct was generated. The *let-858* promoter of the GFP containing plasmid L3786 (#1593) was replaced with a putative *rpt-5* promoter sequence, which mutated the GFP stop codon and replaced the original three prime untranslated region (3'UTR) with a *rpt-5* coding region including introns and the following 3'UTR region. An extrachromosomal transgenic line was created by injecting into the gonads of young N2 adults the plasmid DNA (120–180 ng/mL) with a marker expressing cyan fluorescent protein

(CFP) in pharyngeal muscles under a myosin heavy chain 2 promoter, *Pmyo-2::CFP* (20 ng/mL).

4.1.3 *C. elegans* RNA interference (RNAi)

RNA interference (RNAi) was executed by using the feeding method as described earlier (Timmons et al. 2001) with few changes. The HT115(DE3) bacterial strain expressing the empty pL4440 cloning vector was used as a control. A single bacteria colony was cultured overnight at 37°C in Luria broth (LB) medium with antibiotics (ampicillin 100 µg/mL and tetracycline 12.5 µg/mL). Overnight culture was diluted 100-fold with 2xYT medium supplemented with the antibiotics as mentioned above. The production of the double-stranded ribonucleic acid (RNA) of bacterial cultures reached at the end of log phase (OD600=0.4) were induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Upon three hours' induction, the concentrations of IPTG and antibiotics were doubled prior to bacteria cells were added on RNAi feeding agar plates supplemented with antibiotics (ampicillin 100 µg/mL and tetracycline 12.5 µg/mL) and IPTG (0.4 mM). RNAi clones for the following genes were used: *akir-1(E01A2.6)*, *hda-1(C53A5.3)*, *let-418(F26F12.7)*, *lin-53(K07A1.12)*, *lin-40(T27C4.4)*, *chd-3(T14G8.1)*, *mep-1(M04B2.1)*, *dcp-66(C26C6.5)*, *ama-1(F36A4.7)*, *ima-1(T19B10.7)*, *ima-2(F26B1.3)*, *ima-3(F32E10.4)*, *imb-2(R06A4.4)*, *imb-3(C53D5.a)*, *T16G12.6*, *tsr-1(F53G2.6)*, *xpo-1(ZK742.1)*, *xpo-2(Y48G1A.5)*, *xpo-3(C49H3.10)*, *ubql-1(F15C11.2)*, *F36D4.5*, *imb-1(F28B3.8)*, *C35A5.8*, *Y69A2AR.16*, *pas-5(F25H2.9)*, *pas-6(CD4.6)*, *skn-1(T19E7.2)*, *or gfp*. Combined RNAi (*pas-5* and *skn-1*) was performed by mixing bacterial cultures in 1:1 volume ratio before seeding, in addition, *pas-5* and *skn-1* bacterial cultures were separately mixed with control bacteria (pL4440) in the same volume ratio. Sequencing was used to verify the RNAi clone target. Bacterial cultures for the genome-wide RNAi screen (identifying *akir-1*) with the J. Ahringer RNAi library described in (Kamath et al. 2003) was performed slightly differently compared to the above-mentioned protocol. A single bacteria colony was cultured overnight at 37°C in 2xYT medium supplemented with the antibiotics as mentioned above (no IPTG induction during liquid culture) and seeded on RNAi feeding agar plates containing antibiotics and IPTG as mentioned above. Age-synchronized *YD90* (intestinal polyubiquitin-binding reporter strain) animals at L1 larval stage were subjected for RNAi until being manually scored after three days as young adults for visible changes in fluorescence with a Leica MZ16 FA fluorescence stereomicroscope with a GFP Plus filter.

4.1.4 *C. elegans in vivo* imaging and Hoechst nuclear staining

Age-synchronized animals were mounted on 3% agarose pads and immobilized using levamisole in M9 (0.5 mM or 1 mM). Animals were imaged with the microscopes and objectives listed in the **Table 3** (in the table, "NA" refers to numerical aperture and "EC" for enhanced contrast). All the microscopes run a Zeiss Zen 2 software. Tissue-specifically expressed UbG76V-Dendra2 or Dendra2 was photoconverted by using 405-nm ultraviolet (UV) light. The red fluorescence signal intensity of the photoconverted proteins and the green fluorescence signal intensity of the unconverted proteins were monitored before and immediately after the photoconversion. The intestinal expression was monitored 16-18 hours after the photoconversion and the expression in body-wall muscle cells was monitored after 24 hours. For nuclear staining, age-synchronized animals were washed with M9 buffer, fixed with 100% ethanol for two minutes, permeabilized with Triton X-100 in phosphate buffered saline (PBS) (0.5% or 1%), and stained with Hoechst 33342 stain (4 µg/ml).

Table 3 Microscopes and objectives used in this thesis.

Microscope	Objective	Used in publication(s)
Zeiss Axio Imager 1 or 2 upright wide-field light microscope	EC Plan Neofluar NA 10 x 0.3	II
	Plan Apochromat NA 20 x 0.8	II
	EC Plan Neofluar NA 40 x 0.75	I
	Plan Apochromat NA 63 x 1.4	I
Zeiss LSM 780 inverted confocal microscope	Plan-Neofluor NA 40 x 1.3	II
	Plan-Apochromat NA 63 x 1.40	II
Zeiss LSM 880 inverted confocal microscope	Plan-Apochromat NA 40 x 1.40	II
	Plan-Apochromat NA 63 x 1.4	II

4.1.5 Lifespan assay and progeny count

Lifespan experiments were performed at 20°C. Age-synchronized L1 larvae were plated on RNAi plates. Animals' responses for a gently touch was tested daily, animals failing to react were classified as dead, moreover, animals were censored at the time of their death, if they crawled off the plate, died of an extruded gonad, or carried internally hatched offspring. Basic survival analysis was performed using an online tool, OASIS 2 (Han et al. 2016). For counting progeny, age-

synchronized L1 larvae were plated individually on RNAi feeding plates and daily moved to fresh RNAi plates until they stopped laying eggs. Total viable offspring per animal was counted. Offspring that crawled off the plate or died of an extruded gonad or internally hatched offspring were censored.

4.1.6 Immunohistochemical analysis

Age-synchronized animals were washed with M9 buffer. Animals were fixed in 10% (v/v) phosphate buffered formalin, embedded in agar (2% w/v in Milli-Q), and fixed again in 10% (v/v) phosphate buffered formalin for overnight prior to the agar blocks were processed into paraffin. Paraffin-embedded agar blocks were cut into 4-µm sections, fixed on slides, and dried at 37°C overnight. Xylene deparaffinized sections were rehydrated through gradually decreasing concentrations of ethanol to distilled water. PreTreatment module and 0,05 M Tris-HCl buffer with a pH 8,5 was used for short antigen retrieval at 98°C before immunohistochemistry. Immunohistochemical staining was performed using Dako REALTM EnVisionTM Detection System, Peroxidase/DAB+, Rabbit/Mouse kit (Dako) as reported earlier (Koskensalo et al. 2012). An antibody against proteasome 20S α-subunits (MCP231) was used in 1:2000 dilution and an anti-GFP antibody in 1:5000 dilution. Immunoreactivity was produced with a chromogen, 3,3'-diaminobenzidine tetrahydrochloride (DAB), resulting in a brown reaction product. Mayer's hematoxylin solution was used as a counterstain. Samples were rinsed in tap water and dehydrated through gradually increasing concentrations of ethanol to xylene prior to being mounted with Pertex® mounting medium. The specificity of the anti-20S antibody was tested by pre-incubating the antibody with purified human 20S proteasome in 1:100 dilution before being subjected to the sections. The slides were imaged either with a Zeiss Axio Imager 1 or an Axio Imager 2 upright epifluorescence microscope (see the objectives from the Table 3). Micrographs were processed with Photoshop CS6 software (Adobe Systems). Evaluation of the immunostaining was done independently by two investigators scoring the staining intensity visually as 0 for negative, 1 for mild, 2 for moderate, and 3 for strong positive immunoreactivity.

4.1.7 Immunofluorescence with dissected *C. elegans*

Age-synchronized animals were washed with M9 buffer and immobilized with levamisole in M9 (1 mM). Animals' intestine and gonad were extruded by making an incision close to the pharynx with 27-gauge syringe needles. Samples were fixed with 2xRFB (160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine, 30 mM PIPES pH 7.4, 50% methanol, and 1% formaldehyde). An additional 100% methanol fixation was utilized when the anti-20S antibody was used. Triton X-100

in PBS (0.5% or 1%) was used for permeabilization. Antibodies against polyubiquitinated proteins, FK1, and the proteasome 20S alpha subunits, MCP231, were used in 1:200 dilution. The following Alexa Fluor 594 conjugated anti-mouse IgM and IgG secondary antibodies in 1:200 and 1:100 dilution, respectively, were used for visualization. Hoechst 33342 stain (4 μg/ml) was used to label DNA. Dissected animals were mounted with SlowFade Diamond Antifade prior to imaging.

4.2 *In vitro* methods, fluorescence signal quantification, and statistics

4.2.1 Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from animal pellets using a Nucleospin RNA kit and a Maxima First Strand cDNA synthesis kit for RT-qPCR was utilized for complementary DNA (cDNA) synthesis. Quantitative real-time PCR (qPCR) was performed with Maxima SYBR Green/Rox qPCR Master Mix and LightCycler 480 quantitative PCR machine. The qPCR data for *akir-1* were normalized to the geometric mean expression of three reference genes (*act-1*, *cdc-42*, and *pmp-3*). A comparative Ct (ΔΔCt) method was used to quantify relative expressions of *akir-1* messenger RNA (mRNA). The following oligo sequences were used: 5'-gatatgcgaacgtctgctca, 5'-ggaatagtcatccccagtgc (*akir-1*); 5'-tcggtatgggacagaaggac, 5'-catcccagttggtgacgata (act-1); 5'-ctgctggacaggaagattacg, 5'-ctcggacattctcgaatgaag (cdc-42); and 5'-gttcccgtgttcatcactcat, 5'-acaccgtcgagaagctgtaga (pmp-3).

4.2.2 Western blot analysis

Animal pellets were lysed with a lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA) supplemented with N-ethylmaleimide (NEM) (20 mM), MG-132 (10 μM), and a Pierce Protease Inhibitor Mini Tablet. Extracted proteins were separated on a sodium dodecyl-sulphate (SDS)- polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane using Trans-Blot Turbo Transfer System. The antibodies against 20S proteasome α-subunits (MCP231), polyubiquitinated proteins (FK1), and α-tubulin were used in blotting in 1:1000, 1:500, and 1:10000 dilutions, respectively. The following secondary horseradish peroxidase (HRP)-conjugated anti-mouse IgM and IgG antibodies were used in 1:10000 dilutions. ECL signals were visualized with Odyssey FC Imaging System and quantified either with ImageJ or Image Studio software. The α-tubulin signal was used for normalization.

4.2.3 Native gel analysis

Animal pellets were lysed using a Dounce homogenizer and native gel lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM Mg2Cl2, 0.5 mM EDTA, and 1 mM ATP). Native gel electrophoresis and the in-gel proteasome activity assay were performed as described in (Elsasser et al. 2005, Matilainen et al. 2013) with a few changes. Gels were run in an ice bath for 30 minutes using 20 milliampere and then for 2 hours using 40 milliampere. The gels were either blotted onto a nitrocellulose membrane to detect native proteasome complexes with the anti-20S α-subunits antibody in dilution 1:1000 or incubated in developing buffer containing fluorogenic proteasome substrate succinyl-leu-leu-val-tyr-7-amino-4-methylcoumarin, Suc-LLVY-AMC (160 μM). Gels were exposed to ultraviolet light (UV) and imaged either with MultiImage Light Cabinet using FluorChem 8900 software or Gel Doc XR+ System with Image Lab Software. Colloidal Blue staining kit was used for total protein detection in gels after in-gel proteasome activity measurement and Ponceau S staining in nitrocellulose membrane after blot transfer. Image analyses were made with Fiji software.

4.2.4 Fluorescence signal quantification

The original images in Carl Zeiss Image (CZI)-file format or images in tagged image file format (TIFF) were used to quantify fluorescence signal with Zeiss ZEN 2 software or Fiji software, respectively. In Fiji, background was subtracted by using a sliding paraboloid algorithm. An adequate threshold selecting fluorescence signal and measure function was used to quantify the mean fluorescence intensity. To determine the nuclear/cytoplasmic ratio of the photoconverted UbG76V-Dendra2 signal, a Microscopy Image Browser software (Belevich et al. 2016) was used to quantify mean fluorescence intensity separately from the nuclei and the cytoplasm. Fiji or Adobe Photoshop was used to adjust the brightness of the fluorescence signal in some images. Similar adjustments were used for images, which were presented together for comparisons, unless otherwise stated in the figure legend. For dissected animals, the immunostaining in the nucleus was visually estimated as none, if nuclear staining intensity was similar or less compared to cytoplasmic staining intensity, and if the nuclear staining was stronger compared to the cytoplasm, nuclear staining was visually estimated as weak or strong.

4.2.5 Statistical analysis

The statistical significance was determined using Welch's t-test (two-tailed distribution and unequal variance) with Microsoft Excel 2016 spreadsheet. The

statistical significance in lifespan analysis was determined with a Mantel-Cox (log rank) test using R programming language. The used lifespan data was stratified into three independent experiments.

5 RESULTS AND DISCUSSION

5.1 Tissue-level proteasome expression in *C. elegans* (I)

5.1.1 Endogenous proteasome expression varies between subcellular compartments in wild-type *C. elegans*

Our group has shown in previous studies that UPS activity varies between different cell-types in *C. elegans* (Hamer et al. 2010, Li et al. 2011, Matilainen et al. 2013). The degradation of photoconverted UbG76V-Dendra2 reporter in neurons located in the tail of *C. elegans* (dorso-rectal ganglion) was three times faster than in the intestinal cells, and even eight times faster compared to the degradation rate in body-wall muscle cells (Hamer et al. 2010, Matilainen et al. 2013). There might be several reasons causing the differences in UPS degradation capacity in different cell types, for example the existence of cell type-specific regulators, differences in the expression of E3 ligases, and variation in proteasome subunit composition (Hamer et al. 2010, Matilainen et al. 2013). Additionally, differences in UPS activity might originate from variation in proteasome abundance. Therefore, the first aim of my thesis was to study the tissue expression of endogenous proteasomes in *C. elegans*. Although being a multicellular organism, protein abundance in *C. elegans* is commonly studied from whole animal lysates using Western blotting and mass-spectrometry-based quantitative proteomics. Unfortunately, these methods exclude information regarding tissue- and cell-level expression of the protein. Thus, we decided to apply the well-known and widely used immunohistochemistry method on *C. elegans* for studying proteasome expression at cell- and tissue-resolution level. Interestingly, at the time, our study was the first one, to our knowledge, applying immunohistochemistry on *C. elegans* to examine an endogenously expressed protein, whereas, in a few previous studies immunohistochemistry on *C. elegans* had been used to demonstrate the expression and localization of exogenous proteins, such as human tau and Aβ proteins (Kraemer et al. 2003, McColl et al. 2012).

To study proteasome expression on formalin-fixed, paraffin-embedded (FFPE) *C. elegans* sections, we decided to target the core particle as a readout for

proteasome expression. The core particle is structurally more stable complex compared to the regulatory particle, which conformation is highly dynamic (reviewed in (Mao 2021)). We targeted the core particle by using an antibody recognizing six out of the seven α-subunits of the human 20S core particle, of note there is no antibody recognizing several β-subunits of the core particle. It is believed that α-subunits are present in cells only as incorporated in the 20S complexes (Pack et al. 2014), however, there is also a study reporting an accumulation of some, possible free, α-subunits in senescent human fibroblasts (Chondrogianni et al. 2003). Thus, we first demonstrated that the positive immunoreactivity in *C. elegans* section corresponds to proteasome complexes instead of free native α-subunits. We showed that the antibody detected native α-subunits only when being parts of the 20S, 26S, or 30S proteasome complexes when we immunoblotted proteasome complexes separated by native-gel electrophoresis **(I: Supplementary Figure 1B)**. This suggests the non-existence or undetectable expression of free, native α-subunits in *C. elegans* lysates and sections. Pre-absorption of the antibody with purified human 20S proteasome clearly reduced the immunoreactivity in the sections supporting the specificity of the antibody against the *C. elegans* 20S proteasome **(I: Supplementary Figure 1D)**. We also demonstrated that enzymatic peroxidase-based immunohistochemistry is in general suitable for detecting changes in protein expression in *C. elegans* by showing that the anti-GFP immunoreactivity pattern on sections of *C. elegans* expressing GFP ubiquitously corresponded to the *in vivo* immunofluorescence patter **(I: Supplementary Figure 2A and 2B)**, moreover, the anti-GFP immunoreactivity reduced upon *gfp* targeted RNAi **(I: Supplementary Figure 4A)**.

Our immunohistochemical analysis on 1-day old adult *C. elegans* revealed a positive proteasome immunoreactivity in most tissues and cell types, and the extracellular cuticle was the only part lacking a positive staining **(I: Figure 1B)**. We observed positive expression in intestinal cells, body-wall muscle cells, and in the gonad, including germ cells, oocytes, and embryos **(I: Figure 1C)**. Visual quantifications of the proteasome immunoreactivity revealed that proteasome expression varied between cell types and moreover between cellular compartments as in most cell types the nucleus and/or perinuclear space displayed stronger immunoreactivity compared to the immunoreactivity pattern in the cytoplasm **(I: Figure 1D)**. Our results were in line with a previous study using proteasome immunohistochemistry on whole-fly (*Drosophila melanogaster*) section and showing a generalized cytoplasmic and nuclear proteasome immunoreactivity in all tissues (Tsakiri et al. 2013). They also observed, similarly to our results, variation in staining intensity between different tissues (Tsakiri et al. 2013). Immunohistochemical staining of human liver tissue demonstrated that

proteasomes are distributed both in the nucleoplasm and cytoplasm in hepatocytes (Tanaka et al. 1989). Interestingly, in accordance with our observation, they observed a denser proteasome distribution in the nucleoplasm compared to the cytoplasm in hepatocytes (Tanaka et al. 1989). When utilizing fluorescence correlation spectroscopy, a similar subcellular distribution of the GPF-tagged subunit of the core particle has been observed also in actively dividing yeast cells, in which the absolute concentration of the subunit is higher in the nucleus compared to the concentration in the cytoplasm (Pack et al. 2014). We have furthermore showed a similar subcellular distribution of proteasomes in our later studies by immunostaining dissected intestines and oocytes of wild-type animals with the anti-20S antibody. Consistently with proteasome immunoreactivity in sections, the nuclei of intestinal cells and oocytes displayed higher proteasome immunofluorescence compared to the cytoplasm **(II: Figure 3A)**. A recent study also demonstrated that the 20S core particle subunit α6 immunofluorescence was stronger in the nuclei compared to the cytoplasm in dissected *Drosophila* intestines (Livneh et al. 2023). Moreover, we and other groups have utilized GFP-tagged proteins to study proteasome expression and localization in *C. elegans*, and these results are in line with our immunohistochemical analysis. Fernando and colleagues have for instance shown ubiquitous expression of GFP-tagged 19S RP lid subunits, RPN-7, RPN-8, RPN-9, and RPN-12, in somatic and germline cells, in which nuclei displayed bright fluorescence compared to the dim expression in the cytoplasm (Fernando et al. 2021, 2022). GFP-tagged subunit of the 20S proteasome PAS-1 was also present throughout the germline and predominantly localized to the nucleus (Kumar and Subramaniam 2018). We have shown, by using a strain expressing extrachromosomal arrays of GFP-tagged 19S RP base subunit RPT-5 under endogenous *rpt-5* promoter and by using a CRISPR-engineered strain (Martinez-Fernandez et al. 2023) expressing GFP-tagged to a proteasome-associated DUB, UBH-4, the *C. elegans* ortholog of the human UCHL5, that the fluorescence signal is stronger in the nuclei of intestinal cells compared to the signal displayed in the cytoplasm **(II: Figure 3B)**.

Taken together, regardless of the used methods, observations on proteasome basal expression in *C. elegans* by us and other groups are in line with each other. Immunohistochemistry, however, have some advantages compared to whole animal immunostaining or *in vivo* imaging of fluorescent-tagged proteasome subunits. Immunohistochemical staining of *C. elegans* sections enables more efficient antibody access to the internal cells and proteins, and is less invasive compared to whole animal immunostaining, where chemical or mechanical disruption of the cuticle protecting the larvae, adults and egg shelf surrounded embryos is needed despite the risk of distorting or damaging the worm body.

Moreover, compared to *in vivo* fluorescence imaging of GFP-tagged proteins, where the expression and localization of a singular proteasome subunit can be studied at a time, immunohistochemistry with proteasome antibody enables the detection of several endogenous α-subunits of the proteasome core particle. In conclusion, immunohistochemical detection of proteasome in *C. elegans* sections provides us a method to study changes in endogenous proteasome expression at the cell- and tissue-level in a multicellular organism.

5.1.2 Similar proteasome expression in long-lived *daf-2* mutants and wild-type animals

The intensity of immunoreactivity might be affected by cell-specific features, such as size and morphology and therefore this semi-quantitative method is suitable for comparing intensity variations in the same cell types or tissues upon different treatments or in different strains with similar body size and general appearance. As our group has shown in a previous study that long-lived *daf-2(e1370)* mutants display higher UPS activity compared to wild-type animals (Matilainen et al. 2013), I decided to examine by immunohistochemistry whether potential changes in tissue-level proteasome expression could explain the observed differences in UPS activity between wild-type animals and *daf-2(e1370)* mutants. The *C. elegans daf-2* gene encodes the human insulin/insulin-like growth factor 1 (IGF-1) transmembrane receptor. Animals carrying mutated *daf-2* display compared to wild type animals a more than two-fold increase in their lifespan, which is mediated by the FOXO transcription factor DAF-16 (Kenyon et al. 1993).

In that previous study performed by our group, it was demonstrated that compared to wild-type animals long-lived *daf-2(e1370)* mutants displayed approximately two times faster *in vivo* degradation of photoconverted UbG76-Dendra2 proteins in the intestine and significantly enhanced degradation rate of the UPS reporter in body-wall muscle cells (Matilainen et al. 2013). In the study, it was further showed that the faster UPS-mediated degradation in the intestine and body-wall cells of *daf-2(e1370)* mutants did not stem from a general increase in proteasome abundance, as no difference in the proteasome levels were observed between *daf-2(e1370)* mutants and wild-type animals when proteasome 20S α-subunits were immunoblotted from whole animal lysates (Matilainen et al. 2013). This was in line with a previous study, in which no changes in the abundance of 20S proteasome subunits between *daf-2* and wild-type animal at day 1 of adulthood were observed when the protein abundance changes occurring between *daf-2* mutants and wild-type animals were analyzed by quantitative mass spectrometry (Dong et al., 2007). Interestingly, another study utilizing quantitative proteomics showed that the abundance of some 20S proteasome

subunits reduced in *daf-2* mutants compared to wild-type animals at the first day of adulthood (Stout et al. 2013), whereas a study that profiled proteome changes in *C. elegans* during the lifespan and aging process, showed that *daf-2* mutants display a more pronounced upregulation of proteasomal subunits at the day 6 of adulthood than the wild-type animals (Walther et al. 2015). This study, however, did not show a comparison between the proteomes of *daf-2* mutants and wild-type animals at the day 1 of adulthood, and therefore, their findings are not directly comparable with the previous studies by other groups and by our group. Due to the variable outcomes of the proteome profiling studies and the interesting observation made by our group that *daf-2(e1370)* animals display higher UPS activity in the intestine and body-wall muscle cells without systemic change in proteasome abundance, I decided to utilize immunohistochemistry on *daf-2(e1370)* mutants to examine proteasome protein expression more accurately at cell and tissue resolution level.

The immunohistochemical analysis revealed that adult, 1-day old *daf-2(e1370)* mutants displayed a similar positive proteasome immunoreactivity in intestinal cells, body-wall muscle cells, and in the gonad, including germ cells, oocytes, and embryos as shown with age-matched adult wild-type animals **(I: Figures 3A and 1C)**. In addition, *daf-2(e1370)* mutants displayed in accordance with the wild-type a stronger proteasome immunoreactivity in the nucleus compared to the cytoplasm **(I: Figures 3A and 1C)**. Visual scoring of the proteasome immunoreactivity confirmed that there were no significant differences at tissue level proteasome expression between 1-day old *daf-2(e1370)* and wild-type animals **(I: Figure 3B)**. In conclusion, my results with immunohistochemistry support our previous observations that the faster UPS-mediated degradation in the intestinal cells and body-wall muscle cells in *daf-2(e1370)* mutants originates from changes in activity per se and not from more abundant proteasomes.

5.1.3 Proteasome subunit knockdown results in tissue-specific responses and in a SKN-1-dependent proteasome expression upregulation in the intestine

Next, we were curious to examine whether tissues respond differently on a systemic knockdown of a single proteasome subunit. In mammalian cells, inhibition of the proteasome causes a compensatory upregulation of proteasome subunit gene expression (Meiners et al. 2003). This "bounce-back" response is conserved in *C. elegans*, and our group have previously in collaboration with Li and colleagues shown that impaired proteasome activity executed by RNAi against the essential proteasome subunits *pas-5* or *rpn-2* causes *in vivo* upregulation of the pbs-4, rpt-5, and rpn-11 transcriptional reporters as well as of the translational fusion protein RPN-11::GFP in the intestine and body-wall muscle cells (Li et al. 2011). In addition, I showed that RNAi against 20S proteasome α-subunit *pas-5* increased the total level of α-subunits compared to control RNAi, when proteasome 20S α-subunits were immunoblotted from whole animal lysates **(I: Figures 2A and 2B)**.

Immunohistochemically stained sections of *pas-5* RNAi-treated adult *C. elegans* followed by visual quantification of the immunoreactivity revealed that systemic knockdown of a single proteasome subunit resulted in cell type–specific responses, as proteasome immunoreactivity decreased in germ cells, oocytes, and in the nuclei of embryos, whereas an increase in proteasome immunoreactivity was observed in somatic cell types, such as intestinal and body-wall muscle cells compared to the control RNAi treatment **(I: Figure 2C and 2D)**. The most prominent increase in proteasome immunoreactivity was observed in the intestine, especially the cytoplasmic expression of proteasome increased upon *pas-5* RNAi **(I: Figure 2D)**. RNAi against another 20S proteasome α-subunit *pas-6* showed a similar prominent increase in proteasome immunoreactivity in the intestine **(I: Supplementary Figure 3A and 3B)**. It is worth noticing that most proteasome subunits are essential and RNAi against 20S proteasome core particle subunits started at L1 larval stage results in developmental arrest (Takahashi et al. 2002, Kamath et al. 2003), therefore *pas-5* or *pas-6* RNAi were started on animals at L3 larval stage to avoid developmental arrest. We also examined the sensitivity of intestinal cells and body-wall muscle cells to RNAi treatment by exposing transgenic *C. elegans* animals expressing GFP ubiquitously to *gfp* RNAi. The knockdown of *gfp* decreased the anti-GFP immunoreactivity in both intestinal and body-wall muscle cells **(I: Supplementary Figure 4A)** highlighting that both tissues response to gene silencing via RNAi.

In mammalian cells, the compensatory upregulation of proteasome subunit gene transcription upon impaired proteasome function, is mediated by the transcription factors NRF1 and NRF2 (Kraft et al. 2006, Radhakrishnan et al. 2010, Steffen et al. 2010), and our group have previously shown that in *C. elegans* the similar compensatory response is mediated in by SKN-1 transcription factor, the *C. elegans* ortholog of mammalian NRF1/2/3 (Li et al. 2011). Accordingly, I showed that the prominent increase in proteasome immunoreactivity in the cytoplasm of intestinal cells upon *pas-5* RNAi was dependent on the SKN-1, as concurrently targeting *pas-5* and *skn-1* with RNAi resulted in diminished enhancement in proteasome immunoreactivity **(I: Figure 2E and 2F)**.

Taken together, my results demonstrated for the first time that a multicellular organism display tissue- and cell-type specific variations in endogenous proteasome expression upon systemic proteasome impairment. Interestingly, our group has recently shown that also systemic knockdown of *C. elegans* autophagy genes, *bec-1* and *lgg-2*, decreases the immunoreactivity of endogenous 20S proteasomes in a tissue-specific manner (Jha and Holmberg 2020). In conclusion, my immunohistochemical analysis revealing proteasome expression at a cell- and tissue-level resolution in different backgrounds highlights that a complementary approach with preserved tissue resolution should be utilized in addition to the commonly used whole animal lysate-based methods when examining the expression of proteasome or other proteins in metazoans.

5.2 A nuclear protein AKIR-1 coordinates proteasome function and subcellular localization in *C. elegans* (II)

5.2.1 Tissue-specific variation in the nuclear accumulation of proteasomal substrates in AKIR-1 depleted animals

The second part of my study (II) is based on a genome-wide *C. elegans* RNAi screen conducted previously by our group to identify new UPS modifiers (unpublished results). Results presented here focus on one of the identified hit gene encoding a small nuclear protein AKIR-1 and its role as a proteasome regulator in *C. elegans*. The performed RNAi screen and the rest of the identified hit genes are not presented in this thesis, however, briefly, our group performed a genome-wide RNAi screen using our previously generated polyubiquitin-binding reporter animals expressing in the intestinal cells a fluorescent reporter that binds endogenous polyubiquitinated proteins (Matilainen et al. 2013). These reporter animals were exposed to feeding RNAi from L1 larval stage until 1 day of adulthood when changes in their fluorescence signal compared to control RNAi treatment were visually scored.

RNAi against one gene *akir-1* (E01A2.6) resulted in drastic change in the pattern of the fluorescence signal compared to control RNAi. The knockdown of *akir-1* increased the polyubiquitin-binding reporter fluorescence and accumulated the evenly distributed fluorescence signal of the reporter into a more punctuated pattern **(II: Figure 1A)**. DNA staining confirmed that the polyubiquitin-binding reporter concentrated mainly into the nucleus in the intestinal cells upon *akir-1* knockdown **(II: Figure 1B)**. This subcellular redistribution of polyubiquitin-binding reporter in the intestinal cells upon *akir-1* knockdown reflected an accumulation of cellular polyubiquitinated proteins into the nuclei of intestinal cells. To confirm this, we studied endogenous polyubiquitinated proteins by immunostaining dissected intestines of *akir-1* RNAi-treated wild-type animals with an antibody against polyubiquitin and visually scored the nuclear immunostaining intensity as none, weak, or strong. In addition to RNAi, which downregulated *akir-1* mRNA levels efficiently though not fully **(II: Supplementary figure 1A)**, we also used an *akir-1(gk528)* null mutant (Clemons et al. 2013). The total number of animals displaying a positive nuclear immunostaining of endogenous polyubiquitinated proteins increased from 60% to 90% upon *akir-1* RNAi **(II: Figure 1C)** and from 44% to 90% in *akir-1*(gk528) mutants compared to controls **(II: Figure 1D)**. Interestingly, the total number of animals with strong nuclear immunostaining increased from 14% in control to 72%

in *akir-1* RNAi treated animals **(II: Figure 1C)** and from 16% in wild-type animals to 74% in *akir-1* mutants **(II: Figure 1D)**. Western blot analysis of the total levels of endogenous polyubiquitinated proteins from whole animal lysates revealed no systematic change in lysates of *akir-1* mutants compared to control lysates **(II: Supplementary Figure 1B)**. Interestingly, a very recent study demonstrated that in Hela cells the loss of human AKIRIN2 (the ortholog of *C. elegans* AKIR-1) resulted in a similar nuclear accumulation of proteasome substrate, the mutant A53T α-synuclein (Park et al. 2024). In conclusion, we showed that *akir-1* depletion causes a nuclear accumulation of cellular polyubiquitinated proteins in the intestine in *C. elegans*.

C. elegans akir-1 belongs to a family of Akirin genes found only in metazoan genomes (Macqueen and Johnston 2009). The number of Akirin genes found within species varies from one to eight, a single Akirin gene being present in *Drosophila* and *C. elegans* (*akir-1*), and its two homologs, Akirin1 and Akirin2 are found in vertebrates. Each Akirin gene encodes a highly conserved small protein primarily located in the nucleus (reviewed in (Bosch et al. 2020)). Akirin proteins regulate the immune responses in several organisms, including *Drosophila* and *C. elegans*, in which Akirin/AKIR-1 coordinates the expression of genes related to innate immune response (Goto et al. 2008, Polanowska et al. 2018), and in mouse, in which Akirin2 is required for B-cell differentiation and cytokine production (Tartey et al. 2015). In addition to the conserved role of Akirin proteins in immune response, Akirins are also required for muscle development and function in Drosohila, *C. elegans*, and rodent model systems (Marshall et al. 2008, Salerno et al. 2009, Nowak et al. 2012, Chen et al. 2017, Bosch et al. 2019, Bowman et al. 2020). Proper function of Akirin2 is also needed for normal neurodevelopment process (Bosch et al. 2016, Liu et al. 2017, Peek et al. 2022). Studies on vertebrate Akirin2 have revealed its importance and potential clinical relevance in different cancers, neurodegenerative diseases, as well as in ischemic stroke (Leng et al. 2019, Fekete et al. 2020, Peek et al. 2022, Sun et al. 2022, Kong et al. 2024). In *C. elegans*, AKIR-1 has been shown to function in germline by regulating the disassembly of the synaptonemal complex, which mediates homologous chromosome association before crossover formation in meiosis (meiotic prophase I) (Clemons et al. 2013, Bowman et al. 2019). Akirins mediate many of their functions by regulating gene expression, both activating and repressing gene expression in a context-dependent manner (reviewed in (Bosch et al. 2020)). Akirins regulates gene expression through its interaction with transcription factors and the chromatin remodeling machineries, multi-protein complexes opening compact DNA. However, Akirin proteins lack a DNA-binding domain and, thus, possible act as a bridging protein to stabilize the interaction between the

chromatin remodeling complexes and transcriptional regulators (Nowak et al. 2012).

Besides the intestinal cells, we examined how depletion of *akir-1* affected polyubiquitinated proteins in body-wall muscle cells and in oocytes. To study the effect of *akir-1* downregulation on polyubiquitinated proteins in muscles, we utilized another our previously generated transgenic strains expressing the fluorescent polyubiquitin-binding reporter in body-wall muscle cells in wild-type (N2) or in the RNAi-sensitive *rrf-3* mutant background (Pispa et al. 2020, Jha and Holmberg 2020). Compared to control RNAi, knockdown of *akir-1* did not change the mean fluorescence intensity nor changed the fluorescence pattern itself of the polyubiquitin-binding reporter expressed in body-wall muscle cells in neither background **(II: Supplementary Figure 5B)**. Thus, interestingly, in body-wall muscle cells downregulation of *akir-1* did not cause a nuclear accumulation of polyubiquitinated proteins as in intestinal cells. We speculated that the different phenotype in muscle reporter animals could be due to remaining maternal AKIR-1. Thus, to exclude this possibility, we subjected the *rrf-3* animals expressing the reporter to *akir-1* RNAi from the L1 larval stage and examined their F1 progeny at first day of adulthood for changes in reporter fluorescence. The number of body-wall muscle cell nuclei positive for polyubiquitin-binding reporter increased from 14% in control to 39% in *akir-1* RNAi treated animals **(II: Figure 5B)**. We also tried to determine the changes in cellular polyubiquitinated proteins by immunostaining whole *akir-1* mutants with anti-polyubiquitin antibody but faced some technical issues. This could be overcome in future once we have optimized the use of anti-polyubiquitin antibody on formalin-fixed, paraffin-embedded *C. elegans* sections. Taken together, compared to intestinal cells body-wall muscle cells displayed a delayed onset of nuclear accumulation of polyubiquitin-binding reporter upon *akir-1* knockdown.

As we do not have a transgenic worm strain expressing the polyubiquitin-binding reporter in gonad, we examined the expression of endogenous polyubiquitinated proteins by immunostaining dissected oocytes of *akir-1* mutants and *akir-1* RNAi-treated animals with the anti-polyubiquitin antibody. Interestingly, *akir-1* mutants, *akir-1* RNAi-treated animals, as well as control animals showed weaker immunostaining of polyubiquitinated proteins in the nuclei compared to the cytoplasmic expression in oocytes **(II: Figure 5A and Supplementary Figure S5A)**, occasionally *akir-1* mutants displayed a polyubiquitin-positive immunostaining at the nuclear membrane **(II: Figure 5A, lower panel)**. Thus, on the contrary to intestinal cells, endogenous polyubiquitinated proteins did not accumulate in the oocyte nuclei upon AKIR-1

depletion. Taken together, AKIR-1 depletion affected proteasome substrates differently in the intestinal cells, oocytes, and body-wall muscle cells.

5.2.2 Knockdown of *akir-1* alters proteasome activity

To examine possible reasons causing the accumulation of polyubiquitinated proteins upon AKIR-1 depletion, we first defined the abundance of proteasomes by immunoblotting 20S α-subunits from whole animal lysates. We did not detect any clear change in the amount of 20S α-subunits in *akir-1* mutants or *akir-1* RNAi treated animals compared to controls **(II: Figure 2 A and 2B)**. Next, we examined if knockdown of *akir-1* alters the enzymatic activity of the proteasome by using an in-gel activity assay, in which proteasome complexes are separated on a native gel, and the hydrolysis capacity of the 20S's chymotryptic-like site is determined by measuring the release of a fluorescent compound AMC from the Suc-LLVY-AMC substrate. The in-gel activity assay from whole animal lysates revealed that the substrate hydrolysis was slightly increased in *akir-1* RNAi treated animals compared to control RNAi, and the change in the hydrolysis seemed to mainly originate from the enhanced degradation capacity of the free 20S core particles **(II: Figure 2C)**. Next, we immunoblotted native gel-separated proteasome complexes with the antibody against 20S α-subunits and no clear change in the amount of free 20S CP complexes was observed between *akir-1* RNAi- and control RNAi-treated animals **(II: Figure 2D)**. This suggested that the degradation capacity of the free 20S CP is increased in *akir-1* RNAi treated animals compared to control RNAi treated animals and did not stem from changes in the amount of free 20S CP complexes.

In addition to the *in vitro* in-gel activity assay, we also used our UPS-reporter strains expressing the photoconvertible proteasomal substrate UbG76V-Dendra2 in the intestine or in body-wall muscle cells in *C. elegans* (Hamer et al. 2010, Li et al. 2011) to examine if *akir-1* downregulation changes UPS activity *in vivo*. With these transgenic strains we were also able to better study if knockdown of *akir-1* affects the proteasome's ability to degrade polyubiquitinated proteins, which resemble more the physiological substrates of the proteasome than the Suc-LLVY-AMC peptide. We measured the fluorescence of photoconverted UbG76V-Dendra2 from whole animals, and no difference was detected in the degradation capacity in the intestine **(II: Figure 2E)** or body-wall muscle cells **(II: Supplementary Figure 5C)** in *akir-1* RNAi-treated animals compared to control RNAi-treated animals.

As the most striking phenotype, i.e., drastic nuclear accumulation of cellular polyubiquitinated proteins upon loss of AKIR-1, was observed in the intestine, we measured the degradation of photoconverted UbG76V-Dendra2 at single-cell level, in this study for the first time the nuclear and cytoplasmic fluorescence signal was quantified separately. Interestingly, in accordance with the nuclear accumulation of polyubiquitinated proteins, the nuclear-cytoplasmic ratio of photoconverted UbG76V-Dendra2 fluorescence intensity increased in the intestinal cells upon *akir-1* RNAi compared to control RNAi **(II: Figure 2F)**. This suggests a slower *in vivo* UPS-mediated protein degradation in the nuclei of intestinal cells. Taken together, knockdown of *akir-1* slightly increased the enzymatic activity of the proteasome complexes, especially the activity of the free 20S core particles in whole animal lysates. The *in vivo* UPS activity in the intestine or body-wall muscle cells, however, did not change in *akir-1* RNAi treated animals, suggesting that *akir-1* RNAi-induced increase in the enzymatic activity *in vitro* seems to originate from some other cell type(s) existing in the whole animal lysates. Although there was no change in the *in vivo* UPS activity in the intestine, interestingly at the subcellular level, the proteasome-mediated degradation capacity in the intestinal cells seemed to redistribute between the nucleus and cytoplasm upon *akir-1* RNAi.

5.2.3 AKIR-1 regulates nuclear localization of proteasome in intestinal cells and oocytes

As we observed that downregulation of *akir-1* resulted in a slower *in vivo* UPS-mediated protein degradation in the nuclei of intestinal cells along with the drastic nuclear accumulation of cellular polyubiquitinated proteins, we examined if AKIR-1 depletion affects the subcellular distribution of the proteasome in the intestine. We immunostained dissected intestines of *akir-1* RNAi-treated wild-type animals and *akir-1* mutants with the antibody detecting 20S α-subunits and measured the immunofluorescence intensity from the cytoplasm and the nucleus. We used dissected tissues to improve antibody access to the tissue. Interestingly, 20S immunofluorescence intensity reduced in the nuclei of intestinal cells both in *akir-1* mutants and *akir-1* RNAi treated animals compared to controls, which displayed a strong 20S immunofluorescence in the nuclei of intestinal cells **(II: Figures 3A and Supplementary Figure S3)**. To further confirm our observation, we studied the subcellular localization of GFP-tagged RPT-5 (subunit of the 19S regulatory particle) and UBH-4 (proteasome-associated DUB) in the intestine. Animals exposed to *akir-1* RNAi displayed reduced GFP::RPT-5 and UBH-4::GFP fluorescence in the intestinal nuclei when compared to control RNAi treated animals **(II: Figure 3B and 3C)**, which was in accordance with the 20S immunofluorescence result.

While immunostaining dissected intestines, we noticed that simultaneously dissected oocytes of *akir-1* RNAi-treated wild-type animals and *akir-1* mutants also displayed clearly reduced 20S immunofluorescence in the nuclei compared to a strong nuclear 20S immunofluorescence displayed in control animals **(II: Figure 4A and Supplementary Figure S4)**. A similar reduction in levels of nuclear proteasomes in oocytes was observed when animals expressing UBH-4::GFP were exposed to *akir-1* RNAi **(II: Figure 4B)**. This clear reduction in nuclear proteasomes in oocytes upon loss of AKIR-1 was intriguing as we did not observe an accumulation of polyubiquitinated proteins in the nuclei of oocytes either in control animals or upon AKIR-1 deletion **(II: Figure 5A and Supplementary Figure S5A)**.

C. elegans oocytes might display higher proteasome capacity compared to intestinal cells, which could explain, why we did not detect accumulation of polyubiquitinated protein in the oocyte nuclei. It has been for example shown that dissected gonads from young *Drosophila* flies display elevated proteasome capacity compared to somatic tissues. On the other hand, contradicting results have been obtained in a study examining proteasome mediated protein degradation in germline by utilizing a transgenic *C. elegans* strain, which expresses the uncleavable mutated ubiquitin (UbG76V) fused to a GFP-tagged histone H2B under the germline promoter *pie-1* (Kumar and Subramaniam 2018). In this study, they detected UbG76V::GFP::H2B expression in oocyte nuclei in wild-type animals, which was assumed to be due to the absence or low levels of proteasome activity in oocytes (Kumar and Subramaniam 2018). However, it could also reflect the reporter expression rate in oocytes, the rate of the reporter's polyubiquitination in these cells, and/or the reporter's slow delivery to the proteasome for degradation.

Differences in the pool of proteasomal substrates in oocytes and intestinal cells could also potentially explain why polyubiquitinated proteins accumulated in the nuclei of intestinal cells, but not in oocytes upon loss of *akir-1*. This might also affect how well the used anti-polyubiquitin antibody recognizes those proteins and their ubiquitin modifications. It has also been reported that ubiquitinated proteins are delivered from the nucleus to the cytoplasm upon proteasome inhibition via UBIN-POST system in human cells and *C. elegans* (Hirayama et al. 2018). However, our unpublished data suggest that the possible function of this system in oocytes does not coordinate the nuclear export of polyubiquitinated protein in *akir-1* mutants. Taken together, despite the differences in subcellular distribution of polyubiquitinated protein in different cell types, both downregulation and loss of *akir-1* resulted in a clear reduction in nuclear proteasomes in intestinal cells and oocytes.

5.2.4 Knockdown of importins resemble AKIR-1 depletion-induced phenotype

To examine the mechanism(s) underlying the observed phenotypes in animals lacking AKIR-1, we tested if AKIR-1 regulates the nuclear accumulation of polyubiquitin-binding reporter and possible the subcellular localization of the proteasome in the intestinal cells through its previously identified functions. Akirin proteins have been shown to regulate developmental processes in several animal models, in *C. elegans* AKIR-1 regulates the development of gonad, the embryonic development of muscles, and is required for muscle integrity in the adult *C. elegans* (Bowman et al. 2019, 2020). We demonstrated that the nuclear accumulation of polyubiquitin-binding reporter was not related to developmental defects, as *akir-1* RNAi started at L4 larval stage resulted in a similar nuclear accumulation of the polyubiquitin-binding reporter in the intestine at day 3 of adulthood **(II: Figure 6A)** as we reported with 1-day old adults exposed to *akir-1* RNAi from L1 larval stage **(II: Figure 1A)**.

Akirins mediate several cellular functions by regulating transcription. In *C. elegans* AKIR-1 interacts with the subunits of nucleosome remodeling and deacetylase (NuRD I, II, and MEC) chromatin remodeling complexes to regulate expression of genes related to innate immunity response (Polanowska et al. 2018). In addition, AKIR-1 has been shown to regulate worm movement and body size by acting together with the NuRD chromatin remodeling complex (Bowman et al. 2020). To disturb general transcription in *C. elegans* and to study its effect on the polyubiquitin-binding reporter in the intestine, we downregulated an *ama-1* gene encoding a subunit of the RNA polymerase II, which catalyzes transcription. No nuclear accumulation of the polyubiquitin-binding reporter in the intestinal cells were observed upon *ama-1* RNAi. Next, we examined if RNAi against each member of the NuRD complexes and MEC complexes affected on polyubiquitin-binding reporter in the intestinal cells, but *akir-1* RNAi-induced phenotype was not observed upon any of the RNAi treatments **(II: Supplementary Figure 6)**. Thus, our results indicate that AKIR-1 might regulate some cellular functions independently of transcription. This was also supported by another study that recently identified human AKIRIN2, the ortholog of *C. elegans* AKIR-1, as a negative regulator of the MYC transcription factor in human cancer cell lines and showed that AKIRIN2 regulates MYC through a post-translational mechanism (de Almeida et al. 2021). Moreover, we showed that *akir-1* depletion did not affect the levels of proteasome subunits or the amount of assembled proteasome complexes **(II: Figure 2A, 2B, and 2D)** supporting the role of AKIR-1 regulating proteasome function in a transcription-independent manner.

In *C. elegans*, AKIR-1 has been shown to interact with importin α2 and α3, IMA-2 and IMA-3, respectively (Polanowska et al. 2018). In another study, *C. elegans* AKIR-1 and IMA-2 was shown to physically interact with each other and to function during meiosis by importing and loading the cohesin complex proteins on chromosomes (Bowman et al. 2019). Furthermore, as Akirin proteins, including *C. elegans* AKIR-1, are known to have a highly conserved N-terminal bipartite nuclear localization signal (NLS) (Goto et al. 2008, Macqueen and Johnston 2009, de Almeida et al. 2021), we hypothesized that the *akir-1* depletion-induced phenotypes could be due to its role in coordinating protein transportation between the cytoplasm and the nucleus. Therefore, we tested if RNAi against *C. elegans* karyopherin family members including importins and exportins resulted in nuclear accumulation of polyubiquitin-binding reporter in intestinal cells. There are six importins and seven exportins in *C. elegans*, most of them having a human homolog(s) **(II: Figure 7A)**. Interestingly, a clear nuclear accumulation of polyubiquitin-binding reporter was observed upon RNAi against *ima-3* **(II: Figure 7B and 7C)**. RNAi against importin β1, *imb-1*, also resembled *akir-1* RNAi-mediated phenotype in the intestine, but not as clearly and strongly as *ima-3* downregulation **(II: Supplementary Figure 7B).** In addition, *imb-1* downregulation caused a severely sick phenotype preventing further studies. As mentioned above, the *in vivo* interaction between AKIR-1 and IMA-3 has been shown previously (Polanowska et al. 2018). Here, we demonstrated a genetic interaction between AKIR-1 and IMA-3 by showing that *ima*-3 RNAi in *akir-1* mutants drastically delayed growth and reduced body size compared to the effect of *ima-3* RNAi in wild-type animals or *akir-1* deletion alone on animals' growth and body size **(II: Figure 7D)**. Thus, AKIR-1 and IMA-3 may function together and coordinate some cellular processes, such as nuclear protein import through the same pathway or parallel pathways. Next, we demonstrated that compared to IMA-3, AKIR-1 coordinates the nuclear import of specific cellular proteins. Downregulation of *akir-1* did not affect the nuclear import of NLS-tagged GFP protein expressed ubiquitously under *sur-5* promoter (sur-5p::NLS-GFP), whereas *ima-3* RNAi clearly reduced the GFP signal in the nuclei **(II: Figure 7E)**. There are no previous studies showing a role for IMA-3 or IMB-1 in regulating nuclear import of proteasomes, however, in *Drosophila* it has been shown that nuclear import of proteasome components during spermatogenesis is mediated by an Importinβ/karyopherin family member Importin-9 (Ipo9) (Palacios et al. 2021), which, of note, does not have an ortholog in *C. elegans* (Adam 2009). In addition, importin 5 (IPO5) has been shown to facilitate the nuclear import of 20S proteasomes in human melanoma cells (Spits et al. 2019). In yeast, the karyopherin-α protein Srp1, a homolog of the three α-importins in *C elegans*, regulates the nuclear import of fully assembled proteasomes (Adam 2009, Budenholzer et al. 2020). Taken together, our results suggest for the first time a

possible role for IMA-3 as well as IMB-1 in regulating proteasome nuclear localization in *C. elegans* together with AKIR-1.

Remarkably, while we were preparing the manuscript describing this new role for AKIR-1 in regulating proteasome nuclear translocation in *C. elegans*, a study in Nature journal by de Almeida and colleagues described a similar function for human AKIRIN2 in human cancer cells (de Almeida et al. 2021). They demonstrated that AKIRIN2 homodimers interact directly with fully assembled 20S and 26S proteasomes and mediate their nuclear re-importation after being excluded from the nucleus during mitosis (de Almeida et al. 2021). In addition, they suggested that AKIRIN2 could be a functional homolog of Sts1 in yeast and Cut8 in fission yeast. Sts1 and Cut8 have been shown to function as adaptor proteins mediating the nuclear import of proteasomes together with importins (Tatebe and Yanagida 2000, Takeda and Yanagida 2005, Chen et al. 2011, Takeda et al. 2011, Budenholzer et al. 2020, Breckel et al. 2024). While AKIRIN2 differs structurally from Sts1 and shares no sequence homology with it, both Sts1 and AKIRIN2 bind to 26S proteasomes, coordinate their nuclear import through the highly conserved N-terminal bipartite NLS, interact with karyopherins (importins), contain protein regions of predicted disorder, and are short-lived proteins degraded by the proteasomes (Budenholzer et al. 2020, de Almeida et al. 2021, Breckel et al. 2024).

Our study, demonstrating a similar role for *C. elegans* AKIR-1 as human AKIRIN2 and yeast Sts1, revealed that the role of Akirin proteins in regulating nuclear transport of proteasomes is evolutionarily conserved. However, there seems to be some differences between the human AKIRIN2 and *C. elegans* AKIR-1. First of all, in human cancer cells, the AKIRIN2 knockdown leads to apoptosis (de Almeida et al. 2021), whereas in *C. elegans* the complete loss of *akir-1* leads to developmental defects but a viable phenotype. Secondly, AKIR-1 regulates nuclear transport of proteasomes in non-dividing, post-mitotic intestinal cells compared to AKIRIN2, which was shown to participate in the nuclear re-importation of proteasomes upon the reformation of the nuclear envelop in the late mitosis (de Almeida et al. 2021). Lastly, in *C. elegans* AKIR-1 seems to control the nuclear import with different importins compared to human AKIRIN2, which interacts with an importin 5 (IPO5). Downregulation of the IPO5 homolog in *C. elegans*, *imb-3*, did not resemble the *akir-1* RNAi-induced nuclear accumulation of polyubiquitinated protein in the intestinal cells **(II: Supplementary Figure 7C)**. It is also worth noticing, that there is not a complete loss of nuclear proteasomes upon AKIR-1 depletion in the intestinal cells, suggesting that in *C. elegans* there are also some other factors coordinating the subcellular distribution of proteasomes.

A very recent study demonstrated in more detail the role of yeast Sts1 in regulating 26S proteaome nuclear import (Breckel et al. 2024). They revealed that Sts1 mediates the nuclear import of the 26S proteasome with a karyopherin α/β heterodimer (Srp1/Kap95). Furthermore, they showed that Sts1 binds to the 26S proteasome with its six-helix bundle region and that the proteasome-mediated degradation of Sts1 is blocked when Sts1 is bound to the karyopherin heterodimer. Sts1 degradation *in vitro* is initiated when nuclear RanGTP removes karyopherins from the Sts1 and the unstructured amino-terminal domain is accessible. In addition, the Sts1 degradation is dependent on successful completion of proteasome nuclear import (Breckel et al. 2024). In conclusion, our study demonstrated a new role for *C. elegans* AKIR-1 in regulating proteasome subcellular function. Together with the recent findings regarding human AKIRIN2 our study revealed a conserved Akirins-mediated mechanism regulating the nuclear localization of the proteasome. In addition, we revealed that importins, IMA-3 and IMB-1, potentially regulates proteasome import in *C. elegans*. Further, we demonstrated that different tissues responded differently to *akir-1* depletion and that Akirins seem to display a broader role in maintaining cellular protein homeostasis in a multicellular organism.

6 CONCLUSIONS AND FUTURE PROSPECTS

The ubiquitin-proteasome system is an essential cellular process coordinating intracellular protein degradation and protein homeostasis. We have shown in our previous studies how versatile the animal model roundworm *C. elegans* is in revealing mechanisms regulating proteasome function in a multicellular organism.

The first part of my thesis demonstrated tissue-specific responses in proteasome expression, which were revealed by developing the well-known immunohistochemical method on paraffin-embedded *C. elegans* sections. We showed that in wild-type animals the immunoreactivity of endogenous proteasomes is stronger in the nucleus compared to the cytoplasmic immunoreactivity in all examined cell types. We also demonstrated that systemic proteasome impairment resulted in tissue- and cell-type specific responses in endogenous proteasome expression, however, the mechanisms regulating these cell-type specific responses are only partially known, so further studies are required. In future, in addition to the endogenous expression of proteasomes, immunohistochemistry in *C. elegans* could be utilized to study the cell- and tissue-level expression of endogenous polyubiquitinated substrates by using the antibody against polyubiquitinated proteins. This would expand our ability to study proteasome function in more detail in cell types, which might be difficult to examine by whole worm immunostaining, such as body-wall muscle cells. Moreover, immunohistochemistry in *C. elegans* could be used to study the expression and localization of other proteins regulating proteasome function. Taken together, the development of immunohistochemistry for the multicellular *C. elegans* enables us to study the tissue-level expression of endogenous proteins in this genetically highly engineerable animal model. This promotes the possibility to uncover crucial factors and mechanisms regulating cellular proteostasis, mechanisms that likely will be relevant also in human cells.

The second part of my thesis characterized the role of newly identified AKIR-1 on regulating proteasome function in *C. elegans*. We revealed that loss of AKIR-1

causes a nuclear accumulation of endogenous polyubiquitinated proteins in the intestinal cells but not in oocytes, even though AKIR-1 regulates the nuclear localization of the proteasome in both cell types. The reason(s) causing this interesting phenotype is currently unknown. Further studies are required to dissect the mechanisms regulating the subcellular localization of proteasomal substrates or causing differences in the proteasome function/activity in these two cell types. Together with the recent study on human AKIRIN2, our findings with *C. elegans* AKIR-1 revealed that the role of Akirins in coordinating proteasome translocation into the nucleus is conserved between human cells and in *C. elegans*. Moreover, we demonstrated that AKIR-1 regulates proteasome nuclear translocation in postmitotic, non-dividing cells suggesting a broader role for Akirins in regulating nuclear transport of proteasomes. Future studies are required to uncover whether AKIR-1/AKIRIN2 has a role in regulating nuclear export of proteasomes or in retaining proteasomes in the nucleus. In conclusion, my thesis has broadened our understanding of the mechanisms regulating proteasome function and protein homeostasis in a multicellular organism.

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