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FROM QUALITY CONTROL TO NEURODEGENERATION: REGULATION OF AUTOPHAGY AND THE DNA DAMAGE RESPONSE BY UBIQUITIN-MODIFYING ENZYMES

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Stockholm 2020

Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7831-848-3

From Quality Control to Neurodegeneration: Regulation of Autophagy and the DNA Damage Response by Ubiquitin-Modifying Enzymes

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Being a scientist is like being an explorer.
You have this immense curiosity, this stubbornness,
this resolute will that you will go forward
no matter what other people say.

- Sara Seager
(astrophysicist and planetary scientist)

ABSTRACT

Protein homeostasis and genome integrity are safeguarded by a variety of cellular quality control pathways. While protein quality is controlled by a delicate balance between protein biosynthesis, folding and degradation, the DNA is subject to tightly regulated surveillance processes that sense, signal and repair DNA lesions. Many of these processes are regulated by the conjugation of the small modifier ubiquitin to substrates and effector proteins.

A polyglutamine-repeat expansion in the gene encoding the deubiquitylating enzyme ataxin-3 is the underlying cause of the neurodegenerative disorder Machado-Joseph disease. Similar to other polyglutamine disorders, the disease is characterized by intracellular inclusions of the mutant protein. Interestingly, wild-type ataxin-3, which disassembles both K63-linked and K48-linked ubiquitin chains, has been reported to be neuroprotective. By means of its catalytic activity, it suppresses toxic aggregation of polyglutamine proteins, including its own mutant counterpart. Ataxin-3 has further been implicated in cellular pathways that regulate protein quality control, transcription and DNA repair.

The work presented in this thesis identified additional functions of ataxin-3 in cellular quality control. In **paper I**, we identified ataxin-3 as a novel interactor of the ubiquitin-like autophagy proteins LC3C and GABARAP and demonstrated that ataxin-3 is required for efficient autophagic degradation in both the nematode *Caenorhabditis elegans* and mammalian cells. Loss of ataxin-3 did not only result in aberrant accumulation of autophagic vesicles in mammalian cells but also decreased survival of nematodes upon nutrient deprivation, a condition that relies on functional autophagy for survival, and aggravated the accumulation of protein aggregates and aggregate-related motility defects.

In **paper II**, we engineered an inducible, K63-specific ubiquitin ligase and an associated reference substrate, which allow studying the signaling capacity of K63-linked ubiquitin chains in different cellular contexts. This chain type has frequently been linked to inclusion bodies, autophagic degradation and neurodegeneration. Using a mitochondria-localized substrate, we demonstrate that K63-linked ubiquitylation is sufficient to induce perinuclear clustering of the mitochondria, even in the absence of mitochondrial damage, a phenotype that has previously only been described during clearance of damaged mitochondria by mitophagy.

In **paper III** we identified a novel role for ataxin-3 in the regulation of the cellular response to DNA double-strand breaks. We demonstrate that ataxin-3 is recruited to DNA lesions and consolidates the DNA damage response by preventing premature chromatin-extraction of DNA repair proteins by the SUMO-targeted E3 ubiquitin ligase RNF4. We show that ataxin-3 counteracts RNF4-mediated MDC1-ubiquitylation and chromatin-extraction, promoting recruitment of RNF8 and RNF168 and subsequent damage-induced ubiquitin signaling. Consequently, loss of ataxin-3 impaired recruitment of the repair factors 53BP1 and BRCA1 and therefore both non-homologous end-joining and homologous recombination. Similar to

the recruitment of RNF4, recruitment of ataxin-3 to DNA lesions was dependent on damage-induced SUMOylation.

In **paper IV** we show that ataxin-3 recruitment, in contrast to RNF4, additionally depends on DNA damage-induced poly(ADP-ribos)ylation, thereby restricting the actions of ataxin-3 to the early phase of the DNA damage response. Differential recruitment of ataxin-3 and RNF4 to DNA double-strand breaks thereby promotes the sequential actions of these enzymes on shared substrates and explains how both proteins promote efficient DNA repair despite their opposing activities.

Collectively, the studies presented in this thesis corroborate the importance of ataxin-3 for maintaining genome and protein quality control and furthermore underline the importance of considering the homeostatic functions of wild-type ataxin-3 in the design of therapeutic strategies for Machado-Joseph disease. Additionally, the data presented in this thesis demonstrate the significance of K63-linked ubiquitylation in mediating mitochondrial clustering, not unlike its function in inclusion body formation, and provide a tool to further study the cellular function of these ubiquitin chains.

LIST OF SCIENTIFIC PAPERS

I. **Herzog LK**, Kevei É, Marchante R, Böttcher C, Bindesbøll C, Lystad AH, Pfeiffer A, Gierisch ME, Salomons FA, Simonsen A, Hoppe T and Dantuma NP. (2020). The Machado-Joseph disease deubiquitylase ataxin-3 interacts with LC3C/GABARAP and promotes autophagy. *Aging Cell*, 19:e13051

II. Richard TJC, **Herzog LK**, Suryo Rahmanto A, Sangfelt O, Salomons FA and Dantuma NP. An inducible, engineered K63-ubiquitin ligase mimics Parkin-mediated sequestration of mitochondria in the absence of mitochondrial damage. *Manuscript (submitted)*

III. Pfeiffer A*, Luijsterburg MS*, Acs K, Wiegant WW, Helfricht A, **Herzog LK**, Minoia M, Böttcher C, Salomons FA, van Attikum H and Dantuma NP. (2017). Ataxin-3 consolidates the MDC1-dependent DNA double-strand break response by counteracting the SUMO-targeted ubiquitin ligase RNF4. *The EMBO Journal*, 36, 1066-1083

IV. Pfeiffer A, **Herzog LK**, Luijsterburg MS, Shah RG, Stoy H, Kühbacher U, van Attikum H, Shah GM and Dantuma NP. Poly(ADP-ribos)ylation limits SUMO-dependent ataxin-3 recruitment to DNA double-strand breaks to the early phase of the DNA damage response. *Manuscript (submitted)*

* these authors contributed equally

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Dantuma NP and **Herzog LK**. (2020). Machado-Joseph Disease: A Stress-Combating Deubiquitylating Enzyme Changing Sides. In: Barrio R., Sutherland J., Rodriguez M. (eds) Proteostasis and Disease. Advances in Experimental Medicine and Biology, vol 1233: 237–260. Springer, Cham

CONTENTS

LIST OF ABBREVIATIONS

53BP1	p53-binding protein 1
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinas
Atg	Autophagy-related
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
AUTACs	Autophagy-targeting chimera
BRCA1	Breast cancer type 1 susceptibility protein
BRCT	BRCA1 C-terminal
CBP	CREB-binding protein
CHFR	Checkpoint with forkhead and RING finger domains
CMA	Chaperone-mediated autophagy
CREB	cAMP-responsive element binding
DDR	DNA damage response
DFCP1	Double-FYVE-containing protein 1
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DRPLA	Dentatorubral-pallidoluysian atrophy
DSB	Double-strand break
DUB	Deubiquitylating enzyme
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FHA	Forkhead-associated
GABARAP	γ-aminobutyric acid receptor-associated protein
GABARAPL	γ-aminobutyric acid receptor-associated protein-like
HAT	Histone acetyltransferase
HD	Huntington's disease
HDAC	Histone deacetylase
HECT	Homologous to E6AP C-terminus
HR	Homologous recombination
Hsp	Heat shock protein

JAMM	Jab1/Mpr1/Mov34 proteases
JMJD2	Jumonji domain-containing protein 2
L3MBTL1	Lethal(3)malignant brain tumor-like protein 1
L3MBTL2	Lethal(3)malignant brain tumor-like protein 2
LC3	Microtubule-associated protein 1A/B light chain 3
LIR	LC3-interacting region
MAP1LC3	Microtubule-associated protein 1A/B light chain 3
MDC1	Mediator of DNA damage checkpoint 1
Mfn	Mitofusin
MINDY	Motif interacting with ubiquitin-containing novel DUB family
MIU	Motif interacting with ubiquitin
MJD	Machado-Joseph disease
MMP-2	Matrix-metalloprotease-2
MOM	Mitochondrial outer membrane
MPP	Mitochondrial processing peptidase
mTOR	Mammalian target of rapamycin
NCoR	Nuclear receptor co-repressor 1
NHEJ	Non-homologous end joining
OPTN	Optineurin
OTU	Ovarian tumor protease
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) glycohydrolase
PARL	Presenilins-associated rhomboid-like protein
PARP	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PINK1	PTEN-induced kinase 1
PI3K	Phosphoinositide-3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PLEKHM1	Pleckstrin homology domain-containing family M member 1
PNKP	Polynucleotide kinase 3′-phosphatase
ProxE3	Proximity-induced E3 ubiquitin ligase
PTM	Post-translational modification
RBR	RING-between-RING

RING	Really interesting new gene
ROS	Reactive oxygen species
RPA	Replication-associated protein A
SBMA	Spinal and bulbar muscular atrophy
SCA3	Spinocerebellar ataxia type 3
SIM	SUMO-interacting motif
SNP	Single nucleotide polymorphism
SNX17	Syntaxin-17
SQSTM1	Sequestosome 1
SSB	Single-strand break
ssDNA	Single-stranded DNA
STUbL	SUMO-targeted ubiquitin ligase
SUMO	Small ubiquitin-like modifier
TDP-43	TAR DNA-binding protein 43
UBA	Ubiquitin-associated (domain)
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like
UBXD1	UBX-domain containing protein
UBZ	Ubiquitin-binding zinc finger
UCH	Ubiquitin C-terminal hydrolase
UDR	Ubiquitin-dependent recruitment
UIM	Ubiquitin-interacting motif
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific protease
UV	Ultraviolet
VAMP8	Vesicle-associated membrane protein 8
VBM	VCP-binding motif
VDAC1	Voltage-dependent anion channel 1
V-ATPase	Vacuolar H^+-ATPase
WIPI2	WD repeat domain phosphoinositide-interacting protein 2
XPC	Xeroderma pigmentosum group C-complementing protein
ZUFSP	Zinc finger and UFM1-specific peptidase domain protein

1 INTRODUCTION

Cellular pathways safeguarding protein homeostasis (proteostasis) and genomic stability are of paramount importance for cellular fitness, integrity and survival. In order to maintain these vital functions, cells rely on a series of quality control pathways. Proteostasis is maintained by a tightly regulated network of pathways governing protein synthesis, protein folding and protein degradation while genomic integrity is maintained by spatial and temporal regulation of DNA damage sensing and repair pathways. All of these pathways are subject to age-related decline in their efficiency and quality (Hipp et al., 2019; Wolters and Schumacher, 2013). Dysregulation of these pathways has furthermore been linked to a variety of human pathologies including cancer and neurodegeneration.

Neurodegenerative disorders affect the function and viability of neurons in the human central nervous system, primarily the brain, and are characterized by the progressive loss of neuronal function in the affected regions. Given the age-related gradual decline in the function of cellular quality control mechanisms, increasing age is the single largest risk factor for neurodegeneration (Hindle, 2010; Niccoli and Partridge, 2012). However, many of the diseases have underlying genetic causes, and while many of these are sporadic in nature, several have been shown to be hereditary (Price et al., 1998). While the cause as well as the affected parts of the central nervous system differ largely between neurodegenerative diseases, several of these disorders nonetheless display unifying characteristics. The accumulation of disease proteins with altered conformation, resulting in a protein with increased propensity to aggregate, is one such feature. These diseases are commonly referred to as conformational diseases or proteinopathies (Carrell and Lomas, 1997). A subgroup of proteinopathies are polyglutamine diseases, whose underlying cause is the expansion of a CAG repeat in the affected gene, giving rise to proteins with expanded polyglutamine stretches that render these proteins particularly aggregation prone (Orr and Zoghbi, 2007).

Examples of proteinopathies include amyotrophic lateral sclerosis (ALS) (Scotter et al., 2015), Parkinson's disease (PD), Alzheimer's disease (AD) (Irvine et al., 2008) and Huntington's disease (HD) (Davies et al., 1997). In addition to protein aggregation, deregulation of proteome and genome quality control pathways, evidenced by disturbance of the cellular protein degradation machineries (Dantuma and Bott, 2014; Wong and Cuervo, 2010) as well as altered transcription (Mohan et al., 2014) and accumulation of persistent DNA damage (Abugable et al., 2019), has been linked to proteinopathies and may therefore present a common feature in their pathogenesis.

A polyglutamine repeat expansion in the *ATXN3* gene, encoding the deubiquitylating enzyme (DUB) ataxin-3, is causative for Machado-Joseph diseases (MJD) (Kawaguchi et al., 1994). Similar to other polyglutamine diseases, MJD pathology is complex and the etiology largely unclear, despite the monogenetic cause of the disease. To date, there are no disease-modifying interventions that slow or halt disease progression, but several potential therapeutic strategies are being explored in pre-clinical settings, including modulating ataxin-3 expression (Dantuma and Herzog, 2020).

While the majority of studies in the past have focused on characterizing the cellular phenotypes evoked by expression of polyglutamine-expanded ataxin-3 in patient samples, animal models and cell lines, the functions of the wild-type protein have gained increasing interest in recent years. Interestingly, wild-type ataxin-3 has been documented to exert a neuroprotective function against polyglutamine-expanded disease proteins, including its own mutant counterpart, in *Drosophila melanogaster* (Warrick et al., 2005), and has furthermore been shown to be involved in the regulation of a variety of pathways whose deregulation has been implicated in MJD pathogenesis (Dantuma and Herzog, 2020; do Carmo Costa and Paulson, 2012). Increased knowledge of the native functions of ataxin-3 may thus not only contribute to a better understanding of the molecular mechanisms underlying MJD pathology and uncover additional cellular pathways that may be targeted for disease modification, but it may also be an important aspect to consider when designing therapeutic interventions for MJD targeting ataxin-3 protein levels, to prevent potentially detrimental consequences of tempering with the steady-state levels of this enzyme.

1.1 PROTEIN QUALITY CONTROL

A condition that poses a particular threat to cellular proteostasis is the misfolding of proteins as a result of different stress conditions, stoichiometric imbalance of protein complex subunits or mutations that render the affected protein instable. As misfolding may result in the exposure of regions that would otherwise be shielded in the folded protein, it increases the risk for inappropriate protein-protein interactions, including protein aggregation. The complex protein quality control network is therefore a central component of the cellular pathways safeguarding proteostasis (Morimoto, 2008). A first-in-line cellular defense mechanism against misfolded proteins is the cellular protein folding machinery, which is comprised of both constitutively expressed and stress-induced molecular chaperones that assist in folding, re-folding and unfolding of proteins to maintain cellular proteostasis (Hartl et al., 2011). If the molecular chaperone network is unable to eliminate the misfolded protein,

this machinery can additionally assist in transferring the protein to the second cellular resource in the handling and suppression of misfolded and aggregation-prone proteins: protein degradation (Kriegenburg et al., 2012). In mammalian cells, this is executed by two major proteolytic machineries, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (Rubinsztein, 2006). As central elements of the work presented in this thesis, cellular protein quality control, in particular protein degradation, and its link to neurodegeneration will be explained in detail in the following chapters.

1.1.1 Protein quality control and neurodegeneration

A unifying characteristic of proteinopathies is the generation of proteins with altered chemical and physical properties, and, as a result, an increased propensity to misfold and aggregate. Accordingly, the cellular protein folding machinery has been intimately linked to the etiology of these diseases (Gestwicki and Garza, 2012). Alterations in the expression of molecular chaperones, aberrant interaction of disease proteins with chaperones as well as their sequestration in disease-typical protein inclusions has been reported (Chai et al., 1999a; Huen and Chan, 2005; Yu et al., 2014). Underlining a role for altered protein folding, upregulation of quality control capacity, e.g. by increasing the levels of the molecular chaperones heat shock protein (Hsp) HspA1 (previously referred to as Hsp70) and DnaJB1 (formerly Hsp40) has been reported to suppress the formation of insoluble protein fibrils of huntingtin, the protein underlying HD (Lotz et al., 2010; Muchowski et al., 2000). When chaperones fail at eliminating proteins that pose a threat to the intracellular environment, these proteins can be directed to the cellular protein degradation machineries (Kriegenburg et al., 2012). Many of the neurodegeneration-relevant mutated proteins, however, are themselves poor substrates for the UPS and have instead been reported to interfere with the function of this proteolytic pathway (Bennett et al., 2007; Thibaudeau et al., 2018). Additionally, several proteins that are causative for neurodegeneration have been implicated, in their native conformation, in the regulation of this degradative pathway (Doss-Pepe et al., 2003; Wójcik et al., 2006). Alterations in the second degradative pathway, autophagy, evidenced by the aberrant accumulation of autophagic structures and dysregulation of key proteins regulating this pathway, have also been described as a common feature of proteinopathies, including HD and AD (Martinez-Vicente et al., 2010; Nixon et al., 2005; Onofre et al., 2016). Furthermore, several aggregation-prone proteins associated with neurodegenerative diseases are themselves substrates for autophagy, a pathway that has

therefore also become a prominent target in the exploration of potential therapeutic intervention strategies (Krainc, 2010; Menzies et al., 2010; Ou et al., 2016).

1.2 UBIQUITIN

Despite the complexity and diversity of proteinopathies, a common cellular hallmark observed in these diseases is the accumulation of ubiquitin in the disease-typical inclusions in affected neurons (Alves-Rodrigues et al., 1998). Ubiquitin is a small, 76-amino acid protein that is ubiquitously expressed and serves as a post-translational modification (PTM). As such ubiquitin is a central element in the cellular quality control pathways, despite their diverse functions, and modifies a myriad of proteins involved in protein and genome quality control (Hershko, 1983). Consequently, its role in the induction and coordination of cellular pathways for protein degradation and DNA damage sensing and repair has been studied extensively (Komander and Rape, 2012). Its cellular importance is further underlined by the fact that ubiquitin is highly conserved even among distant eukaryotic species and a ubiquitin-like protein has also been discovered in prokaryotes (Pearce et al., 2008; Zuin et al., 2014).

1.2.1 Ubiquitylation

Ubiquitin can be attached to substrate proteins either as a single moiety, multiple single moieties, or as polymeric ubiquitin chains, resulting in mono-, multiple mono- and polyubiquitylation, respectively (Hershko and Ciechanover, 1998). Conjugation to a substrate protein or another ubiquitin moiety is achieved through the formation of an isopeptide bond between the carboxy-terminus (C-terminus) of ubiquitin and the ε-amino group of a lysine residue in the substrate protein or ubiquitin itself (Pickart, 2000), or, less commonly, the amino-terminal (N-terminal) methionine in substrate proteins (Ciechanover and Ben-Saadon, 2004). Utilization of the N-terminal methionine in ubiquitin has also been described (Kirisako et al., 2006; Rittinger and Ikeda, 2017).

This reaction is catalyzed by an enzymatic cascade of the successive actions of three enzyme classes (**Figure 1**) (Pickart, 2001). In a first step, ubiquitin is activated through C-terminal adenylation by the ubiquitin-activating E1 enzyme. The human genome encodes two E1 enzymes, while most other species express only a single E1 enzyme. Activated ubiquitin is subsequently transferred to an E2 enzyme, also referred to as ubiquitin-conjugating enzyme, giving rise to an E2-ubiquitin thioester intermediate. This class of enzymes, that is comprised

of about 40 different enzymes in humans, subsequently forms a complex with ubiquitin ligases, E3 enzymes. This third enzymatic class primarily increases the rate of ubiquitin-transfer onto the substrate protein (Pickart, 2001; Scheffner et al., 1995). In some cases, extension of existing ubiquitin chains has been shown to additionally depend on the action of chain elongating factors, which have been termed E4 enzymes (Hoppe, 2005; Koegl et al., 1999).

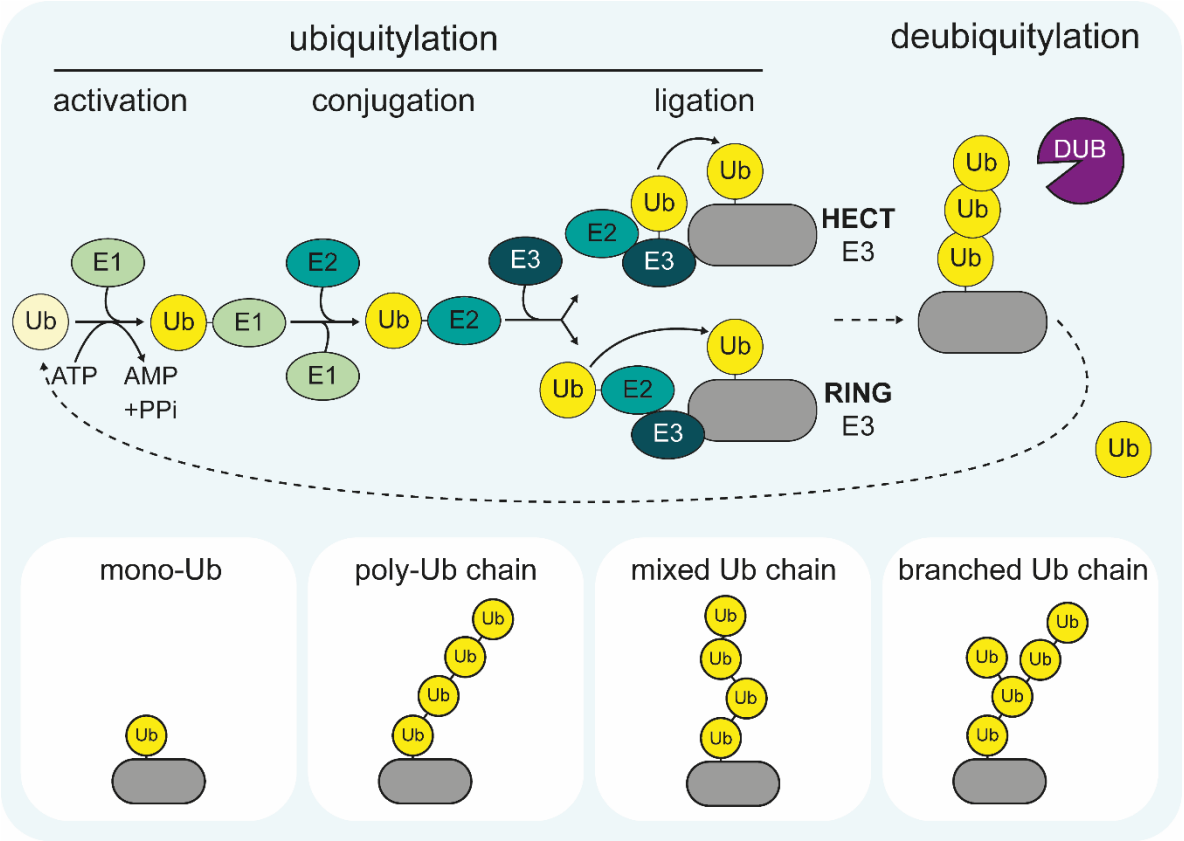

Figure 1. Ubiquitylation. The conjugation of ubiquitin (Ub) is executed by an enzymatic cascade. The E1 activating enzyme activates ubiquitin, in an ATP-dependent manner, and subsequently transfers ubiquitin to the E2 conjugating enzyme. The E2 associates with the E3 ubiquitin ligase for the final transfer of ubiquitin onto the substrate protein. In the case of HECT ligases, ubiquitin is transferred in a two-step mechanism, first to the active site of the HECT ligase and then onto the substrate protein, catalyzed by the ligase itself. RING ligases lack enzymatic activity and associate with the E2, thereby also conferring substrate specificity, while ubiquitin is transferred directly from the E2 onto the substrate. Substrates can be monoubiquitylated or polyubiquitylated, as a result of successive rounds of ubiquitylation. Chains can consist of similar or mixed linkages and branched ubiquitin chains can be generated by the ubiquitylation of a ubiquitin moiety on two sites. Ubiquitin modifications can be cleaved by deubiquitylating enzymes (DUBs).

Substrate specificity of the ubiquitylation reaction is determined by the E3 enzymes. It is therefore not surprising that the mammalian genome encodes more than 600 putative E3 enzymes to allow for the specific ubiquitylation of a broad range of substrates in the myriad of pathways that are regulated by ubiquitin (Zheng and Shabek, 2017). Based on their conserved domain structure as well as the mechanism by which ubiquitin is transferred from

the E2 enzyme to the substrate, E3 enzymes can be grouped into three distinct families (Metzger et al., 2012).

Enzymes containing a Homologous to E6AP C-terminus (HECT)-domain belong to the HECT-family of E3 ligases and are characterized by intrinsic activity, thus transferring ubiquitin in a two-step mechanism from the E2 to the active site of the HECT E3 ligase, harboring a catalytic cysteine residue, and subsequently onto the substrate (Rotin and Kumar, 2009). The presence of a Really interesting new gene (RING)-domain characterizes the family of RING-type E3 ligases. Rather than having intrinsic enzymatic activity, these ligases act as substrate-specific scaffold proteins that allow direct transfer of ubiquitin from the E2 onto their substrate. In the case of RING ligases, it is therefore the pairing between specific E2 and E3 enzymes that determines and governs the linkage of the generated ubiquitin chain. In contrast, HECT ligases dictate the specificity of the linkage themselves (Deshaies and Joazeiro, 2009). The third family is made up of RING-between-RING (RBR) ligases that form a hybrid class of enzymes and combine HECT and RING ligase characteristics. Similar to HECT-ligases, ubiquitin-transfer onto the substrate is preceded by thioester-formation with the ligase (Spratt et al., 2014).

Ubiquitin itself contains seven lysine (Lys; K) residues (K6, K11, K27, K29, K33, K48 and K63), all of which can be used for the formation of polyubiquitin chains, and all of which result in chains with distinct characteristics, thereby creating a complex ubiquitin code (Akutsu et al., 2016; Peng et al., 2003). Additionally, linear ubiquitylation that employs the ε-amino group of the amino-terminal (N-terminal) methionine in ubiquitin has been described (Kirisako et al., 2006; Rittinger and Ikeda, 2017). Adding to the complexity of the ubiquitin code, both mixed chains, containing different linkages within one chain (Nakasone et al., 2013), and branched ubiquitin chains, in which several ubiquitin moieties are conjugated to another ubiquitin, can be generated (Meyer and Rape, 2014).

The complex code generated by the different types of ubiquitin-modifications as well as substrates modified by ubiquitin, allows for spatial and temporal regulation of a variety of cellular pathways. The most studied types of ubiquitin chains are K48- and K63-linked ubiquitin chains. While the former are the primary chain type targeting substrate proteins for degradation by the proteasome (Chau et al., 1989), all chain linkages have been reported to be able to target proteins for proteasomal destruction (Xu et al., 2009). K11-linked ubiquitin chains have primarily been implicated in cell cycle regulation (Matsumoto et al., 2010). Additionally, these chains are involved in endoplasmic reticulum (ER)-associated degradation (ERAD) (Locke et al., 2014). K27- and K29-linked ubiquitin chains have

frequently been associated with lysosomal targeting (Chastagner et al., 2006; Ikeda and Kerppola, 2008; Zotti et al., 2011). However, with their cellular abundance being limited compared to the two major chain types, a lot remains to be elucidated concerning the cellular signaling functions of these remaining linkages.

K63-linked ubiquitin chains do not seem to target proteins for proteasomal degradation in the intracellular environment (Nathan et al., 2013). Instead, these chains are primarily thought to function in signaling cascades, thus affecting protein localization and interaction, a function that is well-documented and characterized in DNA repair (Chen and Sun, 2009; Hofmann and Pickart, 1999; Spence et al., 1995). In the context of DNA damage signaling, K63-linked ubiquitin chains, catalyzed by the E3 ligase RNF168, have been shown to be attached to histones and serve as important recruitment signals for repair proteins that recognize this chains type through specific ubiquitin-binding domains (UBDs). Impairment of the damage-induced K63-linked ubiquitin signal impedes DNA repair, supporting the important signaling function of these chains (Stewart et al., 2009).

In recent years, K63-linked ubiquitin chains have additionally been linked to inclusion body formation and selective degradation of protein aggregates and organelles by autophagy (Lim et al., 2005; Tan et al., 2008). In line with this, and suggesting a particular significance of K63-linked ubiquitin in neurodegeneration, K63-chains were detected in the characteristic lesions in postmortem brain samples of, among others, HD and PD patients (Paine et al., 2009) and accumulation of both K48- and K63-linked ubiquitylation detected in a mouse model of HD using a mass spectrometry approach to quantify the abundance of these chains (Bennett et al., 2007). Similarly, K63-linked ubiquitin chains were found to be upregulated in the insoluble proteome of cells expressing the ALS-associated TAR DNA-binding protein 43 (TDP-43) (Seyfried et al., 2010).

1.2.2 Deubiquitylating enzymes

Reversibility of the modification of proteins with ubiquitin is achieved by the actions of DUBs, ubiquitin-specific proteases, which hydrolyze the isopeptide-bond between ubiquitin and the substrate protein or between two ubiquitin moieties (**Figure 1**) (Amerik and Hochstrasser, 2004; Wilkinson, 1997). By removing ubiquitin modifications from substrate proteins, DUBs can therefore change the fate of substrates, alter their activity and modify protein-protein interactions. As the cellular ubiquitin homeostasis is maintained by an equilibrium of free and conjugated ubiquitin (Dantuma et al., 2006; Kimura and Tanaka, 2010), DUBs furthermore contribute to the recycling of ubiquitin to replenish the free pool of

this important signaling molecule. Additionally, trimming of ubiquitin chain length and editing of chain composition have been described in some contexts (Lee et al., 2011; Winborn et al., 2008), further underlining the complexity of ubiquitin modifications and their effect on substrate fate. DUBs exhibit specificity towards both the substrate and ubiquitin chain linkage and have also been implicated in processing of the ubiquitin precursor (Amerik and Hochstrasser, 2004). In mammals, ubiquitin is encoded by four genes, all of which result in the synthesis of polyubiquitin precursor proteins as well as a ubiquitin-fusion with the 40S ribosomal protein S27a and are subsequently cleaved by DUBs to give rise to mature ubiquitin moieties (Bianchi et al., 2015; Grou et al., 2015).

Approximately 100 DUBs are encoded by the mammalian genome. Based on their catalytic activity, DUBs can be divided into cysteine proteases and metalloproteases (Nijman et al., 2005). While the ubiquitin-specific metalloproteases are comprised by a single family, the Jab1/Mpr1/Mov34 (JAMM) proteases, ubiquitin-specific cysteine proteases can be subdivided into six families: Ubiquitin C-terminal hydrolases (UCH), Ubiquitin-specific proteases (USP), Ovarian tumor proteases (OTU) and Josephin-domain proteases (also referred to as Machado-Josephin domain proteases, MJD) (Nijman et al., 2005) as well as the motif interacting with Ub-containing novel DUB family (MINDY) (Abdul Rehman et al., 2016) and the Zn-finger and UFSP domain protein proteases (ZUFSP), that have been identified more recently (Hermanns et al., 2018; Kwasna et al., 2018).

1.2.3 The proteasome

The most well-known and -documented function of ubiquitin is the targeted destruction of modified substrates by the proteasome, the central component of UPS. The proteasome is a large proteolytic complex consisting of the 20S catalytic core and regulatory 19S particles (Bhattacharyya et al., 2014). The 20S core is built up of four heptameric, stacked rings, with the two inner rings composed of β-subunits and the outer rings formed by α-subunits. The channel formed by these rings harbors the catalytic activity of this multi-subunit proteolytic enzyme. The proteolytic chamber is indirectly controlled by channel accessibility, restricted by the narrow pore on each end of the core (Coux et al., 1996; Groll et al., 1997). The active sites of six protein hydrolases with three distinct protease activities are exposed inside the 20S core, cleaving proteins translocated through the channel into small peptides to recycle short peptides and amino acids for further cellular use. The chymotrypsin-like, trypsin-like and caspase-like activities of these proteases enable proteolysis of a broad range of substrates (Heinemeyer et al., 1997; Kisselev et al., 2006; Rivett, 1989). Proteasomes can exist in their

uncapped state, consisting only of the 20S core particle as well as single and double capped states with 19S regulatory particles (da Fonseca and Morris, 2008). The 19S regulatory particles are made up of the base consisting of six ATPases, two ubiquitin receptors and two organizing subunits. The ATPase units regulate channel gating, substrate unfolding and translocation of the substrate into the catalytic core (Bhattacharyya et al., 2014). The ubiquitin receptors are required for recognition of ubiquitylated cargo that is delivered to the proteasome by ubiquitin shuttling factors. Shuttling factors are themselves not integral parts of the proteasome but instead shuttle ubiquitylated cargo to the proteasome and, thus, transiently associate with this complex. This shuttling function is enabled by a ubiquitin-associated (UBA) domain, localized in the C-terminal or central region of the protein, which interacts with ubiquitylated cargo and an N-terminal ubiquitin-like (UBL) domain that, by means of its ubiquitin-like fold, allows interaction with the proteasome (Ciechanover and Stanhill, 2014). The lid of the 19S particle consists of nine subunits, including the DUB POH1 (also referred to as PSMD14 or Rpn11). POH1-mediated deubiquitylation of substrates is required for efficient degradation as well as for maintaining the dynamic ubiquitin equilibrium (Bhattacharyya et al., 2014).

1.3 AUTOPHAGY

In addition to the UPS, cells possess a second major route for protein degradation, termed autophagy, that has also frequently been linked to ubiquitin. Autophagy is characterized by the lysosomal degradation of cellular components and can be divided into three subtypes with distinct modes of delivery to the lysosomes (Ravikumar et al., 2009). While substrates of microautophagy are delivered into lysosomes by invagination of the lysosomal membrane to engulf the substrate (Li et al., 2012), chaperone-mediated autophagy (CMA) relies on the concerted action of the cytosolic chaperone Hsc70 and subsequent transport of the substrate through the lysosome-localized receptor LAMP2A. Specificity for degradation by CMA is conferred by a canonical targeting motif (KFERQ) in substrate proteins (Kon and Cuervo, 2010). Macroautophagy constitutes the third subtype of autophagy. This pathway is characterized by the formation of double-membrane vesicles, termed autophagosomes, that engulf cellular constituents and subsequently fuse with lysosomes to deliver the cargo (Cuervo, 2004; Ravikumar et al., 2009).

1.3.1 Macroautophagy

Macroautophagy (hereafter referred to as autophagy), in contrast to other cellular degradation pathways, is not limited to the degradation of single protein entities. Instead, its degradative capacity extends to multimeric protein complexes, protein aggregates as well as damaged organelles and invading microorganisms (Bernales et al., 2007; Kim et al., 2007; Kraft et al., 2008; Sakai et al., 2006). While this pathway is constitutively active at basal levels, it can additionally be triggered in response to a variety of stimuli: Autophagy is activated in response to nutrient deprivation (He and Klionsky, 2009), organelle damage (Jin and Youle, 2012) and accumulation of protein aggregates (Lamark and Johansen, 2012) to maintain cellular proteostasis and ensure survival. Similarly, invading pathogens are targeted by autophagy to eliminate the threat they pose to the cell (Knodler and Celli, 2011). Interestingly, albeit being a cytoplasmic pathway, autophagy has also been shown to be activated in response to DNA damage to promote efficient repair (Eliopoulos et al., 2016). Although autophagy is largely viewed as a pro-survival mechanism it has also frequently been linked to a distinct type of cell death, often referred to as autophagic cell death (Kroemer and Levine, 2008).

Autophagy can further be divided into bulk autophagy and selective autophagy. As the names of these mechanisms imply, bulk autophagy degrades cytosolic components in a non-selective manner. Selective autophagy on the other hand, describes the selective degradation of specific cargo, such as damaged organelles, and requires, in addition to the canonical autophagy machinery, both, specific signals on the substrate as well as a class of proteins referred to as autophagy receptors (Khaminets et al., 2016; Kirkin and Rogov, 2019).

Three distinct signaling complexes are essential for the initiation of autophagy. The first major signaling node is the mammalian target of rapamycin (mTOR), a serine-threonine kinase that senses and integrates, among other signals, cellular nutrient levels (Jung et al., 2010; Raught et al., 2001). In nutrient rich conditions, mTOR has an inhibitory effect on autophagy and prevents activation of the pathway by inhibitory phosphorylation of the autophagy-related (Atg) protein Atg13 and its interaction partner, the serine/threonine kinase ULK1 (Chan et al., 2009; Chang and Neufeld, 2009; Kim et al., 2011). Nutrient deprivation results in the deactivation of mTOR through phosphorylation by the AMP-activated protein kinase (AMPK). AMPK also phosphorylates ULK1 at activating sites (Kim et al., 2011), resulting in activation of the ULK1 complex, which additionally consists of Atg13, the scaffold protein FIP200 and the Atg13-interacting protein Atg101, all of which are substrate to ULK1-mediated phosphorylation (**Figure 2**) (Zachari and Ganley, 2017).

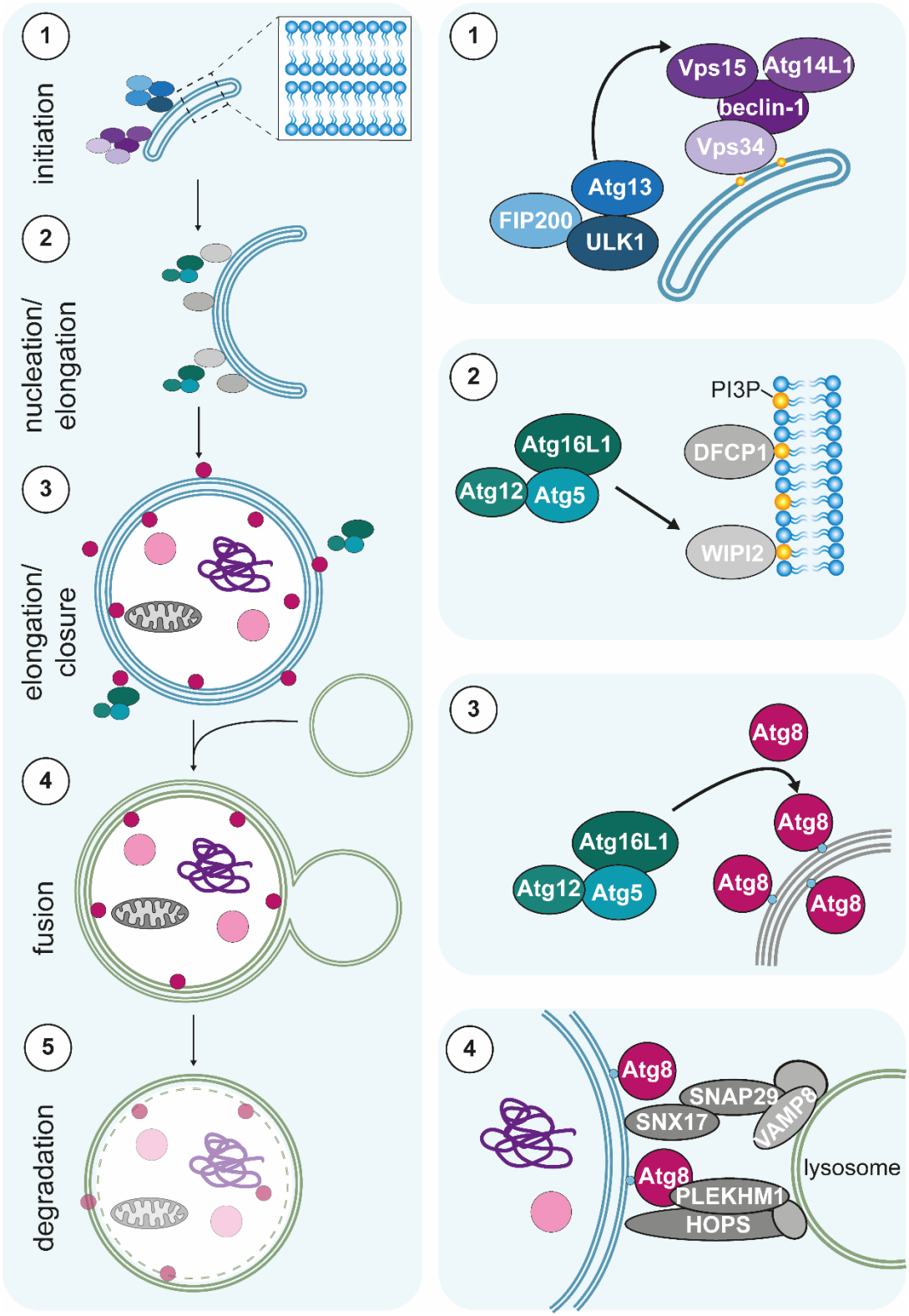

Figure 2. Macroautophagy. (1) Initiation of macroautophagy is mediated by the ULK1 complex, consisting of ULK1, FIP200, Atg13 and its binding partner Atg101, which activates the class III Phosphoinositide-3-kinase (PI3K) complex composed of Vps34, Vps15, beclin-1 and Atg14L1. The PI3K complex is tethered to the site of autophagosome formation, where it phosphorylates the lipid phosphatidylinositol, giving rise to phosphatidylinositol-3-phosphate (PI3P), on the isolation membrane. (2) PI3P is subsequently recognized by DFCP1 and WIPI2. WIPI2 is required for recruitment of the Atg16L1 complex that promotes elongation of the phagophore. (3) The Atg16L1 complex acts as the E3 enzyme for the conjugation of the ubiquitin-like protein Atg8 to phosphatidylethanolamine (PE), further promoting elongation and maturation of the autophagosome. (4) Atg8-proteins are also involved in mediating the fusion of autophagosomes with lysosomes by interaction with PLEKHM1, resulting in the recruitment of the HOPS complex. Additionally, SNX17, SNAP29 and VAMP8 are required for this step. (5) In the lysosome, autophagic cargo is degraded by acidic hydrolases.

The ULK1 complex subsequently activates the class III Phosphoinositide-3-kinase (PI3K) complex, composed of Vps34, Vps15, beclin-1 and Atg14L1 by phosphorylation of beclin-1 (Russell et al., 2013). Together, these two complexes promote nucleation of the isolation membrane, a cup-shaped, double-membrane structure that is also referred to as the phagophore, which gradually expands and eventually matures into an autophagosome (Lamb et al., 2013).

Interaction of beclin-1 with the ER-resident protein VMP-1 tethers the PI3K complex to the ER membrane, which has been suggested to mark the primary site of phagophore formation (Molejon et al., 2013). The membrane-tethered PI3K complex subsequently phosphorylates the lipid phosphatidylinositol, that primarily resides on the cytosolic side of cellular membranes, giving rise to phosphatidylinositol-3-phosphate (PI3P). This modification serves as a recruitment signal for double-FYVE-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) (Axe et al., 2008; Polson et al., 2010), both of which contain a FYVE-domain that enables binding to PI3P (Stenmark et al., 2002). WIPI2 is required for recruitment of the Atg16L1 complex to the forming autophagosome (Dooley et al., 2014; Polson et al., 2010). This complex is essential for elongation of the autophagosome membrane but detaches prior to autophagosome closure and is not detected on mature autophagosomes (Mizushima et al., 2003; Mizushima et al., 2001).

Autophagosome formation is further dependent on Atg8 proteins, that are recruited to the growing membrane by the Atg16L1 complex (Kirisako et al., 1999). Both the Atg16L1 complex and Atg8 proteins will be discussed in the next chapter in more detail. In addition to the maturation of autophagosomes, Atg8-proteins have also been shown to play a crucial role in the fusion of autophagosomes with endolysosomes and lysosomes. Atg8-dependent recruitment of Pleckstrin homology domain-containing family M member 1 (PLEKHM1) has been suggested to recruit the HOPS complex consisting of Vps33A, Vps16, Vps11, Vps18, Vps39 and Vps41 to mediate tethering of the autophagosome to lysosomes (Jiang et al., 2014; McEwan et al., 2015). Fusion of the vesicles additionally requires the actions of the SNARE proteins syntaxin-17 (SNX17), Synaptosomal-associated protein 29 (SNAP29) and Vesicle-associated membrane protein 8 (VAMP8) and assembly of these factors has also been shown to be facilitated by Atg8 (Hegedűs et al., 2013; Itakura et al., 2012; Jiang et al., 2014).

Ubiquitin-like proteins in autophagy

There are two ubiquitin-like systems involved in the autophagy pathway (**Figure 3**), with the Atg16L1 complex being the first to be recruited to the forming autophagosome. Similar to the ubiquitylation cascade, the ubiquitin-like protein Atg12 is conjugated to Atg5, catalyzed by the concerted actions of an E1 and E2 enzyme, while the involvement of a typical E3 enzyme has not been described (Mizushima et al., 1998; Shintani et al., 1999; Tanida et al., 1999). Complex formation of Atg12-5 with Atg16L1 triggers oligomerization of the complex, giving rise to an 800 kDa protein complex, that is essential for autophagosome formation (Mizushima et al., 2003). Atg16L1 furthermore acts as an E3 enzyme for the conjugation of Atg8, the second ubiquitin-like protein in the autophagy (Fujita et al., 2008; Hanada et al., 2007).

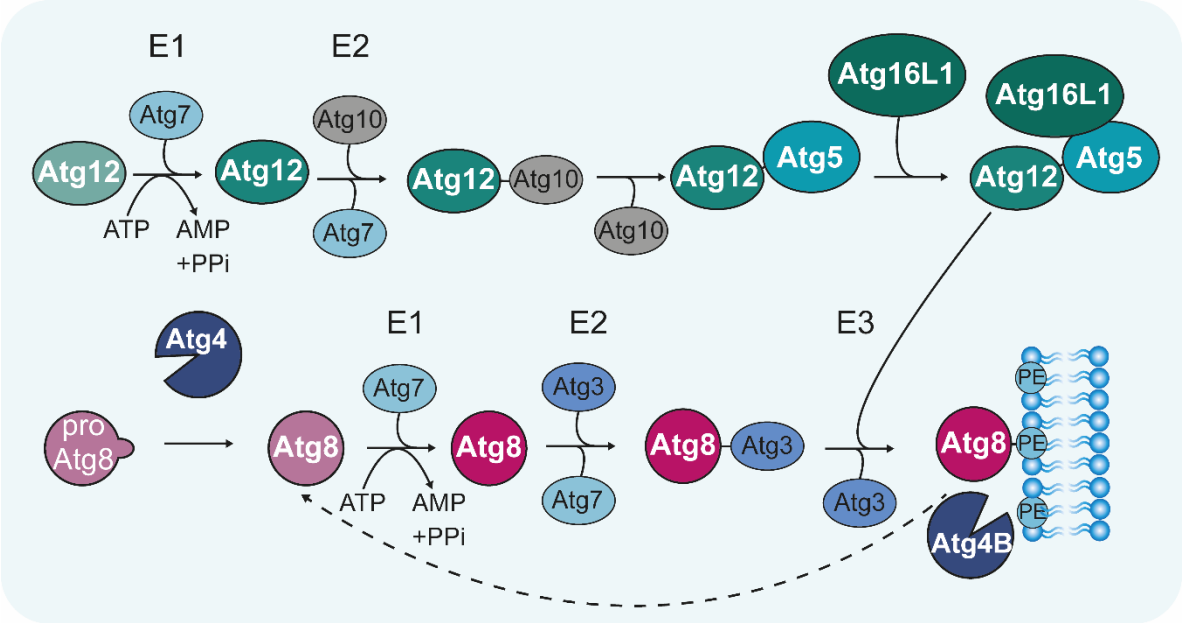

Figure 3. Ubiquitin-like protein conjugation in macroautophagy. Two ubiquitin-like conjugation systems are involved in autophagy. The ubiquitin-like protein Atg12 is activated, in an ATP-dependent manner, by Atg7 (E1). Activated Atg12 is transferred onto Atg10, the E2 enzyme of this conjugation cascade, and subsequently conjugated to Atg5. The Atg12-5 conjugate forms a complex with Atg16L1, giving rise to the Atg16L1 complex. In the second ubiquitin-like conjugation system of autophagy, the ubiquitin-like precursor protein proAtg8 is C-terminally cleaved by Atg4 proteases and subsequently activated by the E1 Atg7 in an ATP-dependent manner. Activated Atg8 is transferred onto the E2 Atg3. Conjugation of Atg8 to phosphatidylethanolamine (PE) in the autophagosome membrane is mediated by the Atg16L1 complex that exhibits E3-like activity. Deconjugation of Atg8 is executed by Atg4B.

The mammalian genome encodes six homologs of the Atg8 family: microtubule-associated proteins 1A/B light chain 3A (MAP1LC3A, more commonly referred to as LC3A), LC3B and LC3C, γ-aminobutyric acid receptor associated protein (GABARAP), GABARAP-like protein 1 (GABARAPL1), and GABARAP-like protein 2 (GABARAPL2, also referred to as

Golgi-associated ATPase enhancer of 16kDa (GATE-16) (Kabeya et al., 2004; Shpilka et al., 2011). All of these ubiquitin-like proteins are synthesized as precursor proteins that are activated by C-terminal proteolytic cleavage by the Atg4 family of proteases, which consists of Atg4A, Atg4B, Atg4C and Atg4D (Fass et al., 2007; Tanida et al., 2004b). This activated form is often referred to as LC3-I or GABARAP-I, respectively, and is, upon autophagy activation, conjugated to the phospholipid phosphatidylethanolamine (PE) in the membrane of the growing autophagosome and thereafter referred to as LC3-II or GABARAP-II (Fass et al., 2007).

This conjugation is executed by an enzymatic cascade that, similar to that of ubiquitin itself, consists of three enzymes. Atg7 is the activating enzyme in this reaction, also referred to as E1, and subsequently transfers the activated Atg8 protein to the E2 enzyme, Atg3. In the final step, the Atg8 protein is conjugated to PE in the autophagosomal membrane, catalyzed by the Atg16L1 complex that acts as the E3 enzyme of this reaction (Ichimura et al., 2000). Interestingly, LC3s and GABARAPs are conjugated both on the inner and the outer membrane of the growing autophagosome. Given this localization on autophagic vesicles, Atg8 proteins are commonly used as markers for autophagosomes and to assess the functionality of the autophagy pathway (Tanida et al., 2008). While the fraction localized on the inner membrane, facing the autophagosome lumen, is degraded in the lysosome together with the cargo, lipidated Atg8 facing the cytosol is deconjugated by Atg4B prior to fusion with the lysosome to replenish the cellular pool of these proteins (Kabeya et al., 2000; Kirisako et al., 2000; Nair et al., 2012; Satoo et al., 2009; Tanida et al., 2004a).

Regulation of autophagy by ubiquitylation

In addition to ubiquitin-like proteins that are critical for the regulation of autophagy, several studies have shed light on the ubiquitylation-dependent regulation of activity, stability and recruitment of proteins involved in autophagy. Initiation of autophagy was shown to be regulated by the E3 ligase SCF-β-TrCP that targets DEPTOR, an inhibitory interactor of mTOR, for proteasomal degradation in a growth-factor dependent manner. Upon release of this inhibitory interaction, mTOR is activated and induces autophagy inhibition (Duan et al., 2011; Gao et al., 2011).

Interestingly, RNF5 was reported to link the action of ubiquitin and ubiquitin-like proteins and negatively regulate autophagy. RNF5 associates with and ubiquitylates Atg4B, resulting in its proteasomal turnover (Kuang et al., 2012). As Atg4B is the primary protease recycling

lipidated Atg8-proteins, it is crucial for the maintenance of a continuous flow of autophagosomes (Kirisako et al., 2000).

The E3 ligase TRIM13 has been implicated in the activation of autophagy in response to ER-stress. Interestingly, this effect has been reported to be independent of its catalytic RING domain. Instead, it activates autophagy by a poorly understood mechanism through interaction with the autophagy receptor SQSTM1/p62 and co-localization with DFCP1 (Tomar et al., 2012).

Additionally, the autophagy initiator beclin-1 and other proteins of the initiation complexes have been shown to be prominent targets for ubiquitin-dependent regulation. Both TRAF6 and Parkin have been demonstrated to regulate beclin-1 levels by modification of its inhibitory interaction with Bcl-2 (Chen et al., 2010; Shi and Kehrl, 2010). While TRAF6 induces dissociation of beclin-1 from Bcl-2 by ubiquitylation of phosphorylated beclin-1 on K117 (Shi and Kehrl, 2010), Parkin promotes inhibition of beclin-1 by monoubiquitylation and stabilization of Bcl-2 (Chen et al., 2010). TRAF6 was additionally reported to K63-ubiquitylate and stabilize ULK1 by interaction with Ambra1 (Nazio et al., 2013). Further, the HECT ligase NEDD4 has been shown to regulate beclin-1 by K11- and K63-linked ubiquitylation, promoting its proteasomal turnover in the absence of PI3K-activation, thereby limiting beclin-1-mediated autophagy activation in the absence of additional cues (Platta et al., 2012).

Deubiquitylating enzymes in the regulation of autophagy

Given the involvement of ubiquitin and E3 ligases in autophagy regulation it is not surprising that several DUBs have been shown to modulate ubiquitin signaling in the context of autophagy. In line with this, a general effect of DUB inhibitors on autophagy has been reported. Similar to E3 ligases, the main target of DUBs in autophagy appear to be the initiation complexes, and beclin-1 in particular. The DUB A20, which belongs to the OTU family was reported to antagonize TRAF6-mediated ubiquitylation of beclin-1, preventing its dissociation from Bcl-2 (Shi and Kehrl, 2010). Similarly, both USP10 and USP13 were shown to modulate the ubiquitylation status of several components of the Vps34 complex, preventing their proteasomal degradation (Liu et al., 2011). Interestingly, spautin-1, a compound identified in small-molecule screen for autophagy inhibitors, was demonstrated to selectively inhibit both USP10 and USP13. USP10 and USP13 were further shown to regulate each other. Their steady-state levels were also regulated by components of the Vps34

complex, including beclin-1, thus demonstrating a complex interplay between the expression levels and activity of these proteins (Liu et al., 2011). The DUB ataxin-3 has also been implicated in the regulation of beclin-1 and autophagy activation (Ashkenazi et al., 2017). Ataxin-3 was reported to interact with and deubiquitylate beclin-1, preventing its proteasomal degradation. This interaction was shown to be dependent on the polyglutamine tract of ataxin-3 and was outcompeted by mutant ataxin-3 and huntingtin with expanded polyglutamine repeats, the proteins causative for MJD and HD, respectively (Ashkenazi et al., 2017).

1.3.2 Ubiquitin-dependent selective autophagy

Selective autophagy has been described for a number of different cellular structures and organelles. The different types of selective autophagy pathways are therefore typically named after their particular substrate. Selective autophagy functions during physiological conditions, such as the degradation of parts of the nuclear envelope and other nuclear components by nucleophagy, as well as in response to stress conditions, such as the degradation of parts of the ER in response to ER stress, a process termed ERphagy (Bernales et al., 2007; Mijaljica et al., 2010). The degradation of protein aggregates in response to proteotoxic stress is referred to as aggrephagy (Lamark and Johansen, 2012). Interestingly, selective autophagy is also one of the major cellular defense mechanisms against invading pathogens, resulting in their targeting and degradation by xenophagy (Knodler and Celli, 2011; Marshall et al., 2015). Additionally, selective autophagy also plays a role in maintaining cellular homeostasis by degradation of damaged organelles such as mitochondria by mitophagy (Kim et al., 2007) or degradation of inactive proteasomes by so-called proteaphagy (Marshall et al., 2015). Several other homeostatic functions of selective autophagy have been described including pexophagy, the selective degradation of damaged or superfluous peroxisomes (Sakai et al., 2006), lipophagy, the degradation of lipid droplets (Liu and Czaja, 2013), and ribophagy, the degradation of ribosomes (Kraft et al., 2008).

While certain types of selective autophagy employ ubiquitin-independent signals for cargo recognition, ubiquitylation, both in the form of monoubiquitylation and polyubiquitin chains, has been linked to the majority of these selective autophagy pathways and a specific targeting function of ubiquitin-signals in this context has been suggested (Khaminets et al., 2016). The selective degradation of specific substrates by autophagy utilizes the same core autophagy machinery as non-selective bulk autophagy. However, in addition to this machinery it employs a series of autophagy receptor proteins that enable selectivity for distinct cargo

(Johansen and Lamark, 2020; Kirkin and Rogov, 2019). Several autophagy receptors have been shown to contain a C-terminal UBA domain (Kim et al., 2016), that mediates interaction with the ubiquitylated substrates. The most well-characterized selective autophagy-receptor is sequestosome-1 (SQSTM1)/p62 (Bjørkøy et al., 2005; Kirkin and Rogov, 2019; Pankiv et al., 2007). SQSTM1/p62 primarily binds linear and K63-linked ubiquitin chains over K48-linked ubiquitin via its UBA-domain, and two ubiquitin-moieties have been shown to be the critical chain length required for recognition by this receptor (Seibenhener et al., 2004). Interestingly, the ability of SQSTM1/p62 to oligomerize, mediated by its N-terminal PB1 domain, is crucial for its targeting-ability (Moscat et al., 2006; Wurzer et al., 2015). In addition to ubiquitin-binding, the most common characteristic of autophagy receptors is their ability to bind to the Atg8 proteins LC3 and GABARAP on the autophagosomal membrane with varying specificity, allowing tethering of cargo to the forming autophagosome. This binding is mediated by an LC3-interacting region (LIR), which was first identified in the C-terminus of SQSTM1/p62 (Pankiv et al., 2007). Similar LIR motifs have since been identified in other selective autophagy receptors, with the canonical motif being defined by two critical amino acids: W/F/Y-X-X-L/I/V (WxxL). This core motif is additionally flanked by residues that affect affinity and specificity and is commonly preceded by a series of acidic amino acids. Phosphorylation of these residues has been shown to modify LC3/GABARAP-binding (Johansen and Lamark, 2020). LIR motifs are, however, not limited to autophagy receptors. Instead, these motifs also facilitate the recruitment of other autophagy effector proteins to autophagic structures (Kraft et al., 2012; Skytte Rasmussen et al., 2017). While receptors typically accompany the substrate and become degraded in the process, recruitment of these other proteins is transient.

It was recently shown that the autophagy receptors p62 and NDP52 also directly interact with factors involved in the early steps of autophagy initiation, such as ULK1 and FIP200 (Ravenhill et al., 2019; Turco et al., 2019; Vargas et al., 2019), suggesting that autophagosome formation is initiated in proximity to the cargo by recruitment of these initiation factors. The receptors subsequently tether the expanding phagophore around the cargo by binding to LC3 in the membrane (Ravenhill et al., 2019; Turco et al., 2019).

Additionally, the autophagy adaptor autophagy-linked FYVE protein (ALFY) has been shown to promote selective autophagy. ALFY acts as scaffold protein, linking the cargo to the growing autophagosome membrane by direct interaction with SQSTM1/p62 and core components of the autophagy machinery (Isakson et al., 2013).

Deubiquitylating enzymes in selective autophagy

As a role for ubiquitin in the regulation of selective autophagy has been demonstrated, the involvement of DUBs in this process has also been established. Several DUBs have been implicated in the selective degradation of protein aggregates. USP36, UCHL-1 and USP9X have been demonstrated to promote aggregate formation or clearance of aggregation-prone proteins by autophagy (Cartier et al., 2012; Taillebourg et al., 2012). USP9X was reported to promote autophagic degradation of α-synuclein by removing the monoubiquitin modification that destines it for proteasomal degradation and thereby promoting α-synuclein degradation by autophagy (Rott et al., 2011). Surprisingly, deubiquitylation of α-synuclein by USP8 instead prevents its autophagic clearance and promotes Lewy body formation, likely by removal of K63-linked ubiquitin chains that would otherwise target this protein for autophagy (Alexopoulou et al., 2016).

PINK1/Parkin-mediated mitophagy

As a central element of cellular energy production, a healthy mitochondrial network is of vital importance for cellular integrity and survival. In order to maintain a functional mitochondrial network and to be able to adapt to changing conditions and energy demands, cells dynamically regulate mitochondrial fusion and fission. Mitochondria are also one of the major intracellular sources of reactive oxygen species (ROS), and damaged entities therefore need to be rapidly and selectively eliminated, a task that is executed by mitophagy (Ashrafi and Schwarz, 2013; Baker et al., 2011). Accordingly, mitochondrial dysfunction as well as dysregulated mitophagy have been linked to a variety of pathologies, including neurodegenerative disorders (Johri and Beal, 2012).

The best characterized type of mitophagy, in which ubiquitin furthermore plays a central role, is mediated by the PTEN-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin (**Figure 4**). This pathway is triggered in response to mitochondrial damage or stress involving loss of mitochondrial membrane potential, e.g. in response to chemical uncouplers, such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Jin and Youle, 2012). Interestingly, autosomal recessive mutations in both PINK1 and Parkin, have been identified as the underlying genetic cause of familial types of PD, resulting in impaired mitophagy (Bonifati et al., 2002; Geisler et al., 2010b).

The mitochondrial outer membrane (MOM)-resident serine-threonine kinase PINK1 is constitutively imported into the mitochondria by the TOM/TIM complex and subsequently

cleaved and thereby inactivated by a series of mitochondrial proteases including the mitochondrial processing peptidase (MPP) (Greene et al., 2012) and presenilin-associated rhomboid-like protease (PARL) (Deas et al., 2011; Meissner et al., 2011). However, disruption of the mitochondrial membrane potential disables the function of the TOM/TIM complex and results in accumulation of active PINK1 on the MOM. PINK1 becomes further activated by autophosphorylation and proceeds to phosphorylate a number of proteins on the mitochondrial membrane, including ubiquitin, which is phosphorylated on serine 65 (Koyano et al., 2014). Another substrate of PINK1 is the E3 ubiquitin ligase Parkin, which is recruited to mitochondria by phosphorylated ubiquitin and is subsequently phosphorylated itself, resulting in enhanced ubiquitin ligase activity (Kim et al., 2008b; Matsuda et al., 2010; Narendra et al., 2010b).

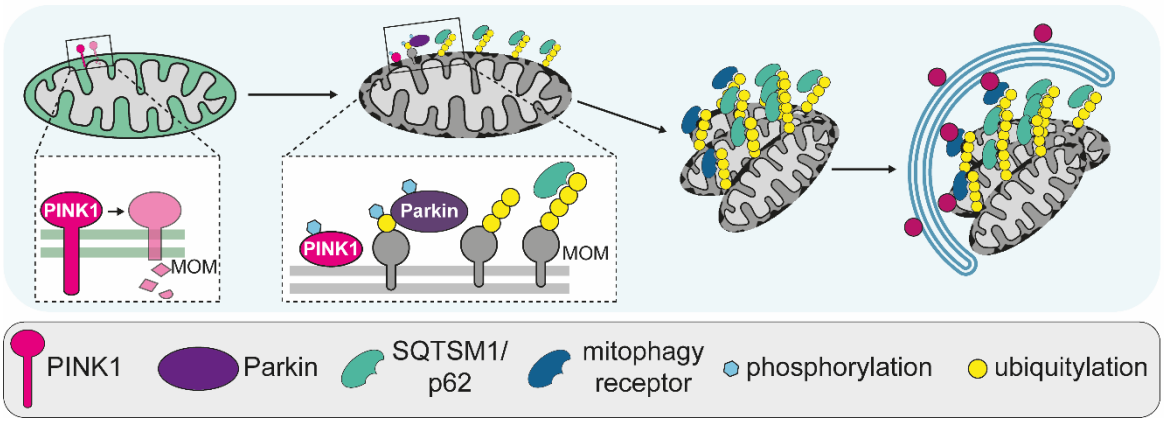

Figure 4. PINK1/Parkin-mediated mitophagy. The kinase PINK1 is constantly imported into the mitochondria and cleaved by mitochondrial proteases. Upon loss of mitochondrial membrane potential PINK1 accumulates on the mitochondrial outer membrane (MOM) and phosphorylates ubiquitin on MOM proteins. The E3 ligase Parkin is recruited to damaged mitochondrial by this modification and phosphorylated by PINK1, resulting in its increased activity. Parkin ubiquitylates numerous proteins on the mitochondrial surface. Recruitment of the autophagy receptor SQSTM1/p62 induces perinuclear clustering of the damaged mitochondria. Specific mitophagy receptors recruit components of the autophagic machinery and promote sequestration of the damaged mitochondria in autophagosomes by interaction with lipidated Atg8-proteins in the membrane.

Parkin ubiquitylates numerous substrates on the MOM, thereby decorating the surface of the mitochondria with ubiquitin. The mode of action of Parkin is complex and the ligase is able to generate several different linkages, including K27-, K48- and K63-linked ubiquitin chains, all of which have been implicated in mitophagy and reported on mitochondrial proteins (Chan et al., 2011). Additionally, autoubiquitylation of Parkin with K6-linked chains has been reported (Durcan et al., 2014). To prevent fusion of damaged mitochondria with healthy ones and thereby spread of the damage throughout the mitochondrial network, initial

substrates of Parkin include mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) (Gegg et al., 2010) as well as the voltage-dependent anion channel 1 (VDAC1) (Geisler et al., 2010a). The rapid proteasomal degradation of these proteins results in fragmentation of the mitochondrial network and isolation of damaged entities (Tanaka et al., 2010). PINK1 further primes the MOM-localized atypical GTPase Miro1 for Parkin-mediated ubiquitylation and subsequent degradation (Wang et al., 2011). As Miro1 is involved in anchoring of mitochondria to kinesin complexes that are required for organellar transport along the microtubule network, its degradation triggers the release from kinesin and further supports the protective mechanism of mitochondrial isolation (Wang et al., 2011). Additionally, Parkin induces the proteasomal degradation of a variety of other MOM proteins, including several components of the TOM-complex (Yoshii et al., 2011).

Proteasomal degradation of mitochondrial proteins requires the ubiquitin-dependent AAA+ ATPase valosin-containing protein (VCP)/p97, that mediates retrotranslocation of mitofusins and other mitochondrial membrane proteins (Kim et al., 2013). Spatial and temporal regulation as well as substrate-specificity of VCP/p97 is achieved by a number of specific co-factors that bind to VCP/p97 in different cellular contexts (Hänzelmann and Schindelin, 2017). It has been reported that the VCP/p97 co-factor UBX-domain containing protein 1 (UBXD1) translocates to mitochondria upon loss of membrane potential in a Parkin-dependent manner, where it promotes VCP/p97 recruitment (Bento et al., 2018). Interestingly, it has also been shown that proteasomal degradation of MOM proteins induces rupture of the MOM, though this process has been suggested to be dispensable for mitophagy (Yoshii et al., 2011). In contrast, it has been described that MOM rupture leads to the exposure of prohibitin-2 (PHB2), which was reported to be required to induce mitochondrial degradation. PHB2 contains an LC3-selective LIR motif and was therefore suggested to act as a mitophagy receptor (Wei et al., 2017).

K63-ubiquitylation of mitochondrial proteins by Parkin results in recruitment of the autophagy receptor SQSTM1/p62 (Narendra et al., 2010a). Oligomerization of SQSTM1/p62 bound to ubiquitylated proteins has been shown to be a driver for the formation of protein condensates, so-called p62 bodies, that are subsequently targeted for autophagic degradation to promote cell survival (Bjørkøy et al., 2005). Analogous to this, SQSTM1/p62 recruitment to depolarized, ubiquitylated mitochondria results in dramatic changes in mitochondrial localization, causing their accumulation in distinct perinuclear clusters. Surprisingly, mitophagy was reported to be independent of SQSTM1/p62-mediated clustering (Narendra et al., 2010a). Therefore, additional work will be required to fully comprehend the significance

of this mechanism as well as the targeting potential of ubiquitin and, more specifically, the contribution of individual ubiquitin-linkage types in the context of mitophagy.

Interestingly, NBR1, another autophagy receptor, was shown to be dispensable for Parkin-mediated mitophagy (Shi et al., 2015), whereas optineurin (OPTN) and NDP52 were reported to amplify mitophagy signalling and to be required for this pathway, while their actions appear to be largely redundant (Lazarou et al., 2015; Padman et al., 2019). The mechanism that underlies NDP52- and OPTN-amplified mitophagy signalling, has been suggested to be a two-step process. Interestingly, while recruitment of these receptors was dependent on ubiquitin-phosphorylation, the LIR motifs in NDP52 and OPTN were dispensable for the initial recruitment of Atg8 proteins to damaged mitochondria, unlike what has been suggested to be the classical function of autophagy receptors (Padman et al., 2019). Once autophagy has been initiated, Atg8 proteins promote further recruitment of NDP52 and OPTN, in a LIR-dependent manner, resulting in an Atg8-dependent positive feedback loop driving further autophagosome biogenesis and thereby promoting efficient mitophagy (Padman et al., 2019).

Given the robust and diverse ubiquitin signals generated on damaged mitochondria by the E3 ligase Parkin, it is not surprising that the majority of the DUBs implicated in selective autophagy has been shown to function in this particular pathway. USP15, USP30 and USP35 were found to oppose Parkin-mediated ubiquitylation. USP15 deubiquitylates substrates of Parkin, thereby preventing the accumulation of autophagy receptors, such as SQSTM1/p62 on mitochondria (Cornelissen et al., 2014), USP30, a mitochondria-localized DUB, preferentially removes K6- and K11-linked ubiquitin chains, was shown to prevent mitophagy initiation (Bingol et al., 2014; Cunningham et al., 2015; Wang et al., 2015). Interestingly, USP30 overexpression was reported to delay translocation of Parkin to damaged mitochondria (Wang et al., 2015). In contrast, USP35, which also opposes Parkin, appears to employ a different mechanism, as this DUB localizes to mitochondria under physiological conditions, while uncoupling of mitochondrial membrane potential induces its re-translocation to the cytosol (Wang et al., 2015). Parkin-dependent mitophagy has been shown to be stimulated by USP8, which removes K6-linked ubiquitin chains from Parkin itself, likely allowing association with other proteins that is required for functional mitophagy (Durcan et al., 2014).

1.4 GENOME INTEGRITY

As the storage of genetic information, maintenance of a healthy and stable genome is paramount for cellular survival. The DNA is subject to constant insults by both exogenous factors, such as chemicals and ultraviolet (UV) radiation, as well as endogenous factors including ROS (Jeggo and Löbrich, 2007). In fact, it is estimated that each cell can face as many as 70.000 counts of damage to their DNA on a daily basis (Lindahl and Barnes, 2000). The complex cellular measures detecting, signaling and counteracting insults to the DNA are collectively referred to as the DNA damage response (DDR). As such, the DDR affects many cellular processes and can trigger checkpoint signaling and cell cycle arrest, transcriptional alterations, repair of the DNA lesion as well as induction of cell death by apoptosis. These measures as well as the cellular outcome of DNA damage depend largely on the type of damage that is inflicted (Jeggo and Löbrich, 2007; Polo and Jackson, 2011). The cellular response to DNA damage constitutes a central element of the work presented in this thesis and will therefore be the focus of the following chapters, also with respect to its link to neurodegeneration.

1.4.1 Genome integrity and neurodegeneration

In addition to protein quality control pathways, the disturbance of cellular pathways governing genomic integrity has also been linked to neurodegeneration (Abugable et al., 2019; McKinnon, 2009). Given the high oxygen consumption of the brain compared to other organs as well as the post-mitotic state of neuronal cells, oxidative DNA damage has been associated with the pathogenesis of neurodegenerative disorders (Barzilai, 2007). Elevated levels of oxidative damage to both nuclear and mitochondrial DNA have been demonstrated in AD and PD (Alam et al., 1997; Wang et al., 2005; Zhang et al., 1999). Furthermore, high levels of oxidative stress have been reported to be able to promote somatic expansion of the HD-associated triplet repeat (Kovtun et al., 2007).

Mutations in one of the key proteins governing the DDR have been shown to be causative for the severe, young-onset neurodegenerative disorder Ataxia telangiectasia. Similarly, mutations in several other proteins involved in the DDR as well as the specific response to single-strand breaks (SSBs) or double-strand breaks (DSBs) and other types of lesions have been shown to result in neurological pathologies, some of which are defined as neurodegenerative disorders (McKinnon, 2009).

Conversely, mutated proteins underlying proteinopathies have instead been shown to trigger DNA repair defects (Abugable et al., 2019). In line with this, several polyglutamine proteins, including huntingtin and ataxin-3, have been shown to modulate DDR signaling by direct regulation of repair proteins or sequestration of repair proteins in the disease-typical inclusions (Massey and Jones, 2018).

1.5 THE DNA DAMAGE RESPONSE

Activation of the DDR is coordinated by three major signaling kinases in response to different sensor proteins that sense the presence and type of DNA damage: DNA-dependent protein kinase (DNA-PK), Ataxia-telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related protein (ATR) (Awasthi et al., 2015; Maréchal and Zou, 2013). In contrast to ATR, which mainly responds to and mediates signaling to counteract SSBs, ATM and DNA-PK are activated in response to DSBs, though the sensor proteins recruiting either of these differ (Harper and Elledge, 2007). The Ku70/Ku80 heterodimer recognizes and threads onto the double-stranded DNA ends generated by DSBs and promotes the formation of the DNA-PK holoenzyme, which facilitates DSB repair (Hammel et al., 2010). ATM is recruited by a similar sensing mechanism executed by the MRN complex, consisting of Mre11, Rad50 and Nbs1, which, similar to Ku70/Ku80, recognizes and associates with the DNA ends flanking the break site (Lee and Paull, 2005). The recruitment of ATR to SSBs is mediated by replication protein A (RPA), which binds and coats single-stranded DNA (ssDNA) upon DNA end-resection at DNA SSBs (Zou and Elledge, 2003).

While SSBs make up the large majority of the daily insults to genomic DNA, DSBs pose a particularly severe threat, as these breaks can result in cell death if they are left unrepaired or form the basis for transformation and tumorigenesis, as a result of larger chromosomal alterations, if repaired incorrectly (Jeggo and Löbrich, 2007). There are two major DNA repair pathways resolving DSBs: Homologous recombination (HR) that uses a homologous sequence for repair of the lesion and non-homologous end-joining (NHEJ) that ligates the DNA ends generated by the damage (Ciccia and Elledge, 2010).

1.5.1 DNA double-strand break repair

The final step in the DDR to DSBs is the repair of DNA lesions by HR or NHEJ. As HR relies on a DNA template, thus, the presence of a homologous chromosome or more

commonly a sister chromatid, this type of repair is limited to specific time windows in the cell cycle, namely the S and G2 phases. HR requires initial ssDNA resection mediated by the MRN complex and CtBP-interacting protein (CtIP), followed by coating of the ssDNA by RPA and RAD51-mediated invasion of the homologous sequence (Wright et al., 2018). NHEJ, on the other hand, does not require a template or major resection but is instead based on ligation of the broken DNA ends and can therefore operate throughout the cell cycle, thus constituting the predominant repair type in postmitotic cells (Ciccia and Elledge, 2010). During NHEJ, the Ku70/Ku80 heterodimer recruits the DNA-PK catalytic subunit (DNA-PKcs), resulting in formation of the functional holoenzyme DNA-PK, followed by DNA end-processing by the nuclease Artemis. The subsequently recruited DNA ligase IV complex, consisting of the catalytic subunit DNA ligase IV, its cofactor XRCC4 and XRCC4-like factor (XLF) ultimately ligates the DNA strands (Davis and Chen, 2013).

1.5.2 DNA double-strand break signaling

Given the particular threat that DSBs pose to genomic integrity, their detection, signaling and repair is executed by a tightly controlled, hierarchical cascade of sensor, mediator, transducer and effector proteins. DSBs are sensed by the MRN complex as well as the Ku70/Ku80 heterodimer, that forms a ring-structure and localizes on the DNA ends generated by DSBs. While the dimerization of the MRN component Mre11 stabilizes binding to DNA, the dimerization of Rad50 was shown to tether the DNA ends closer together (Polo and Jackson, 2011). Together with the sensor kinase ATM, the recruitment of these proteins signals the presence of DSBs. Following activation, ATM phosphorylates the histone variant H2AX proximal to the DNA lesion at its C-terminal residue S139. This phosphorylated version of H2AX is commonly referred to as γH2AX (Burma et al., 2001). This modification serves as a robust DNA damage signal for the recruitment of mediator of DNA damage checkpoint 1 (MDC1) and is also commonly used as a marker for DSBs in experimental settings. MDC1-binding to γH2AX is mediated by its phospho-protein binding breast cancer C-terminal (BRCT) motif (Stucki et al., 2005). MDC1, in turn, is also phosphorylated by ATM, creating a binding platform for downstream effector proteins. The occurrence of DSBs as well as the ATM-mediated modification of MDC1 initiate a series of events that trigger the accumulation of different PTMs, including poly(ADP-ribose) (PAR), SUMO and ubiquitin, to coordinate the recruitment of proteins involved in the repair of DNA lesions (Garvin and Morris, 2017; Liu et al., 2017; Schwertman et al., 2016).

Poly(ADP-ribos)ylation in DNA-double strand break signaling

The initial occurrence of DNA damage is sensed by PAR polymerases (PARP), in particular PARP1, the primary enzyme catalyzing DNA-damage induced poly(ADP-ribos)ylation (PARylation) (Benjamin and Gill, 1980; Pion et al., 2005). PARP1 is a highly abundant enzyme that is largely inactive in unchallenged cells and is able to sense both SSBs and DSBs by scanning stretches of chromatin. Detection of SSBs or DSBs activates PARP1 through intramolecular conformational changes and results in robust PARylation, making PARP1 recruitment and PARylation one of the earliest marks of DNA damage (Liu et al., 2017).

PARylation primarily modifies acidic residues such as glutamate and aspartate, but in certain cases also lysine residues, in substrate proteins and is, similar to other PTMs, reversible. PAR chains typically contain around 200 ADP-ribose units and are branched in intervals of 20-50 units (Ray Chaudhuri and Nussenzweig, 2017). Disassembly of PAR chains is primarily mediated by PAR glycohydrolase (PARG) (Schreiber et al., 2006).

Rapid accumulation of PAR is required for recruitment of ATM and further promotes recruitment of components of the MRN complex, thereby driving DNA damage signaling and repair (Haince et al., 2007; Haince et al., 2008). Proteins that are recruited to DSBs in a PARylation-dependent manner frequently contain PAR-binding motifs that mediate non-covalent interaction with PAR (Teloni and Altmeyer, 2016). It has furthermore been reported that forkhead-associated (FHA) and BRCT domains allow interaction with damage-induced PARylation (Li et al., 2013). Thus, PARylation forms a recruitment platform for proteins involved in the early DDR. Additionally, the robust accumulation of negatively charged PAR polymers in proximity to negatively charged DNA has been suggested to promote repulsion-induced relaxation of the lesion-surrounding chromatin, allowing recruitment of DNA-binding DDR factors (Poirier et al., 1982). The chromatin remodelers CHD3 and CHD4 have both been suggested to be recruited to lesions by this mechanism (Smith et al., 2018), similar to HP1 (Smith et al., 2019). In addition to PARG-mediated disassembly, ubiquitin-dependent removal of PARP1 from DSBs contributes to the rapid termination of PAR-signaling. Both, the E3 ligase checkpoint with forkhead and RING finger domains protein (CHFR) and RNF146 (also known as Iduna), have been shown to ubiquitylate PARP1 resulting in its displacement from DSBs and degradation (Kang et al., 2011; Liu et al., 2013).

SUMOylation in DNA-double strand break signaling

DNA damaged-induced signaling is also mediated by modification of DDR proteins with the small ubiquitin-like modifier SUMO. SUMO is, similar to ubiquitin, conjugated to substrate proteins by an enzymatic cascade consisting of E1, E2 and E3 enzymes and subsequently recognized by reader proteins containing SUMO-interacting motifs (SIMs) (Hendriks and Vertegaal, 2015). In mammals, only a single E1, a heterodimeric complex formed by Sae1 and Sae2, as well as a single E2 enzyme, Ubc9, have been identified, while more than ten E3 ligases modulate transfer of SUMO from Ubc9 to substrate proteins (Pichler et al., 2017). In mammals, three isoforms of SUMO have been described (SUMO1, SUMO2, SUMO3) and it has further been reported that SUMO2 and SUMO3 contain internal SUMO-acceptor sites, which enable polymeric SUMO chain formation (Schwertman et al., 2016). Damage-induced SUMOylation is primarily executed by the SUMO ligases PIAS1 and PIAS4 and SUMOylation is required for the recruitment of RNF168, Breast cancer type 1 susceptibility protein (BRCA1) and 53BP1. Further, both BRCA1 and 53BP1 are themselves targets for this modification (Galanty et al., 2009). Additionally, the transducer protein MDC1 has been shown to be modified by SUMOylation at K1480, which is required for recruitment of RPA, CtIP and RAD51 and thus, damage repair by HR (Luo et al., 2012).

Ubiquitylation in DNA-double strand break signaling

Given the multitude of proteins involved in the DDR as well as the requirement for a highly regulated hierarchical action of these proteins, it is not surprising that a central role in the DDR has been assigned to ubiquitin (Schwertman et al., 2016). This is corroborated by the different types of UBDs that have been found in a large number of repair proteins including ubiquitin-interacting motifs (UIMs), motifs interacting with ubiquitin (MIUs) as well as ubiquitin-binding zinc finger (UBZ) domains. Additionally, several repair proteins have been shown to contain tandem UBDs, increasing their interaction with ubiquitylated proteins (Panier et al., 2012).

Interestingly, many of the ubiquitylation factors involved in the DDR are constitutively active and present in the cell and their action is therefore largely, but not exclusively, controlled by their translocation to lesions, in close proximity to their substrates, rather than by modulation of their intrinsic activity (Dantuma and Pfeiffer, 2016). Modification of histones with ubiquitin in response to DNA damage is one of the crucial signals in the DDR and required for efficient DNA repair.

Ubiquitylation is primarily catalyzed by two major E3 ubiquitin ligases in the context of DSB signaling and repair, RNF8 and RNF168, that are recruited to DSBs in a sequential manner (**Figure 5**). RNF8 is recruited by ATM-mediated phosphorylation of MDC1 via its forehead-associated (FHA) domain and, in complex with the K63-specific E2 enzyme Ubc13, ubiquitylates a number of substrates, which leads to the recruitment of RNF168 (Bekker-Jensen et al., 2010). The nature of this recruitment has been debated and different signals have been suggested, all of which evolve around RNF8-mediated ubiquitylation while the substrate of this ubiquitylation, responsible for RNF168 recruitment, varies. One suggested mechanism is priming ubiquitylation of the linker histone H1 by the E3 ligase HUWE1 followed by RNF8-mediated extension of the ubiquitin-chain (Mandemaker et al., 2017; Thorslund et al., 2015). Another study identified Lethal(3)malignant brain tumor-like protein 2 (L3MBTL2) as the protein that links RNF168-recruitment to RNF8 (Nowsheen et al., 2018). RNF168 binds to ubiquitylated proteins via its N-terminal UBD1 as well as its C-terminal UBD2 (Panier et al., 2012) and subsequently propagates the ubiquitin signal at DSBs by ubiquitylation of H2A on K13/K15 as well as H2AX, creating a positive feedback loop (Mattiroli et al., 2012).

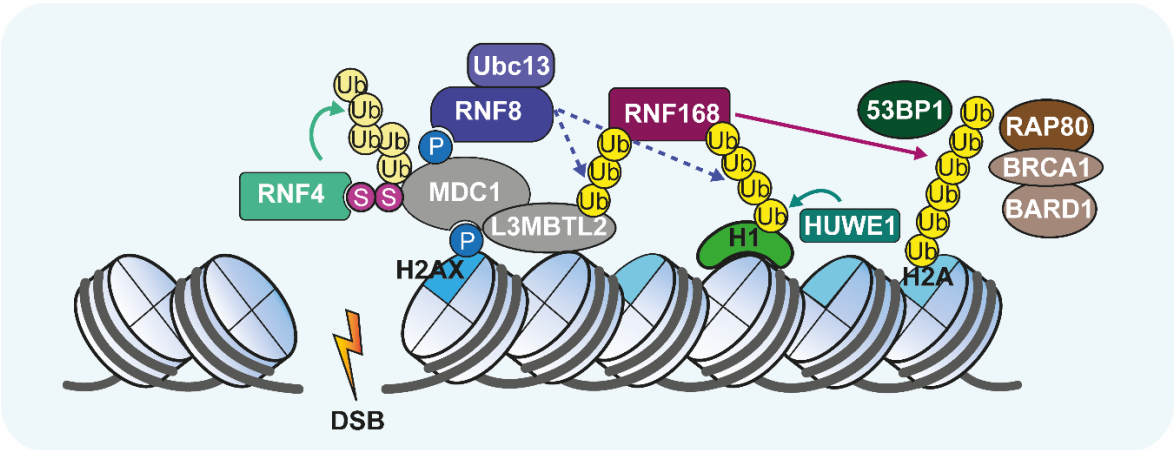

Figure 5. Ubiquitylation in the DNA damage response to double-strand breaks. Ubiquitylation in response to DNA double-strand breaks (DSBs) is primarily catalyzed by the E3 ligases RNF8 and RNF168 that are recruited to lesions in a sequential manner. RNF8 recognizes ATM-phosphorylated MDC1, bound to phosphorylated histone H2AX (γH2AX), and ubiquitylates its substrates in complex with the K63-specific E2 Ubc9. RNF168 is recruited in an RNF8-dependent manner and both ubiquitylation of L3MBTL2 and ubiquitin chain-extension on HUWE1-primed linker histone H1 have been suggested to mediate this recruitment. RNF168 subsequently K63-ubiquitylates H2A resulting in amplification of the damage-induced ubiquitin signal. Ubiquitin chains generated by RNF168 serve as a recruitment platform for repair proteins that interact with these chains via (tandem) ubiquitin-binding domains (UBDs). The SUMO-targeted ubiquitin ligase (STUbL) RNF4 ubiquitylates SUMOylated MDC1 resulting in its removal from the lesion. Timely removal of proteins from lesions is required to allow progression of the DNA damage response and efficient repair.

RNF168 catalyzed ubiquitylation thereby amplifies the ubiquitin signal at DSBs, resulting in a profound ubiquitylation signature. It is noteworthy in this context that tethering of RNF8

and RNF168 to chromatin is sufficient to trigger the DDR even in the absence of actual DNA damage (Acs et al., 2011; Luijsterburg et al., 2012). This damage-induced ubiquitylation serves as a recruitment platform for both, RAP80, via its tandem ubiquitin-interacting motifs (UIMs) (Sobhian et al., 2007; Yan et al., 2007), a protein that is frequently found in complex with BRCA1, as well as p53-binding protein 1 (53BP1), an important mediator of NHEJ (Fradet-Turcotte et al., 2013). While it was assumed that 53BP1 is indirectly recruited by the actions of RNF8/RNF168, as it lacks UBDs, it was recently shown that 53BP1 contains a ubiquitin-dependent recruitment (UDR) motif that recognizes RNF168-catalytzed H2A-ubiquitylation at K15 and thereby promotes stable retention of 53BP1 at lesions (Fradet-Turcotte et al., 2013).

It is the balance between the recruitment of BRCA1 and 53BP1 as well as associated proteins that ultimately determines which DNA repair route will be taken, NHEJ or HR. Once recruited, 53BP1 not only promotes NHEJ but also antagonized HR-mechanisms through the recruitment of RIF1 and REV7 that displace BRCA1 from DSBs during G1 (Ghezraoui et al., 2018). Additionally, the ubiquitin-dependent recruitment of RAP80 and complex formation with BRCA1, including BRCA1-associated RING domain 1 (BARD1), limits the association of BRCA1 with other HR-promoting factors such as CtIP and RAD51, thereby preventing excessive end resection at DSBs (Hu et al., 2011). In contrast, during S-phase, BRCA1 interferes with RIF1 retention to favor break repair by HR (Isono et al., 2017).

In addition to RNF8 and RNF168, a number of other ubiquitin ligases are implicated in the cellular response to DSBs (Dantuma and Pfeiffer, 2016). While the steady-state levels of RNF168 are controlled by both UBR5- and TRIP12-dependent ubiquitylation (Gudjonsson et al., 2012), RNF168 is also antagonized by its paralog RNF169, which also recognizes the ubiquitin-mark on H2A but does not amplify the RNF8-initiated ubiquitin signal and therefore restricts downstream ubiquitin-signaling (Poulsen et al., 2012). In contrast to the majority of other ligases, the E3 ligase RNF138 is recruited to the DNA break itself rather than chromatin-associated proteins flanking the break. RNF138 has been reported to stimulate HR by promoting removal of the Ku70/Ku80 heterodimer (Ismail et al., 2015) as well as ubiquitin-dependent recruitment of CtIP, which, together with the MRN complex, is required for DNA end resection (Schmidt et al., 2015). RNF2-BMI1 was reported to recruit ATM by ubiquitylation of H2AX at K119 (Pan et al., 2011).

While ubiquitylation in the context of the DDR occurs largely via K63-linked ubiquitylation and serves to recruit repair proteins, the ubiquitin-dependent removal of DDR proteins, by displacement or degradation, in a timely manner is crucial for the progression of the

hierarchical signaling events that orchestrate DNA repair . The first wave of ubiquitylation at DSBs is mediated by the ligase CHFR that is recruited to DSBs by PARylation and ubiquitylates PARP1 with both K48- and K63-linked ubiquitylation, resulting in its subsequent displacement (Liu et al., 2013). RNF8-mediated K48-linked ubiquitylation triggers the proteasomal degradation of Jumonji domain-containing protein 2 (JMJD2) A and JMJD2B (Mallette et al., 2012), Ku80 (Feng and Chen, 2012) and Proliferating cell nuclear antigen (PCNA) (Zhang et al., 2008). Similarly, L3MBTL1 removal is regulated by ubiquitylation. This is mediated by the ubiquitin-dependent segregase VCP/p97 (Acs et al., 2011). Chromatin extraction of L3MBTL1 is necessary to expose the H4K20me2-mark that is recognized by the tandem TUDOR domain in 53BP1 in order to enable efficient NHEJ (Botuyan et al., 2006). The degradation of the catalytic subunit PKcs of DNA-PK also relies on ubiquitin-dependent extraction and proteasomal degradation promoted by VCP/p97 (Jiang et al., 2013).

Particularly interesting in the context of this thesis is furthermore the involvement of the SUMO-targeted E3 ubiquitin ligase (STUbL) RNF4 that links the signaling functions of SUMO and ubiquitin. STUbLs are ubiquitin ligase that selectively ubiquitylate SUMOylated proteins, in the case of RNF4, SUMO2/3 modified substrates (Sriramachandran and Dohmen, 2014). RNF4 is recruited to DSBs and interacts with its SUMOylated targets, including MDC1 and RPA, via four SIMs located in its N-terminus, while its catalytic RING domain resides in the C-terminal part of the protein. RNF4-mediated ubiquitylation targets MDC1 and RPA, and likely others, for their timely removal and thereby promotes efficient repair (Galanty et al., 2012; Yin et al., 2012).

Deubiquitylating enzymes DNA-double strand break signaling

With increasing evidence of the importance of ubiquitin signaling in coordination of the DNA damage response and DNA repair, the involvement of DUBs in this process has gained significant interest (Kee and Huang, 2016). A systematic screen for the identification of DUBs involved in DNA repair, combining both accumulation of DUBs at DSBs, siRNA-depletion to identify proteins that are required for DDR and G2/M-checkpoint signaling as well as repair efficiency identified that nearly half of the DUBs encoded by the human genome are recruited to DSBs or in some way involved in DNA damage signaling (Nishi et al., 2014). The majority of DUBs with characterized roles in DNA repair have been shown to antagonize the major E3 ligases RNF8 and RNF168.

POH1, an integral subunit of the 19S particle of the proteasome that has been reported to disassemble K63-linked ubiquitin chains, antagonizes RNF8, thereby inhibiting 53BP1 recruitment and promoting DNA repair by HR instead (Butler et al., 2012). Similarly, the K63-specific DUB BRCC36, has been reported to antagonize RNF8 to prevent unnecessary activation of NHEJ, thus limiting the potential risk of error-prone repair (Ng et al., 2016). Another DUB that antagonizes RNF8 is OTUB2, preventing RNF168-catalytzed ubiquitylation and recruitment of 53BP1 and RAP80 (Kato et al., 2014), while OTUB1 was shown to inhibit Ubc13, the E2-enzyme that enables RNF8-catalyzed ubiquitylation, independent of its catalytic activity (Nakada et al., 2010). Deubiquitylation of H2A by USP44 was also reported to prevent recruitment of RNF168 (Mosbech et al., 2013).

Other examples of DUBs acting on histone-ubiquitylation are USP3 and USP11, both of which deubiquitylate H2AX, thereby preventing both BRCA1 and 53BP1 recruitment (Ting et al., 2019). USP3 was additionally shown to antagonize RNF168-mediated ubiquitylation of H2A at lysine K13/K15 (Sharma et al., 2014). HR was further reported to be promoted by USP26 and USP37. These DUBs promote repair by HR by preventing the inhibitory complex formation of the BRCA1-Abraxas-RAP80-MERIT40 complex and instead promote association of BRCA1 with partner and localizer of BRCA2 (PALB2)-BRCA2-RAD51 (Typas et al., 2015). Additionally, UCHL5 and USP4 have been implicated in regulation of HR by promoting DNA-end resection by the MRN complex and CtIP (Nishi et al., 2014; Wijnhoven et al., 2015). Interestingly, while this is dependent on the catalytic activity of USP4, the substrate of this DUB activity is neither MRN nor CtIP but USP4 itself. Interaction of USP4 with both MRN and CtIP promotes recruitment of CtIP to DSBs (Wijnhoven et al., 2015).

Besides regulation of the recruitment of various repair factors, several DUBs have also been implicated in stabilization or destabilization of the E3 ligases RNF8 and RNF168 and therefore modulate ubiquitin-dependent signaling at DSBs. Both USP34 and USP7 stabilize RNF168 at damage sites by means of deubiquitylation (Sy et al., 2013; Zhu et al., 2015). Regulation of RNF8, on the other hand, was reported to be differentially regulated by the DUB ataxin-3 in complex with the ubiquitin-dependent segregase VCP/p97 under physiological conditions and in response to genotoxic insults. While ataxin-3 was shown to stabilize RNF8 in the absence of DNA damage, it promoted VCP/p97-dependent chromatin extraction and proteasomal turnover of RNF8 at DSBs, preventing appropriate activation of NHEJ (Singh et al., 2019).

1.6 MACHADO-JOSEPH DISEASE

MJD, also known as spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant heritable neurodegenerative disorder and the most common dominantly inherited ataxia (Taroni and DiDonato, 2004). The disease primarily affects neurons in the dentate nucleus of the cerebellum, the substantia nigra, the pons and as well as the striatum and the spinal cord of diseased individuals and causes progressive cerebellar ataxia (Koeppen, 2018). While a wide range of symptoms has been associated with MJD, the most common symptoms include gait and limb ataxia, spasticity, ophthalmoplegia as well as difficulties with swallowing (dysphagia) and speech (dysarthria) (Fowler, 1984). The cause for MJD is a CAG repeat expansion in the *ATXN3* gene, giving rise to mutant ataxin-3 protein containing an expanded polyglutamine repeat (Kawaguchi et al., 1994; Takiyama et al., 1993). As such, MJD is one of nine polyglutamine disorders, all of which constitute progressive neurodegenerative disorders, while the genetic cause, the affected brain region as well as symptoms of the diseases differ broadly. In addition to MJD, five spinocerebellar ataxias (type 1, 2, 6, 7, 17) as well as spinal and bulbar muscular atrophy (SBMA, also known as Kennedy disease), dentatorubral-pallidoluysian atrophy (DRPLA) and HD belong to this family of disorders (Orr and Zoghbi, 2007). Expansion of the polyglutamine tract in the affected proteins results in an increased propensity to aggregate, a toxic gain-of-function mechanism, making inclusion bodies of the mutant proteins the unifying hallmark of these diseases, which are therefore classified as proteinopathies (Zoghbi and Orr, 2000).

The number of CAG repeats as well as the pathogenic threshold has been shown to differ between the affected genes (Shao and Diamond, 2007). Furthermore, the repeat is located close to the N-terminus in all of these proteins with the exception of the protein that is causative for MJD, ataxin-3, in which this feature is located close to the C-terminus. Additionally, while the remaining polyglutamine diseases display a gradual increase in repeats from healthy to affected individuals, a gap in repeat length between these two populations seems to be characteristic for ataxin-3 and MJD. Wild-type encoding repeats in ataxin-3 range between 13 and 36 residues, while affected individuals carry repeats longer than 60 polyglutamine residues (Shao and Diamond, 2007).

Polyglutamine-expanded ataxin-3 is primarily found in intranuclear inclusions in neurons of the affected brain regions of MJD patients. In line with observations made in most proteinopathies, the molecular chaperones HspA1 (Hsp70), DnaJB1 (Hsp40) and HspA8 (Hsc70) are found in these inclusions (Chai et al., 1999a; Seidel et al., 2012), together with components of the 19S particle of the proteasome, while the 20S proteasome was only found

to be sequestered in a subset of these (Schmidt et al., 2002). In contrast, other studies reported a subcellular redistribution of the 26S proteasome into ataxin-3 inclusions in affected neurons (Chai et al., 1999b). Further indicating a general perturbation of the cellular protein quality control pathways, neurons and fibroblasts derived from MJD patients display signs of impaired autophagy, as evidenced by altered expression and localization of autophagy proteins (Onofre et al., 2016). While nuclear deposits, and therefore decreased soluble protein levels, of both beclin-1 and mTOR have been reported, increased cytoplasmic accumulation of Atg5, Atg12 and Atg16 were observed (Nascimento-Ferreira et al., 2011). Similarly, accumulation of autophagic vesicles in post-mortem brain material from MJD patients, accompanied by increased levels of lipidated LC3 suggest impairment of autophagy (Sittler et al., 2018). Recruitment of the autophagy receptor SQSTM1/p62 to inclusions of mutant ataxin-3 further suggests attempted clearance of the aggregates (Mori et al., 2012; Seidel et al., 2010). Accordingly, stimulation of autophagy alleviates MJD phenotypes in animal models and patient-derived induced pluripotent stem cells (Cunha-Santos et al., 2016; Nascimento-Ferreira et al., 2013; Nascimento-Ferreira et al., 2011; Ou et al., 2016).

Collectively, these observations suggest that a general impairment of the cellular pathways for protein degradation is likely one of the mechanisms that contribute to MJD pathology (Nóbrega et al., 2018). Interestingly, effects of polyglutamine-expanded ataxin-3 are not limited to protein quality control. Similar to other proteinopathies, dysregulated transcription as well as accumulation of DNA damage has been reported in both, MJD patient-derived cells and mouse models of the disease (Chou et al., 2008; Gao et al., 2015). It remains, however, to be elucidated whether these effects on protein quality control and genome maintenance are a consequence of the toxic aggregation of polyglutamine-expanded ataxin-3 or represent the loss of native functions of ataxin-3.

1.6.1 Ataxin-3

Ataxin-3 is a ubiquitously expressed DUB that localizes in the nucleus and cytosol and is the founding member of the Josephin-domain family of DUBs (Scheel et al., 2003; Trottier et al., 1998). The catalytic activity of the N-terminal Josephin domain of ataxin-3 is conferred by the catalytic triad of histidine 119 (H119), asparagine 134 (N134) and the catalytic cysteine at position 14 (C14) (**Figure 6**) (Nicastro et al., 2005; Tzvetkov and Breuer, 2007). Ataxin-3 preferentially cleaves polyubiquitin chains consisting of four or more ubiquitin moieties and exhibits a preference for K63-linked ubiquitin chains over K48-linkage. Ataxin-3 has also

been implicated in editing of mixed chains, modulating the composition of chains with mixed linkages, and altering the fate of the modified substrate proteins (Winborn et al., 2008).

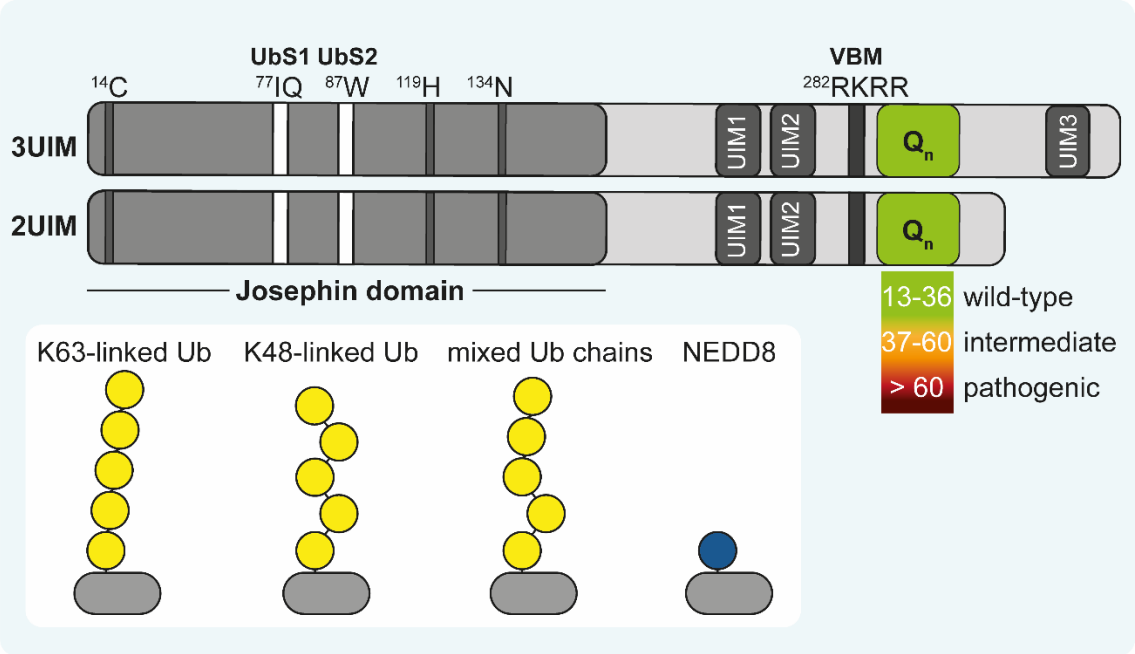

Figure 6. Schematic illustration of the 3UIM and 2UIM splice isoforms of ataxin-3 and the ubiquitin- and ubiquitin-like modifications it targets. The N-terminus of ataxin-3 consists of its catalytic Josephin domain, with the catalytic triad formed by the catalytic cysteine at position 14 as well as histidine 119 and asparagine 134. In addition, two ubiquitin-binding sites (UbS1, UbS2) are located in the Josephin domain. While UbS1 is required for catalytic activity, UbS2 mediates interaction with Rad23 and prevents proteasomal degradation of ataxin-3. The unstructured C-terminus contains the polymorphic polyglutamine tract, which, when expanded over a pathogenic threshold, is causative for the neurodegenerative disorder Machado-Joseph disease (MJD). In addition to the polyglutamine tract, the C-terminal part contains a VCP-binding motif (VBM) as well as ubiquitin-interacting motifs (UIMs). Both isoforms contain tandem UIMs prior to the polyglutamine tract. The 3UIM isoform contains a third UIM at the C-terminus, while alternative splicing of the 2UIM isoform results in a protein with a C-terminal hydrophobic stretch. This isoform displays similar catalytic activity but a higher propensity to aggregate. Ataxin-3 cleaves both K63-linked and K48-linked ubiquitin chains as well as mixed chains and primarily targets chains longer than four ubiquitin moieties. Additionally, ataxin-3 has been reported to possess deNEDDylase activity.

In contrast to its globular Josephin domain, the C-terminus of ataxin-3 is largely unstructured and contains three UIMs, that allow binding to polyubiquitin chains. Located between UIM2 and UIM3 is the polyglutamine tract, which, when expanded over a pathological threshold is causative for MJD. An alternative splice isoform, that instead gives rise to a protein with a C-terminal hydrophobic stretch downstream of the polyglutamine tract and only possesses two UIMs, has been reported. These two splice variants are typically termed 3UIM and 2UIM isoform but are both considered full-length ataxin-3 proteins (Goto et al., 1997). However, differential expression patterns, with the 3UIM being the predominant isoform expressed in the brain, as well as different aggregation properties have been assigned to the isoforms (Harris et al., 2010). Ataxin-3 2UIM was shown to have a higher propensity to aggregate due

to the fact that, in an oligomeric state, the hydrophobic C-termini of several ataxin-3 proteins associate with one another, thus driving a local increase in polyglutamine concentration (Harris et al., 2010; Weishäupl et al., 2019). Several other isoforms, including N-terminal splice variants, have been identified, some of which were exclusively detected in samples of MJD patients (Harris et al., 2010).

The first two tandem UIMs of ataxin-3 facilitate binding to K48-linked ubiquitin chains, while the third UIM domain has been reported to allow selectivity for K63-linked ubiquitin chains (Sims and Cohen, 2009). In addition to UIMs, ataxin-3 also contains two ubiquitin-binding sites (UbS) located in the Josephin domain. While UbS1 that is comprised of isoleucine 77 (I77) and glutamine 78 (Q78) and located in proximity to the catalytic triad in ataxin-3, is required for catalytic activity, likely by positioning of ubiquitin in proximity to the active site, UbS2 was shown to mediate interaction with the UBL domain of Rad23, a ubiquitin shuttling factor that mediates delivery of ubiquitylated substrates to the proteasome (Blount et al., 2014; Nicastro et al., 2010). The interaction with Rad23 has been reported to prevent proteasomal degradation of ataxin-3. Accordingly, mutation of this site results in decreased levels of the protein, which can be prevented by inhibition of the proteasome (Blount et al., 2014). The C-terminus of ataxin-3 additionally contains a VCP-binding motif (VBM) that mediates interaction with the ubiquitin-dependent segregase VCP/p97 (Boeddrich et al., 2006).

A particularly interesting feature of ataxin-3 is that it not only interacts with the ubiquitin-like modifier NEDD8 but also possesses deNEDDylase activity *in vitro*. While it is unclear which domain or motif in ataxin-3 mediates this interaction, its UIM motifs were found to be dispensable for this activity (Ferro et al., 2007).

Given its direct link to neurodegeneration, a lot of the work on ataxin-3 and its function has been focusing on the polyglutamine-expanded mutant ataxin-3, while the physiological role of wild-type ataxin-3 only gained interest in recent years. Ataxin-3 is localized in the nucleus and cytoplasm of mammalian cells and several functions in maintaining cellular protein homeostasis as well as transcriptional regulation and genome integrity have been described (do Carmo Costa and Paulson, 2012; Trottier et al., 1998). Interestingly, many of these pathways have been reported to be defective or dysregulated in MJD, supporting a potential contribution of loss-of-function mechanisms of the wild-type protein, in addition to the gain-of-function of the mutant protein, in the disease etiology (do Carmo Costa and Paulson, 2012). A particular intriguing finding in this context is that wild-type ataxin-3 was found to be neuroprotective and alleviates the phenotypes caused by several polyglutamine proteins,

including its mutant counterpart in a *Drosophila melanogaster* model of MJD, underlining the importance of understanding the functions of wild-type ataxin-3 in order to fully understand the etiology of MJD and develop adequate therapeutic approaches (Warrick et al., 2005).

Regulation of protein quality control and proteostasis by ataxin-3

In accordance with the important signaling function of ubiquitin in protein quality control and degradation, the cellular function of ataxin-3 as a DUB has also been linked to the UPS and protein quality control pathways. Examination of pontine neurons from MJD patients showed that in addition to ubiquitin, components of the 20S core particle and more so components of the regulatory 19S particle of the proteasome localized in nuclear inclusions of mutant ataxin-3 (Schmidt et al., 2002). Additionally, several molecular chaperones have been found in inclusions formed by polyglutamine-expanded ataxin-3 (Chai et al., 1999a; Seidel et al., 2012; Zijlstra et al., 2010). Ataxin-3 furthermore interacts with components of the UPS and was therefore suggested to play a role in degradation of ubiquitylated cargo by the proteasome, likely through editing of the attached polyubiquitin chains and cooperation with Rad23 (Doss-Pepe et al., 2003). Several studies have also revealed the involvement of ataxin-3 in VCP/p97-mediated extraction of ER-resident proteins during ERAD, a quality control pathway that targets misfolded proteins of the ER for proteasomal degradation (Wang et al., 2006; Zhong and Pittman, 2006).

In addition, ataxin-3 has been reported to play a role in the targeting and sequestration of misfolded, aggregation prone proteins to inclusions bodies and aggresomes to counteract aggregate-induced cytotoxicity, a cellular response that has frequently been linked to K63-linked ubiquitin chains (Ouyang et al., 2012). The mechanism by which ataxin-3 promotes sequestration of aggregation-prone proteins in aggresomes has been suggested to be the generation of unanchored K63-linked ubiquitin chains, the free C-termini of which, are subsequently recognized by the histone deacteylase HDAC6, that facilitates transport to the aggresomes (Ouyang et al., 2012). Interestingly, wild-type ataxin-3 was reported to suppress polyglutamine-induced toxicity, including that of its own mutant counterpart. This process was also suggested to be mediated by modification of protein aggregation rather than a degradation-dependent mechanism and was dependent on the catalytic activity of ataxin-3 (Tsou et al., 2013; Warrick et al., 2005).

Further underlining this role in protein quality control is that ataxin-3 interacts with the autophagy regulator beclin-1, a key component of the PI3K complex that is required for autophagy initiation (Ashkenazi et al., 2017). Interaction of ataxin-3 with beclin-1 is mediated by the polyglutamine tract in ataxin-3 and results in beclin-1 deubiquitylation and stabilization. This interaction was reported to be outcompeted by expanded polyglutamine tracts, causing destabilization of beclin-1 by proteasomal degradation and thereby preventing autophagy initiation (Ashkenazi et al., 2017). These data may provide a potential explanation for the derailed autophagy observed in several polyglutamine disorders.

Similar to other DUBs, association of ataxin-3 with several E3 ligases, counteracting substrate ubiquitylation or modifying the ubiquitylation state of the ligases themselves, has been reported (Durcan and Fon, 2013). One example of this is the regulation of the E3 ligase CHIP by ataxin-3. CHIP interacts with molecular chaperones to promote the ubiquitylation and subsequent degradation of misfolded proteins by the proteasome. As such, CHIP was found to exert neuroprotective functions in several neurodegenerative disorders, including polyglutamine disorders, and was reported to ubiquitylate pathogenic, polyglutamine-expanded ataxin-3, targeting it for degradation (Jana et al., 2005). Additionally, also wild-type ataxin-3 is ubiquitylated by CHIP, resulting in its enhanced DUB activity (Todi et al., 2010). Vice versa, ataxin-3 itself is able to deubiquitylate CHIP, affecting its activity rather than its steady-state levels. The E2 enzyme Ube2w catalyses CHIP auto-ubiquitylation at K2, which has been shown to enhance its ligase activity and further stabilize its interaction with ataxin-3. Association of ataxin-3 with CHIP, prevents excessive CHIP-mediated ubiquitylation of substrates by monitoring and limiting the ubiquitin chain length. Substrate ubiquitylation *in vitro* is further controlled by ataxin-3-mediated deubiquitylation of the ligase itself, resulting in a termination of the ubiquitylation reaction (Scaglione et al., 2011). As ataxin-3 has been shown to preferentially trim ubiquitin chains longer than four moieties, this step is particularly intriguing and was stimulated by the presence of a ubiquitylated substrate. These findings also bear importance for MJD, as polyglutamine-expanded ataxin-3 was reported to show dramatically increased affinity for CHIP, while its deubiquitylating activity was unaltered, and CHIP levels were found to be decreased a transgenic mouse model of MJD (Scaglione et al., 2011).

Similarly, ataxin-3 has been shown to interact with the E3 ligase Parkin, which has been implicated in the degradation of defective mitochondria as well as delivery of misfolded proteins to the aggresome (Chin et al., 2010). The interaction of ataxin-3 with Parkin is mediated by UIM2 and UIM3 in ataxin-3 and the UBL-domain of Parkin. While Parkin is

unable to ubiquitylate ataxin-3, both wild-type and pathogenic ataxin-3 were shown to counteract Parkin autoubiquitylation. Interestingly, polyglutamine-expanded ataxin-3 was reported to prevent autoubiquitylation of Parkin more efficiently, inducing its clearance by autophagy and therefore, an increased ability of this mutant to process K27- and K29-linked ubiquitin has been suggested (Durcan et al., 2012; Durcan et al., 2011). By association of ataxin-3 with both Parkin and the E2 enzyme simultaneously, ataxin-3 transfers ubiquitin from the E2 directly onto itself, rather than removing already existing ubiquitin modifications from Parkin (Durcan et al., 2012). Similar E3-like activity has been described for other DUBs (Liu et al., 2002; Wertz et al., 2004). These findings further strengthen a role of native ataxin-3 in protein quality control and disturbance of these homeostatic mechanisms may therefore be involved in the pathogenesis of MJD.

Interestingly, ataxin-3 activity is stimulated by ubiquitylation of the Josephin domain at K117 and impairment of cellular protein quality control pathways or proteotoxic stress have been shown to positively regulate this modification of ataxin-3, corroborating the important function of native ataxin-3 in these pathways (Todi et al., 2009; Tsou et al., 2013).

Ataxin-3 in transcriptional regulation and genome integrity

In addition to these primarily cytosolic functions of ataxin-3 in protein quality control and mechanisms counteracting proteotoxic insults, the nuclear pool of ataxin-3 has been implicated in several nuclear processes such as transcriptional regulation and DNA repair and therefore genome integrity.

Transcriptome analysis of mouse embryonic fibroblasts lacking ataxin-3 showed distinct transcriptomic changes compared to wild-type ataxin-3 expressing cells, suggesting an involvement of ataxin-3 in transcriptional regulation (Zeng et al., 2018). Interestingly, several transcription factors become sequestered in aggregates of polyglutamine-expanded ataxin-3 and cells expressing mutant ataxin-3 also showed a markedly altered transcriptome (Evert et al., 2003). Further evidence suggests that ataxin-3 in fact acts as a transcriptional repressor. While ataxin-3 was found to bind to the transcriptional co-activators cAMP response element (CREB)-binding protein (CBP), p300 and the CBP/p300-associated factor PCAF, mediated by its C-terminal polyglutamine repeat, the N-terminal part of ataxin-3 is able to interact with histone, thereby blocking access and inhibiting the histone acetyl transferases (HATs), required for transcriptional activation (Li et al., 2002). Similarly, other neurodegeneration-associated polyglutamine proteins, including ataxin-7 and huntingtin, have been implicated in

transcriptional regulation (Benn et al., 2008; Gao et al., 2019; Ström et al., 2005). Additionally, opposing roles of mutant and wild-type ataxin-3 on transcriptional regulation of the matrix-metalloprotease-2 (MMP-2) gene have been described, wherein mutant ataxin-3 is unable to recruit HDAC3 and nuclear receptor co-repressor 1 (NCoR) and consequently fails to repress transcription of the MMP-2 gene (Evert et al., 2006). These data therefore suggest a role in the transcriptional regulation of specific genes in addition to general transcriptional repression.

Further, several studies have also implicated both wild-type and mutant ataxin-3 in the repair of DNA damage (Chatterjee et al., 2015; Gao et al., 2015). The polynucleotide kinase 3′-phosphatase (PNKP), a bifunctional enzyme possessing both 3’-phosphatase and 5’-kinase activity, is required for DNA end-processing in response to SSBs as well as DSBs, where it associates with the repair proteins XRCC1 and XRCC4, respectively. Wild-type ataxin-3 stimulates the phosphatase activity of this enzyme, while expression of polyglutamine-expanded ataxin-3 inhibits this activity. PNKP was further found to be sequestered in nuclear inclusions in MJD brain sections. Underscoring the differential regulation of PNKP-mediated DNA repair by mutant ataxin-3, its function was found to be decreased in a mouse model of MJD and accumulation of DNA damage was observed in brain sections of MJD patients (Chatterjee et al., 2015; Gao et al., 2015).

A native role of ataxin-3 in the regulation of DNA damage signaling and repair was further substantiated by the finding that ataxin-3 regulates the levels of the DNA damage-related E3 ligase RNF8, by preventing its VCP/p97-mediated proteasomal turnover under physiological conditions (Singh et al., 2019). In the context of DNA damage, on the other hand, ataxin-3 was reported to co-operate with VCP/p97 to mediate chromatin-extraction of RNF8, thereby fine-tuning the balance between different repair pathways. Accordingly, ataxin-3 depletion was found to result in NHEJ deficiency an increased cellular sensitivity to IR-induced DNA damage in this study (Singh et al., 2019).

A mass spectrometry approach to identify ataxin-3 interactors in the DDR additionally suggests a role for ataxin-3 in the regulation of DNA damage checkpoint signaling (Tu et al., 2017). The study identified Chk1 and DDB2 together with CUL4 and CUL1 as novel interactors of wild-type ataxin-3. The levels of Chk1 are primarily controlled by proteasomal turnover in response to polyubiquitylation by the two SKP1-CUL1-F-box protein (SCF)-E3 ubiquitin ligase complexes comprised of DDB1, CUL4A as well as CUL1 and FBXO6. Degradation of Chk1 consequently allows completion of DNA repair and termination of check point signaling. Ataxin-3 antagonizes the actions of these two E3 ligase complexes,

resulting in stabilization of Chk1 and promoting check point signaling to safeguard genomic integrity (Tu et al., 2017).

Interestingly, several proteins implicated in DNA repair are found in MJD inclusions, including Xeroderma pigmentosum group C-complementing protein (XPC) and Rad23, both of which are required for nucleotide excision repair (Yang et al., 2018). Combined, these data therefore demonstrate that ataxin-3 functions in a variety of nuclear processes involved in genome maintenance and integrity. Given the altered function of mutant ataxin-3 in several of these processes as well as sequestration of both ataxin-3 itself and other proteins involved in these pathways in inclusions, their global disturbance by the polyglutamine-expanded mutant ataxin-3 is therefore likely to be a contributing factor in MJD pathogenesis.

2 AIMS

The work presented in this thesis aimed at understanding the involvement of the DUB ataxin-3 and K63-linked ubiquitin chains in quality control pathways safeguarding the proteome and genome of cells, in particular in the regulation of autophagy and the DDR.

The specific aims addressed in the papers included in this thesis were as follows:

The aim of **paper I** was to explore and characterize the involvement of wild-type ataxin-3 in bulk autophagy in response to starvation.

Paper II aimed at engineering a tool that allows the inducible and targeted generation of K63-linked ubiquitin chains in order to understand whether these chains are sufficient to induce mitochondrial clustering.

Paper III focused on the identification and characterization of a functional involvement of ataxin-3 in DSB signaling and repair.

The aim of **paper IV** was to elucidate the mechanism by which ataxin-3 and the SUMO-targeted E3 ubiquitin ligase RNF4, both of which are recruited to DSBs in a SUMO-dependent manner, promote DNA repair despite their antagonizing functions.

3 RESULTS AND DISCUSSION

The studies included in this thesis aimed at understanding the involvement of ubiquitin-modifying enzymes in quality control pathways safeguarding the proteome and genome of cells. In particular, we examined the function of the DUB ataxin-3 in autophagy (**Paper I**) and developed a tool to study the signaling function of K63-linked ubiquitin chains in mitochondrial sequestration (**Paper II**). Additionally, we investigated the involvement of ataxin-3 in DSB signaling and repair (**Paper III & IV**).

3.1 PAPER I

The Machado–Joseph disease deubiquitylase ataxin-3 interacts with LC3C/GABARAP and promotes autophagy

The DUB ataxin-3 shows a preference for K63-linked ubiquitylation, which has been linked to autophagic degradation both in the context of substrate-targeting and regulation of autophagy proteins. Importantly, autophagy is severely dysregulated in cells derived from MJD patient. We therefore hypothesized that wild-type ataxin-3 may be involved in the regulation of autophagy.

A recent study utilizing the nematode *Caenorhabditis elegans* furthermore identified that loss of the ataxin-3 ortholog, ATX-3, together with one of the VCP/p97 orthologues, CDC48.1, results in a longevity phenotype (Kuhlbrodt et al., 2011). Interestingly, life-span expansion has frequently been linked to autophagic activity (Madeo et al., 2015). In **paper I**, we therefore investigated the functionality of autophagy in the absence of ataxin-3 in both, *C. elegans* and mammalian cells together with our collaborators. To induce autophagy, we utilized nutrient starvation, a condition that yields rapid and robust mTOR-regulated autophagy activation to promote nutrient recycling and ensure survival (Jung et al., 2010). Surprisingly, we found that the long-lived deletion strain *cdc48.1; atx-3* showed increased sensitivity to starvation, which we could attribute to the loss of ataxin-3, not *cdc-48.1*. We demonstrated that curtailing autophagy by RNA-interference (RNAi)-mediated downregulation of the autophagy regulators BEC-1 and UNC-51, the *C. elegans* homologs of beclin-1 and ULK1, respectively, in the *atx-3* deletion strain further sensitized the larvae, thus corroborating a defect in the autophagy pathway.

Another commonly studied and highly relevant pathway relying on functional autophagy is the degradation of misfolded and aggregation-prone proteins, termed aggrephagy (Lamark and Johansen, 2012). We observed that *C. elegans* lacking ATX-3 that were challenged by

the body-wall muscle cell-specific expression of aggregation-prone polyglutamine protein encompassing 40 glutamine residues accumulated increased amounts of insoluble protein and showed significantly reduced motility, thus strengthening the importance of ataxin-3 for functional autophagy.

Similar to *C. elegans*, we found that depletion of ataxin-3 in human cells resulted in aberrant autophagy, illustrated by an increased accumulation of autophagic vesicles. This increase in vesicles was likely due to increased formation of autophagosomes, as it was primarily detected upon inhibition of lysosomal degradation. This was corroborated by a similar accumulation of LC3-positive vesicles in mouse embryonic fibroblasts homozygous for ataxin-3 deletion, while we did not detect differences in the expression of key autophagy proteins. This contrasts with a recent study that showed that ataxin-3 depletion for extended time periods destabilizes beclin-1 resulting in increased proteasomal turnover (Ashkenazi et al., 2017). In this earlier study, interaction with beclin-1 was shown to be mediated by the polyglutamine tract in ataxin-3. While both this finding as well as ours argue for a stimulatory role of ataxin-3 in autophagy, we did not see any effects of ataxin-3 depletion on beclin-1 levels. However, we depleted ataxin-3 for considerably shorter time periods in our study and we can therefore not rule out that long-term depletion of ataxin-3 results in additional or compensatory mechanisms that may lead to a reduction in beclin-1. It is further noteworthy that the *C. elegans* homolog of atx-3 lacks the polyglutamine tract, suggesting that the observed effects are the result of a polyglutamine repeat-independent function, while they do not exclude additional functions of ataxin-3 in the autophagy pathway.

Interestingly, while many neurodegeneration-associated proteins are subject to autophagic degradation in their pathogenic state, some of these proteins have, in their native state, been reported to play regulatory roles in this pathway. One such example is huntingtin, the polyglutamine protein that is causative for HD, which interacts with the autophagy receptor SQSTM1/p62 to enhance tethering of ubiquitylated cargo to autophagosomes during selective types of autophagy. Huntingtin was further shown to interact with ULK1 and therefore proposed to enhance selective autophagy by increasing proximity between cargo and autophagy initiating complexes (Rui et al., 2015). Given the significance of ataxin-3 in functional autophagy and the reported effects of polyglutamine ataxin-3 in this pathway, these data may collectively suggest that MJD pathology is evoked not solely by the formation of toxic protein aggregates but also, at least in parts, by the loss of its wild-type function.

In addition to ubiquitin, ataxin-3 promiscuously interacts with other ubiquitin-like proteins such as SUMO1 and NEDD8, and, *in vitro*, furthermore possesses deNEDDylase activity

(Ferro et al., 2007; Pfeiffer et al., 2017). Adding to this, we found that ataxin-3 interacts with the ubiquitin-like autophagy proteins LC3C and GABARAP and identified two conserved LIR motifs, termed LIR1 and LIR2, in the Josephin domain of ataxin-3 that may mediate this interaction. As members of the Atg8-family, LC3C and GABARAP are required for functional autophagy and play key roles in autophagosome formation, fusion with lysosomes and substrate tethering. However, structural analysis and mutation of the critical residues in the two LIRs revealed that alterations in these motifs likely disturb folding of the Josephin domain and the contribution of these LIRs to autophagy regulation therefore remains elusive and requires additional work. Similar to this, the physiological relevance of ataxin-3 interaction with NEDD8 also remains to be explored (Ferro et al., 2007). In the context of DNA double-strand break signaling and repair, we showed that interaction of ataxin-3 with SUMO is required for its recruitment to DNA double-strand breaks where it deubiquitylates and thereby stabilizes substrates of the SUMO-targeted ubiquitin ligase RNF4 to promote efficient DNA damage signaling (**paper III**) (Pfeiffer et al., 2017). It is tempting to speculate that the interaction of ataxin-3 with LC3C/GABARAP may similarly be required for spatial and/or temporal regulation of the actions of ataxin-3 in the autophagy pathway. Interestingly, we noticed that LIR1 shares a critical residue with UbS1 and binding of LC3C may thus prevent or interfere with positioning and cleavage of ubiquitin. Structural analysis of LIR2, on the other hand, revealed that exposure of this motif may require dramatic conformational changes in the Josephin domain and may therefore be less likely to contribute to LC3C/GABARAP-binding.

Furthermore, the substrate(s) of ataxin-3 in the autophagy pathway, mediating the observed cellular effects remains to be identified. Surprisingly, the increased formation of autophagosomes in the absence of ataxin-3 was accompanied by significantly decreased autophagic degradation of long-lived proteins. Together with the observation that the protein levels of all analyzed autophagy proteins remained unchanged upon depletion of ataxin-3 we therefore speculate that ataxin-3 depletion results in a dilution of the available pool of LC3 (and potentially other autophagy proteins) over an increased amount of autophagic structures, thereby hindering efficient autophagy and restricting the degradative capacity. This hypothesis further opens up the possibility that ataxin-3 acts at the cross-roads of cargo ubiquitylation and autophagosome initiation and suggests that its substrate(s) may be ubiquitylated cargo or autophagy receptors interacting with the cargo. However, additional experiments will be required to investigate this and identify the substrate of ataxin-3.

Of particular interest is also that LC3s and GABARAPs execute specific functions in the autophagy pathway and display only partial redundancy, despite their structural similarity (Lee and Lee, 2016). LC3s but not GABARAPs were shown to be dispensable for bulk autophagy, while selective autophagy may require LC3s. GABARAPs furthermore play crucial roles in the fusion of autophagosomes with lysosomes (Szalai et al., 2015). LC3C, on the other hand, is a particularly interesting member of the Atg8 family as not only specific LC3C-LIR motifs (CLIRs) have been identified but also in regard to its unique role in the clearance of invading *Salmonella* by xenophagy (von Muhlinen et al., 2012). It is therefore also tempting to speculate that the differential interaction of the LIRs identified in ataxin-3 with LC3C and GABARAP may suggest that the actions of ataxin-3 are not limited to a single stage in the autophagy pathway and may furthermore extend to selective types, such as xenophagy.

3.2 PAPER II

An inducible, engineered K63-ubiquitin ligase mimics Parkin-mediated sequestration of mitochondria in the absence of mitochondrial damage

While K63-linked ubiquitin chains do not seem to target for proteasomal degradation in cells, their function in promoting protein-protein interactions in DNA repair and NFκB-signaling has been studies extensively (Chen and Chen, 2013; Spence et al., 1995). Furthermore, a role for K63-linked ubiquitin in inclusion body formation and selective autophagy has been identified (Tan et al., 2008). The most well-studied pathway for selective, ubiquitin-dependent autophagy is PINK1/Parkin-mediated mitophagy in response to mitochondrial uncoupling (Jin and Youle, 2012). The E3 ligase Parkin is able to conjugate different types of ubiquitin chains, including K6-, K11-, K48- and K63-linked chains and ubiquitylates a large number of mitochondrial proteins (Chan et al., 2011). Prior to degradation, this results in perinuclear sequestration of the ubiquitylated mitochondria, a process that is mediated by the autophagy receptor SQSTM1/p62 (Geisler et al., 2010a; Narendra et al., 2010a). However, given the complex nature of the ubiquitin signals generated on mitochondria by Parkin it is currently unclear whether K63-linked ubiquitin chains are sufficient to initiate mitochondrial sequestration.

In **paper II**, we therefore generated a system to study the specific effect of K63-linked ubiquitylation on substrate proteins by engineering a K63-specific ubiquitin ligase based on the HECT-domain of NEDD4, which we termed proximity-induced E3 ubiquitin ligase

(ProxE3). In addition, we engineered a reference substrate based on EGFP (GFP-Sub), that allows inducible dimerization with ProxE3, thereby creating a system that can be temporally controlled. The substrate also allows spatial regulation by targeting to different subcellular localizations. We confirmed the specific and inducible generation of K63-linked ubiquitin chains on the substrate both *in vitro* and in cells. Furthermore, we detected rapid K63-linked autoubiquitylation of the ligase *in vitro*. Surprisingly, in cells, the active ligase was additionally modified with K48-linked ubiquitin chains, resulting in its rapid proteasomal turnover. As this was dependent on the catalytic activity of ProxE3 and not observed when cells were transfected with a catalytically inactive version of this ligase, we cannot exclude that this is autoubiquitylation. However, given the specificity of ProxE3 for K63-linked ubiquitylation and its inability to synthesize K48-linked ubiquitin chains *in vitro*, these results may suggest that other cellular ligases target autoubiquitylated ProxE3. Although speculative, the K63-linked ubiquitin chains on ProxE3 may serve as a recognition signal for a K48-specific ubiquitin ligase, similar to what has been described for SUMO-modified proteins that are subsequently targeted by STUbLs (Sriramachandran and Dohmen, 2014).

To address the question whether K63-linked ubiquitin chains are sufficient to induce mitochondrial clustering, we targeted the reference substrate to the mitochondrial membrane (mitoGFP-Sub) and compared the effect of the expression of ProxE3 to Parkin. Upon dimerization of the ligase with its substrate mitoGFP-Sub, we detected profound K63-linked ubiquitylation on the mitochondria, similar to the effect of Parkin upon treatment with the mitochondrial uncoupler CCCP. In contrast, we only detected marginal K48-signal on the mitochondria in cells expressing ProxE3 while Parkin induced robust K48-linked ubiquitylation. We also noticed dramatic changes in the mitochondrial morphology and localization, with a strong perinuclear clustering of mitoGFP-Sub-positive mitochondria, mimicking the effect of Parkin. In Parkin-mediated mitophagy, this phenotype has been reported to be dependent on the ubiquitin-binding autophagy-receptor SQSTM1/p62 (Narendra et al., 2010a). In line with this, we found that ProxE3-induced K63-linked ubiquitylation of mitoGFP-Sub recruits SQSTM1/p62. Collectively, these data demonstrate that specific K63-linked polyubiquitylation of a mitochondria-localized protein suffices to induce mitochondrial clustering even in the absence of mitochondrial damage. Further, while it would require additional work to confirm this, it is tempting to speculate that the clustering is mediated by SQSTM1/p62.

In a previous study, the effects of tethering a version of ubiquitin that cannot be cleaved by DUBs to the MOM was analyzed, as this has been described to be the minimal signal

sufficient for targeting peroxisomes for autophagy (Kim et al., 2008a; Narendra et al., 2010a). However, despite CCCP-induced mitochondrial damage, clustering of the mitochondria was only observed in a subset of cells and it did not suffice to induce degradation (Narendra et al., 2010a). These data suggest that monoubiquitin, even in combination with mitochondrial damage, is not sufficient to trigger the changes in mitochondrial morphology and distribution that we observed. Instead, it suggests that it is the specific modification of mitochondria with K63-linked ubiquitin chains that is required to induce mitochondrial sequestration and that is sufficient to induce clustering even in the absence of damage. This is also in line also the selectivity of SQSTM1/p62 towards this chain type and preferred binding to chains longer than two ubiquitin moieties (Seibenhener et al., 2004). In another recent study, the use of autophagy-targeting chimera (AUTACs) to induce mitochondrial clearance was tested. AUTACs use a chemical group mimicking S-guanylation, a modification shown to induce K63-linked ubiquitylation during xenophagy, to selectively target substrates for autophagy (Arimoto and Takahashi, 2017). While these probes induced K63-linked ubiquitylation of mitochondria, their localization as well as turnover was unaffected and mitophagy required additional fragmentation of the mitochondria. In contrast, degradation was only observed upon additional fragmentation of mitochondria by genetic alterations in the fission machinery (Takahashi et al., 2019), underlining the complexity of the cellular response triggered by Parkin in response to mitochondrial dysfunction.

Interestingly, K63-linked ubiquitin chains have also been linked to neurodegeneration and are commonly found in insoluble protein inclusions. Several studies have since implicated K63-linked ubiquitin in the formation of these inclusion bodies (Olzmann et al., 2007; Ouyang et al., 2012; Tan et al., 2008). Additionally, SQSTM1/p62 is frequently found in inclusions and was also shown to be involved in inclusion body formation and clearance (Bjørkøy et al., 2005; Zatloukal et al., 2002). The SQSTM1/p62-mediated clustering of ubiquitylated mitochondria is reminiscent of this process. However, it has been debated whether ubiquitylation or protein oligomerization is the driving force of these processes (Bersuker et al., 2016). The data presented in **paper II** suggest that it may indeed be primarily the K63-linked ubiquitin signal that is the initial trigger for this phenotype.

While additional work will be required to determine whether K63-linked ubiquitylation alone is also sufficient to induce the subsequent clearance of the labeled mitochondrial by mitophagy as well as the significance of SQSTM1/p62-mediated clustering for this process, the system developed in this study provides a tool to address these questions. Using the isolated HECT-domain of NEDD4 rather than the full-length ligase, this system circumvents

the need for an E2 enzyme for ubiquitin-linkage specificity (Metzger et al., 2012). Additionally, this likely prevents ubiquitylation of endogenous substrates of the ligase. Similarly, the EGFP-based substrate lacks an endogenous function. Together, these engineered proteins constitute a system that can be controlled both temporally and spatially.

3.3 PAPER III

Ataxin-3 consolidates the MDC1-dependent DNA double-strand break response by counteracting the SUMO-targeted ubiquitin ligase RNF4

The aim of **paper III** was to investigate a potential involvement of ataxin-3 in the cellular response to DSBs. VCP/p97, a known interactor of ataxin-3 in other cellular contexts, has been shown to be involved in chromatin extraction of DNA repair proteins (Acs et al., 2011). We therefore analyzed the recruitment of ataxin-3 to DSBs and effects of ataxin-3 depletion on DNA damage signaling in response to DSBs.

We found that ataxin-3 is rapidly recruited to sites of DNA damage using both micro-irradiation and the inducible endonuclease FokI to inflict DSBs. Surprisingly, ataxin-3 recruitment was independent of its ability to interact with VCP/p97, suggesting a VCP/p97-independent role, and further did not require its catalytic activity or UIMs. As UBDs have been shown to be enriched in DNA repair proteins and required for the recruitment of many of these proteins, including other ubiquitin-modifying enzymes, such as RNF168, to sites of DNA damage (Doil et al., 2009; Panier et al., 2012), this was particularly surprising. Instead, we observed that ataxin-3 recruitment was dependent on the presence of SUMOylation at DSBs and were able to demonstrate binding of a previously predicted SIM in the C-terminus of the Josephin domain (Guzzo and Matunis, 2013) to SUMO1 *in vitro*. Accordingly, inhibition of damage-induced SUMOylation by depletion of the SUMO E2 enzyme Ubc9 abrogated ataxin-3 recruitment to DSBs.

Based on the function of ataxin-3 as a DUB, we therefore hypothesized that ataxin-3 may act on targets that are modified with both SUMO and ubiquitin chains. In the context of the DDR, these two modifications are functionally connected by the STUbL RNF4 that was reported to ubiquitylate SUMOylated MDC1 and RPA resulting in their extraction from chromatin (Galanty et al., 2012; Luo et al., 2012). Indeed, we found that ataxin-3 antagonizes RNF4-mediated MDC1 ubiquitylation. While depletion of RNF4 resulted in decreased ubiquitylation of RNF4, depletion of ataxin-3 increased MDC1 ubiquitylation and thereby decreased the retention time of MDC1 at DSBs. Both these phenotypes were reverted by

simultaneous depletion of RNF4, further underlining the antagonizing actions of these two enzymes. Additionally, the effect of ataxin-3 depletion on MDC1 ubiquitylation was rescued by overexpression of wild-type but not catalytically inactive ataxin-3, indicating that this effect is indeed mediated by the deubiquitylating activity of ataxin-3, and suggesting that MDC1 is a substrate of ataxin-3. In addition to MDC1, ataxin-3 depletion also impaired recruitment of XRCC4, a component of the NHEJ pathway to DSBs. This effect was partially rescued by expression of wild-type but not catalytically inactive ataxin-3. Furthermore, simultaneous RNF4-depletion largely prevented the reduced recruitment of XRCC4 in the absence of ataxin-3.

We consider it likely, that ataxin-3 and RNF4 share additional substrates at DSBs. One obvious candidate for this is RPA (Galanty et al., 2012). Depletion of ataxin-3 resulted in decreased steady-state levels of RPA at lesions, but further work is required to identify whether this is also due to ataxin-3 mediated deubiquitylation and rescue of RPA from RNF4-mediated removal.

In response to DSBs, MDC1 binds phosphorylated H2AX and is subsequently phosphorylated itself by ATM (Pan et al., 2011; Stucki et al., 2005). This modification of MDC1 serves as the recruitment signal for the E3 ligase RNF8, which in turn recruits RNF168 to induce the ubiquitin-dependent recruitment of downstream repair factors (Huen et al., 2007). In accordance with the important function of MDC1 in the response to DSBs, creating a recruitment platform for crucial signaling proteins, we found that ataxin-3 depletion resulted in decreased recruitment of RNF8 and RNF168 and, consequently, significantly decreased ubiquitylation at sites of DNA damage. We further observed significantly reduced recruitment of the repair factors 53BP1 and BRCA1. In line with this, we showed, using two commonly employed reporter cells lines to test the repair capacity of NHEJ and HR, respectively, that ataxin-3 depleted cells display significantly reduced ability to repair DSBs by either of these pathways.

3.4 PAPER IV

Poly(ADP-ribos)ylation limits SUMO-dependent ataxin-3 recruitment to DNA double-strand breaks to the early phase of the DNA damage response

In **paper III**, we reported that ataxin-3 counteracts the STUbl RNF4 to promote efficient DSB signaling and repair. Interestingly, it was reported that RNF4 is also required for

functional DNA repair as its depletion results in impaired replacement of RPA by BRCA2 and RAD51 and therefore persistent γH2AX signal (Galanty et al., 2012).

The question that we addressed in **paper IV** was therefore how both ataxin-3 and RNF4 can promote efficient DNA repair despite their opposing enzymatic actions and despite being recruited by the same PTM. While the opposing actions of E3 ubiquitin ligases and DUBs to fine-tune the DDR is not an uncommon phenomenon, it is surprising and, at first sight, counterintuitive, that two enzymes with antagonizing functions both promote the DDR and, as a result, DNA repair (Kee and Huang, 2016). Despite being recruited by the same PTM, in the case of ataxin-3 and RNF4, a potential explanation for this may be additional mechanisms that define the different time-windows during which each of these enzymes act. While ataxin-3 opposing RNF4 at early time points after DNA damage infliction may be beneficial for appropriate induction of the DDR and ultimately efficient DNA repair, RNF4-mediated removal of proteins acting early in the DDR may be necessary for recruitment of repair factors, as has been shown for the displacement of RPA by BRCA2 (Galanty et al., 2012).

We therefore investigated whether additional signals or PTMs regulate the rapid and SUMO-dependent recruitment of ataxin-3 to DSBs to understand how both ataxin-3 and RNF4 can promote efficient DNA repair despite their opposing activities on shared substrates. Analysis of the kinetics of ataxin-3 recruitment to DSBs following damage infliction revealed that ataxin-3 recruitment was very transient, reminiscent of the accumulation of PARylation at sites of DNA damage.

The primary enzyme catalyzing PARylation in response to DNA damage is PARP1. PARP1 senses DSBs resulting in conformational changes of the enzyme and results robust autoPARylation as well as PARylation of several histones, including the linker histone H1 (Liu et al., 2017). Owing to its negative charge, the accumulation of PAR chains on H1 has been suggested induce chromatin relaxation (Poirier et al., 1982). Additionally, several effector proteins involved in the very early steps of DSB signaling, such as the chromatin remodeler ALC1, are recruited by interaction with PAR-polymers, thus resulting in active remodeling of the chromatin surrounding the lesion (Gottschalk et al., 2009). Subsequently, PAR polymers are rapidly disassembled by the PAR glycohydrolase PARG, making PAR a highly transient PTM at DSBs.

Indeed, we found that the recruitment of ataxin-3 to DSBs was dependent on PARylation as it was suppressed when cells were treated with PARP inhibitor, similar to the recruitment of ALC1. Many of the proteins that are recruited to breaks at these early time points in a

PARylation-dependent manner, possess PAR-binding capacity (Teloni and Altmeyer, 2016). In contrast, we found that ataxin-3 was unable to directly interact with PAR polymers *in vitro* and consider it therefore unlikely that ataxin-3 is recruited to lesions by interaction with PAR-chains. This was further corroborated by the finding that while PARP inhibition immediately after damage infliction resulted in the rapid disappearance of PAR signal in less than 5 minutes, ataxin-3 recruitment persisted for approximately 20 min after PARP inhibition and loss of PAR signal. Collectively, these data argue for an indirect mechanism mediating the PARylation-dependent recruitment of ataxin-3. It is possible that ataxin-3 requires initial PAR-mediated chromatin-relaxation in order to be recruited to DSBs, as has been proposed for the chromatin-remodelers CHD3 and CHD4 as well as for HP1. (Smith et al., 2019; Smith et al., 2018). One explanation for this may be that PARylation-mediated chromatin opening increases access of ataxin-3 to its SUMOylated substrates. In contrast to RNF4, which contains four SIM-motifs and may therefore display higher affinity for SUMOylated proteins, only a single SIM has been identified in ataxin-3. Particularly interesting in this context is furthermore that ataxin-3 has previously been shown to possess DNA-binding capacity and we can therefore not exclude that ataxin-3 may be directly binding to DNA, increasing proximity to its substrates.

We further demonstrated that damage-induced PARylation and SUMOylation are independent signals, as damage-induced PARylation was unaffected by knockdown of the SUMO-conjugating enzyme Ubc9, and vice versa, SUMO1 as well as SUMO2/3 accumulation at lesions was not affected by PARP inhibitor. Similarly, RNF4 recruitment was not dependent on damage-induced PARylation, indicating that these two independent PTMs provide the necessary means for the differential recruitment of ataxin-3 and RNF4. The dependency on two independent PTMs, whose simultaneous presence at DSBs is confined to the early phase of the DDR thereby defines the time window for the actions of ataxin-3 to promote efficient recruitment and retention of early DDR protein, such as MDC1. The SUMO-dependent RNF4 recruitment, on the other hand, persists for longer periods of time, ensuring the subsequent timely removal of DDR protein to allow recruitment of repair proteins and promote progression of the repair pathway. In line with this, we also found that ataxin-3 and PARylation epistatically regulate the accumulation of the repair protein XRCC4.

In addition to ataxin-3 counteracting RNF4-mediated ubiquitylation in the context of DSB signaling and repair, it was recently reported that ataxin-3 also regulates the E3 ligase RNF8 (Singh et al., 2019). Interestingly, differential regulation of RNF8 was shown to take place under physiological conditions and upon infliction of DNA damage. While ataxin-3 prevents

VCP/p97-mediated proteasomal degradation of RNF8 under basal conditions by deubiquitylation, it acts in complex with the ubiquitin-dependent segregase VCP/p97 during DSB repair to extract RNF8 from lesions and thereby balances the choice of repair pathway. In line with this, depletion of ataxin-3 was demonstrated to impair NHEJ. Interestingly, depletion of ataxin-3 in this study resulted in hyperaccumulation of RNF8 several hours after DNA damage infliction. However, while our data do not exclude additional functions of ataxin-3 in the DDR, we demonstrate that ataxin-3 is only very transiently recruited to DSBs and therefore consider it unlikely that this same pool of ataxin-3 mediates the regulation of RNF8. It is noteworthy, that the authors also observed an initial decrease in the recruitment of RNF168 during the first hour following damage infliction, which is in line with the observations we made in **paper III**. It may therefore be interesting to analyze whether the recruitment kinetics of VCP/p97 are also dependent on PARylation and whether ataxin-3 may be recruited to DSBs during a second, later wave by other signals.

4 METHODOLOGICAL CONSIDERATIONS

In the following, important considerations regarding some of the methods used in the studies included in this thesis will be highlighted.

Assessment of autophagy and autophagic flux using LC3

In **paper I**, we investigated the functional status of autophagy upon ataxin-3 depletion. LC3 is a commonly used marker for autophagic structures and frequently used to assess the functionality of the autophagy pathway. Both, immunolabelling of endogenous LC3 in cells and expression of fluorescently labelled LC3 are used to analyze the number of autophagic structures as well as autophagic flux. It is, however, noteworthy, that the number of LC3-labelled structures only gives limited information about autophagic flux, as LC3-positive vesicles increase in number both, in response to autophagy activation as well as upon block or defects in lysosomal degradation (Yoshii and Mizushima, 2017). Lysosomal inhibitors, such as the vacuolar H^+-ATPase (V-ATPase) inhibitor Bafilomycin A1, which prevents acidification of lysosomes and thereby inhibits fusion of autophagosomes with lysosome (Yamamoto et al., 1998), can be used to distinguish between induced and impaired autophagy. LC3-I is conjugated to PE in the autophagosome membrane, giving rise to the lipidated form LC3-II, a fraction of which is degraded in the lysosome together with autophagic cargo (Kabeya et al., 2000). These two forms migrate differently in SDS-PAGE gels, allowing analysis of autophagy by immunoblotting. Even in this approach it is advisable to include autophagy inhibitors to assess (Tanida et al., 2005).

Similarly, the autophagy receptor p62 can be used to assess autophagy by immunoblotting, as it is degraded during autophagy, e.g. in response to starvation. The lysosomal degradation of LC3 and p62 in a given time period can be used to assess autophagic flux (Yoshii and Mizushima, 2017).

In our studies, we used a stable cell line expressing GFP-LC3, immunolabelling of endogenous LC3 and immunoblotting of LC3 and p62 in combination with other autophagy markers to assess autophagy in the absence of ataxin-3. We combined this with Bafilomycin A1 as well as the PI3K inhibitor 3-methyladenine (3-MA) to analyze autophagic flux. We furthermore measure autophagic flux using a tandem-labelled version of LC3 (mRFP-GFP-LC3) that is based on the sensitivity of GFP to acidic environments. While GFP-fluorescence is quenched by acidic pH, mRFP is relatively more stable and the ratio of double-positive versus mRFP-positive structures therefore allows measuring the fraction of autophagosomes and autolysosomes, respectively, and assessment of autophagic flux (Kimura et al., 2007).

In addition to using LC3 as read-out for autophagic flux, we performed long-lived protein degradation assays (LLPDs). These assays employ radioisotope-labeling of long-lived proteins and subsequent analysis of their degradation in response to different treatments (Seglen et al., 1979).

In combination, the different methods to assess autophagy result in a reliable measure of autophagic activity and efficiency.

Induction of DNA damage for microscopic analysis of protein recruitment to DNA double-strand breaks

In **paper III** and **IV**, we investigated the role of ataxin-3 in the cellular response to DNA damage, in particular DSBs. In order to inflict DSBs for microscopic analysis of protein recruitment to DSBs we utilized primarily two different methods.

A method that is widely used to inflict DSBs is laser micro-irradiation (Holton et al., 2017). We used a tunable, pulsed nitrogen laser with a wavelength of 365 nm (UV-A radiation) to damage the DNA in a selected region of the nucleus. The damage caused by this radiation is, however, not limited to DSBs and may additionally result in the generation of other UV-induced damages (Kong et al., 2009). Treatment of cells with photosensitizers such a 5-bromo-2-deoxyuridine (BrdU), a thymidine analog, or Hoechst dye, which binds to the minor groove of double-stranded DNA, can be used to promote photolysis and thereby primarily the formation of DSBs. In our experiments, we treated cells with BrdU for 24 hours prior to damage infliction to allow efficient incorporation of this thymidine analog into the DNA. This method allows both, live imaging as well as fixation of samples at different time points after damage infliction, to investigate different stages of the DDR. A disadvantage of the system employed in our studies is, however, that it requires manual selection of the region that will be micro-irradiated. As a consequence, this method only allows damage of a limited number of cells per sample. Micro-irradiation was therefore carried out during a five-minute time window to prevent substantial timing differences between the cells in one sample. To validate the efficient induction of DSBs, immunolabelling of γH2AX, the product of ATM-mediated phosphorylation and therefore a potent marker of DSBs (Burma et al., 2001), was performed.

Another tool for the induction of DSBs that we used to validate our findings and to study DSB signaling is the endonuclease FokI, that is stably expressed as mCherry-LacI-FokI in a U2OS cell line with stably integrated LacO-repeats. A modified estrogen receptor as well as a destabilization domain fused to FokI allow inducible stabilization and nuclear translocation by treatment of the cells with shield-1 ligand and 4-hydroxytamoxifen (4-OHT), respectively. Nuclear translocation subsequently results in tethering of mCherry-LacO-FokI to the integrated LacO-repeats, thereby enabling the induction of DSBs at a specific locus. However, given the requirement for protein stabilization to induce DSBs, as well as the fact that stabilized, tethered FokI results in the constant generation of DSBs, a particular disadvantage of this system, compared to micro-irradiation, is its limited use for analyzing kinetics at DSBs.

5 CONCLUDING REMARKS

Cellular pathways safeguarding proteostasis and genomic stability are frequently dysregulated in neurodegenerative disorders, evidenced by accumulation of ubiquitylated proteins, impaired function of the cellular protein degradation machineries (Dantuma and Bott, 2014; Wong and Cuervo, 2010) as well as altered transcription (Xiang et al., 2018)and accumulation of DNA damage (Massey and Jones, 2018). A polyglutamine repeat expansion in the gene encoding the DUB ataxin-3 has been identified as the underlying cause for MJD (Kawaguchi et al., 1994), an autosomal dominant neurodegenerative disorder that, to date, lacks adequate therapeutic interventions to slow or prevent disease progression.

One route of potential therapeutic strategies is currently focused on the modulation ataxin-3 expression by targeting ataxin-3 at the mRNA- or protein level (Dantuma and Herzog, 2020). As MJD patients are heterozygous for the CAG repeat-expansion, some of these approaches aim at selectively targeting the polyglutamine-expanded ataxin-3 (Alves et al., 2008; Nóbrega et al., 2013). However, this selectivity may not be achievable with all strategies. Interestingly, wild-type ataxin-3 itself has been documented to be involved in the regulation of the very same pathways whose deregulation has been implicated in MJD pathogenesis (Dantuma and Herzog, 2020). It is therefore of utmost importance to understand the native functions of ataxin-3 to avoid the potentially detrimental repercussions that altering the steady-state levels of this DUB may imply.

Our findings, that ataxin-3 directly interacts with the ubiquitin-like Atg8 proteins LC3C and GABARAP further validate the promiscuous interaction of ataxin-3 with ubiquitin-like proteins, a particular feature of this DUB that has been demonstrated previously by us and others for SUMO1 and NEDD8 (Ferro et al., 2007; Pfeiffer et al., 2017). It is tempting to speculate this this may present a common mechanism for spatial control of the actions of ataxin-3. However, additional work will be required to elucidate the significance of the LIRs and to identify the substrate of ataxin-3 in the context of autophagy

Despite polyglutamine-expanded ataxin-3 primarily forming intranuclear inclusions (Paulson et al., 1997), not much is known about the nuclear functions of wild-type ataxin-3. It is therefore interesting that we and others recently implicated ataxin-3 in genome integrity and demonstrated that ataxin-3 is required for efficient repair of DSBs (Singh et al., 2019). In the context of the DDR, we found that, while ataxin-3 acts on ubiquitylated substrates, its localization to DSBs and its timing are regulated by other PTMs, underlining the complex

code that these PTMs create to precisely govern the actions of the many proteins involved in this pathway.

Collectively, we demonstrate that wild-type ataxin-3 promotes both autophagy, in response to nutrient deprivation, and DNA repair, in response to DSBs, a damage type that constitutes a particular threat to genome integrity. Thus, our findings add to the list of native functions of ataxin-3 that are of crucial importance for cellular proteostasis and genome integrity. While further work is required to evaluate the functionality of polyglutamine-expanded ataxin-3 in these contexts, for the regulation of beclin-1, it has been shown, that polyglutamine-expanded ataxin-3 outcompetes binding of wild-type ataxin-3 to beclin-1 (Ashkenazi et al., 2017). These data suggest that, polyglutamine-expansion in ataxin-3 may not only result in a toxic gain-of-function due to its increased propensity to aggregate but it may additionally result in a dominant-negative effect of the mutant in cellular processes that are regulated by ataxin-3. Similarly, it has been shown that polyglutamine-expanded ataxin-3 deubiquitylates the E3 ligase Parkin more efficiently, resulting in the autophagic degradation of Parkin (Durcan et al., 2011).

When it comes to the function of ataxin-3 in DNA damage signaling, it is furthermore tempting to speculate that the localized recruitment of polyglutamine-expanded ataxin-3 to DNA lesions, resulting in high local concentrations, may serve as a seeding point for intranuclear aggregates.

Another concern is that the sequestration of ataxin-3 in the disease-typical aggregates may further exacerbate the cellular defects evoked by polyglutamine-expanded ataxin-3 through loss or gross alteration of the native functions of ataxin-3 by limiting the soluble pool of this enzyme.

The native functions of ataxin-3 are also highly relevant when designing potential therapeutic strategies that are aimed at reducing the levels of ataxin-3. It may therefore be beneficial to focus on strategies that allow selective targeting of the mutant ataxin-3, while leaving wild-type ataxin-3 levels unaltered. Such possibilities have been demonstrated e.g. using RNAi to selectively suppress expression of polyglutamine-expanded ataxin-3 and thereby neuropathology in mouse and rat models of MJD, by exploiting the presence of a disease-specific single-nucleotide polymorphism (SNP) (Alves et al., 2008; Nóbrega et al., 2013). This particular SNP has been shown to be present in around 70 % of MJD patients (Gaspar et al., 2001) and thus allows targeting mutant ataxin-3 in the majority of patients.

In addition to the identification and characterization of the involvement of ataxin-3 in autophagy and DNA repair, we furthermore developed a tool to characterize the signaling function of K63-linked polyubiquitin chains in cells and demonstrated that specific K63-ubiquitylation of a mitochondria-targeted reference substrate are sufficient to recruit the autophagy receptor SQSTM1/p62 to undamaged mitochondria and furthermore to induce robust changes in mitochondrial morphology and localization, evidenced by perinuclear clustering of the mitochondria.

While additional work will be required to investigate whether K63-linked ubiquitylation is sufficient to induce clearance of mitochondria by mitophagy as well as the contribution of mitochondrial clustering to this process, the system developed by us constitutes a suitable tool to address these questions. Furthermore, this system is not limited to mitophagy or mitochondrial processes and could therefore be used to elucidate additional cellular functions of K63-linked ubiquitin chains.

In conclusion, the work presented in this thesis provides new insights in the regulation of cellular pathways safeguarding protein quality control and genome integrity by K63-linked ubiquitin chains and ataxin-3 and points out important considerations for the understanding and therapeutic modulation of MJD and related pathologies.

6 ACKNOWLEDGEMENTS

In the following, I would like to thank all the people that have been supporting me on this journey over the past years:

First of all, I would like to thank my supervisor **Nico** for seeing potential in me during my time as a master student in the group and giving me the opportunity to perform my PhD studies in his group. I am grateful for the many learning experiences that were offered to me during this time. Thank you for supporting me and helping me become the scientist that I am today.

Florian, I would like to thank you for your constant support during these many years and for training me when I first arrived in the lab. Thank you also for teaching me everything I know about microscopes and for giving me the opportunity to help you share the joy of microscopy and image analysis during several teaching activities. I will miss our morning coffees and scientific discussions.

I would also like to thank my co-supervisor **Aris** for his help and advice.

Anne, thank you for welcoming me into your group. The time spent in your lab and the people I met, taught me a lot. I am grateful for all your support and advice during the autophagy project. Many thanks also to all the members of the **Simonsen lab**. You all made me feel welcome from the first day. Special thanks to **Alf** and **Christian** for contributing with data and helpful input to the autophagy project. **Christian**, thank you also for not only giving me a place to stay but a home away from home. In those few short weeks, you became a good friend. I had a lot of fun with you and will never forget your joyful laughter.

Thank you to all other collaborators that contributed with helpful input, advice and experiments to the work presented in this thesis: **Thorsten Hoppe, Éva Kevei and Ricardo Marchante, Martijn Luijsterburg**, the **van Attikum lab**, **Girish and Rashmi Shah**, **Olle Sangfelt** and **Aldwin Suryo Rahmanto**.

To all the past and present members of the **Dantuma lab**: Thank you for making work a fun and inspiring place, even during tough times. I enjoyed the many nice conversations, lunch and coffee breaks we had over the years. **Maria, Katharina, Shanshan, Thibaud, Laura, Klara and Claudia**, you have all helped me in one way or another on this journey and I am very grateful for that. **Tati,** you have become a really good friend. Thank you for always being there for me. Not being alone on this journey has made it a lot easier and being able to ventilate with you when it was far from easy has probably saved me from a few nervous breakdowns. I will miss working with you. **Daniela**, thank you for all your support, especially during the beginning of my time in the lab. I learned a lot from you. Thank you for allowing me additional questions when Florian gave me a limit of ten questions per day. **Annika**, I really enjoyed our conversations both at and outside work. I am especially thankful for the mamma-meetups with you and Finn and for your support even after you left the lab. You have become a good friend over the past years. **Melania,** thank you for bringing a lot of

fun and laughter to the lab and for the mamma-meetups that both Joar and I enjoyed. **Henri,** working with you has been a lot of fun. Thank you for your help and support and the many great conversations as well as scientific discussions.

I would also like to thank all my colleagues in quarter 7A. I had a good time with you in Biomedicum. I enjoyed our cell culture and lab conversations and our semi-regular After Work events. A special thank you to **Chiara, Davide, Sofie and Eleanor** for your emotional support whenever I was frustrated with the laser.

Thank you also to **Matti** and **Linda** for their invaluable help over the last years and to everyone else at CMB, who contributed to a nice work environment.

Sophia, danke, dass du all die Jahre trotz der weiten Entfernung immer für mich da warst. Ich bin sehr dankbar eine Freundin wie dich an meiner Seite zu haben.

Ein ganz besonderer Dank gilt auch meinem früheren Biologielehrer **Ulrich Vahrenholt**, der maßgeblich dazu beigetragen hat, dass ich mich für das Biologiestudium entschieden habe.

I would also like to say thank you to all the members of my family in Germany and in Sweden.

Susan, tack för din support och tack för att du alltid ställde upp när det behövdes.

Mama und **Papa**, vielen Dank für alles, was ihr mir in meinem Leben ermöglicht habt. Danke auch dafür, dass ihr mir immer das Gefühl gegeben habt, dass ich alle meine Ziele erreichen kann und, dass ihr dabei immer an mich geglaubt habt.

Laetitia, du bist nicht nur meine Schwester, sondern meine beste Freundin. Danke, dass auch du mich immer unterstützt hast.

Jimmy, tack för din villkorslösa kärlek och stöd genom åren. Jag hade nog inte klarat det utan dig. Tack för att du alltid finns här för mig när jag behöver dig och tack för att du alltid finns där för Joar.

Joar, du förgyller varje dag och har visat mig vad som är viktigast i livet. ♥

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