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TRIM proteins in autophagy: A study of TRIM32 and TRIM27

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Summary

TRIM proteins are ubiquitin E3 ligases known to be involved in a number of different processes in the cells, such as intracellular signaling, transcription, autophagy, innate immunity, cell cycle progression and DNA repair. Mutations and dysregulation of TRIM proteins are implicated in many diseases, and a number of TRIMs are described as both tumor suppressors and oncogenes. Furthermore, several TRIM proteins are found to have roles in autophagy.

We identified TRIM32 as a potential autophagic substrate in a double- tag screen including 22 different TRIM proteins. We found that the autophagic degradation of TRIM32 was selective, and that TRIM32 interacted directly with the autophagy receptors p62 and NDP52, either of which was sufficient for directing TRIM32 to lysosomal degradation. Conversely, we identified both p62 and NDP52 to be TRIM32 substrates. Mutations in the C-terminal region of TRIM32 causes limb girdle muscular dystrophy 2H (LGMD2H). The LGMD2H disease mutants were unable to ubiquitylate p62 and NDP52, and unable to undergo autophagic degradation. Moreover, we showed that reintroduction of TRIM32 in TRIM32 KO cells decreased the protein level of the sequestosome-like receptors (SLRs), while reintroduction of TRIM32 LGMD2H disease mutant did not. Furthermore, we found TRIM32 expression in TRIM32 KO cells to enhance ULK1 stability and TBK1 autophosphorylation, while the LGMD2H mutant did not. The positive effect of TRIM32 on selective autophagy was supported by the finding that mitophagy was reduced in TRIM32 KO cells, while it was restored when TRIM32 was reintroduced. These findings suggest that the pathogenic effect of the LGMD2H mutation at least partially may be due to dysregulation of selective autophagy receptors, affecting the protein homeostasis in muscle cells.

We also identified TRIM27 as an autophagy substrate. Similarly as TRIM32, TRIM27 interacted with p62 and NDP52, and its autophagic degradation was dependent on ATG7 and the SLRs. TRIM27 impaired the normal autophagic response of p62 and LC3B when the cells were stressed with plasmid transfection or mimicking of virus transfection. Interestingly, TRIM27 mRNA was significantly upregulated in breast cancer tissues compared to normal tissue, and the expression of TRIM27 in various breast cancer cell lines was inversely correlated with LC3B expression. These results may reveal a link between TRIM27 and dysregulated autophagy in breast cancer.

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Even though most of the work was done at the lab, I still want to thank my friends and family for always being supportive and helpful, and helping me think about other things than work. Thank you for always being there.

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List of papers

Paper I

TRIM32, but not its muscular dystrophy-associated mutant, positively regulates and is targeted to autophagic degradation by p62/SQSTM1.

Overå, K.S., Garcia, J.G., Bhujabal, Z., Jain, A., Øvervatn, A., Larsen, K.B., Deretic, V., Johansen, T., Lamark, T., and Sjøttem, E. (2019).

J. Cell Science. PMID 31828304.

Paper II

TRIM32 – a putative regulator of NDP52 mediated selective autophagy

Overå, K.S., Bhujabal, Z., Garcia, J.G., Sjøttem, E.

Manuscript

Paper III

TRIM27 is an autophagic substrate and putative regulator of LC3B

Garcia, J.G., Overå, K.S., Knutsen, E., Bhujabal, Z., Evjen, G., Lamark, T., Johansen, T., Sjøttem, E.

Manuscript

Abbreviations

Abi2	Abl interactor 2
ADHD	Attention deficit hyperactivity disorder
Ago1	Argonaute1
ALFY	Autophagy-linked FYVE
AMBRA	Activating molecule in BECN1-regulated autophagy protein 1
AMPK	AMP-activated protein kinase
APL	Acute promyelocytic leukemia
ATG	Autophagy related
BAG	BCL2-associated athanogene
BBS11	Bardet Biedl syndrome 11
bHLH	Basic helix-loop-helix
B-NHL	B-cell non-Hodgkin lymphoma
BNIP3	BCL2 and adenovirus E1B 19 kDa-interacting protein 3
BNIP3L	BNIP3-like protein
cGAS	Cyclic GMP-AMP synthase
CHIP	Carboxyl terminus of Hsp70 interacting protein
CK2	Casein Kinase 2
CMA	Chaperone- mediated autophagy
Cul3	Cullin3
DFCP1	Double FYVE containing protein 1
EMT	Epithelial-to-mesenchymal transition
EPC1	Enhancer of polycomb homolog 1
EPG5	Ectopic P granules protein 5
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FIP200	Focal adhesion kinase family interaction protein of 200 kDa
FUNDC1	FUN14 domain-containing protein 1
GABARAP	γ-aminobutyric acid receptor-associated protein
Gal8	Galectin8
GWA	Genome wide association
HCC	Hepatocellular carcinoma
HECT	Homologous to E6AP C-terminus
HOPS	Homotypic fusion and vacuolar protein sorting
HP	Hydrophobic pocket
Hsc70	Heat shock-cognate protein of 70 kDa
IFN	Interferon
IL-1β	Interleukin 1β
IRF	Interferon regulatory factor
KEAP1	Kelch-like ECH-associated protein 1
KIR	KEAP1 interacting region
LAMP2A	Lysosomal-associated membrane-2 protein
LDS	LIR docking site
LGMD2H	Limb girdle muscular dystrophy 2H
LIR	LC3 interacting region
LUBAC	Linear ubiquitin chain assembly complex
MAP1LC3	Microtubule-associated proteins 1A/1B light chain 3
MAVS	Mitochondrial antiviral signaling protein

MEK5	Mitogen activated protein kinase kinase 1
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NAP1	Nucleosome assembly protein 1
NBR1	Neighbor of BRCA1 gene 1
NCOA4	Nuclear receptor coactivator 4
NDP52	Nuclear dot protein-52
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NLS	Nuclear localization signal
NRF2	NF-E2-related factor 2
OPTN	Optineurin
PB1	Phox and Bem1
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PHD	Plant homeodomain
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PINK1	PTEN-induced kinase 1
RB1	Retinoblastoma protein 1
RBR	RING-between-RING
RING	Really interesting new gene
SAR	Selective autophagy receptors
SKICH	SKIP carboxyl homology
SLR	Sequestosome-1-like receptors
SNARE	Soluble *n*-ethylmaleimide-sensitive factor-attachment protein receptors
SNP	Single nucleotide polymorphism
SQSTM1	Sequestosome-1
STAT3	Signal transducer and activator of transcription 3
STBD1	Starch-binding domain-containing protein 1
STING	Stimulator of IFN genes
SUMO	Small ubiquitin-like modifier
TAX1BP1	Human T-cell leukemia virus type 1 binding protein 1
TBK1	TANK-binding kinase 1
TLR4	Toll like receptor 4
TNFα	Tumor necrosis factor α
TOLLIP	Toll-interacting protein
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRIF	TIR domain-containing adaptor molecule 1
TRIM	Tripartite motif
UBA	Ubiquitin-associated domain
UBZ	Ubiquitin binding zinc finger
UDS	UIM docking site
UIM	Ubiquitin-interacting motif
ULK1	Unc-51-like kinase 1
UPS	Ubiquitin proteasome system
UVRAG	UV radiation resistance associated gene
VPS34	Vacular protein sorting 32
WIPI	WD-repeat protein interacting with phosphoinositides

Introduction

Cells require amino acids, lipids and carbohydrates to stay healthy, and therefore they are depending on nutrient uptake and recycling. Furthermore, the cells have to dispose of damaged or otherwise unwanted proteins. The maintenance of the proteome is called proteostasis and it involves the control of the initial production and folding of the proteins, conformational maintenance, control of the abundance and subcellular localization and the disposal of proteins by degradation (Klaips et al., 2018). Each process in the cells is carefully watched by the cells many quality control systems which makes sure that abnormal mRNA will be degraded before translation and misfolded proteins are directed to degradation by chaperones (McClellan et al., 2005; Shyu et al., 2008). Faults in the proteostasis often results in an accumulation of damaged or misfolded proteins, which in turn can cause diseases.

There are two major pathways for protein turnover, the autophagy/lysosome system and the ubiquitin proteasome system (UPS).

Ubiquitin Proteasome system

Misfolded, damaged and redundant proteins destined for degradation are marked with ubiquitin, a 76 amino acid protein, in a process called ubiquitylation. Ubiquitin is a marker for many processes, and proteins marked with ubiquitin chains are directed to the proteasome for degradation (Chau et al., 1989). Ubiquitylation of proteins involves an E1 ubiquitin-activating enzyme, which activate ubiquitin and transfers it to E2 ubiquitin-conjugating enzymes. E3 ubiquitin-protein ligases specifically binds substrate proteins and E2s, and ubiquitin is then transferred from the E2 to the substrates (Figure 1) (Hershko and Ciechanover, 1998; Scheffner et al., 1995). E1 enzymes are encoded by two different genes, E2 by at least 37 different genes and E3 ligases by more than 800 genes and elicit the specificity of the ubiquitylation process. E3 ligases are classified depending on their catalytic site. Really Interesting New Gene (RING) ligases simultaneously binds E2 and substrate (Dou et al., 2012; Plechanovova et al., 2012), while Homologous to E6AP C-terminus (HECT) and RING-between-RING (RBR) ligases receive ubiquitin from E2 before modifying the substrate (Figure 1) (Buetow and Huang, 2016; Scheffner et al., 1995; Wenzel et al., 2011). Successive rounds of E3-catalyzed reactions can produce substrates with polyubiquitin chains linked to them.

Ubiquitin itself can be ubiquitylated on seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) (Peng et al., 2003). The ubiquitylation pattern elicit distinct downstream

signaling and the fate of the protein (Buetow and Huang, 2016). Initially, polyubiquitylation of K48- linked chains was found to target proteins for proteasomal degradation (Chau et al., 1989). However, later studies have found that also K11- and K63- linked ubiquitin chains might target proteins for proteasomal degradation (Saeki et al., 2009; Xu et al., 2009). Additionally, K11-linked ubiquitin chains have a role in cell cycle regulation (Jin et al., 2008; Matsumoto et al., 2010). K6- linkage is involved in regulation of mitophagy (Durcan et al., 2014) and K6- and K33- linked polyubiquitylation increase upon DNA damage, indicating that they have a signaling role in DNA damage response (Elia et al., 2015). K27- linked polyubiquitylation is recently reported to have a role in innate immunity response (Wu et al., 2019) while K29- linked polyubiquitin is involved in stimulation of the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activation (Huang and Baek, 2017). K63- linked polyubiqutin chains confer non-degradative signals for different pathways, for example protein trafficking, ion transport, translation and DNA repair (Back et al., 2019; Liu et al., 2018). However, K63- linked polyubiquitylation is also associated with degradation by autophagy (Tan et al., 2008).

Furthermore, the linear ubiquitin chain assembly complex (LUBAC) is a ubiquitin ligase complex composed of two RING finger proteins which does not form lysine linkages, but linkages between the C- and N-termini of ubiquitin (Kirisako et al., 2006). LUBAC activates canonical NF-κB by binding NF-κB essential modulator (NEMO), and it conjugates linear polyubiquitin chains onto specific lysine residues in NEMO (Tokunaga et al., 2009).

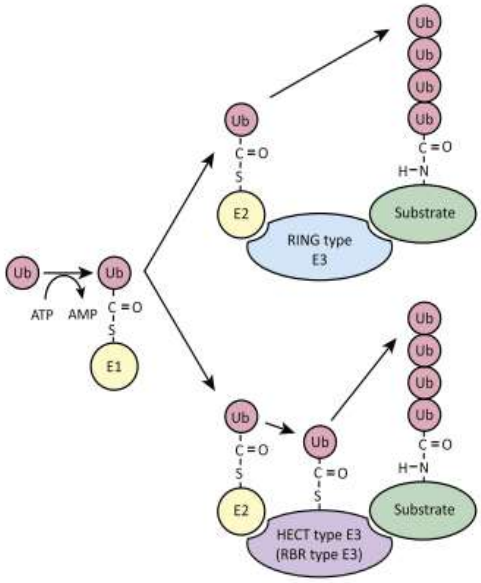

Figure 1: Ubiquitylation of proteins. Ubiquitin is activated by E1 in an ATP dependent process and transferred to E2. E3 ligases bind to both E2 and the substrate and transfer the ubiquitin from E2 to the substrate. Due to the existence of many different E3 ligases, they can bind substrates specifically and they are the main specificity factor of the ubiquitylation process. Substrates can be mono or polyubiquitylated. Abbreviations: RING, Really interesting new gene; HECT, homologous to E6AP C-terminus; RBR, Ring-between-Ring. Figure adapted from Hatakeyama (2017), with permission from Elsevier.

The proteasome is an ATP-dependent protease complex, which consists of two subunits. The 20S catalytic core subunit forms a barrel shape structure with two 19S subunits attached on either end (Figure 2) (Pickart and Cohen, 2004). The 20S subunit is composed of four stacked rings. The outer rings are composed of seven α subunits surrounding a narrow channel through which polypeptides can enter. These α subunits act as gates. The two inner rings are composed of β subunits, which make up the catalytic chamber (Smith et al., 2007). The proteins targeted for proteolysis by attachment of polypeptide chains are rapidly degraded by the proteasome to small peptides, which can be reused by the cell. The ubiquitin is either released and recycled (Ciechanover, 1994; Hershko et al., 1980), or degraded along with the substrate in a "piggyback" mechanism (Shabek et al., 2009).

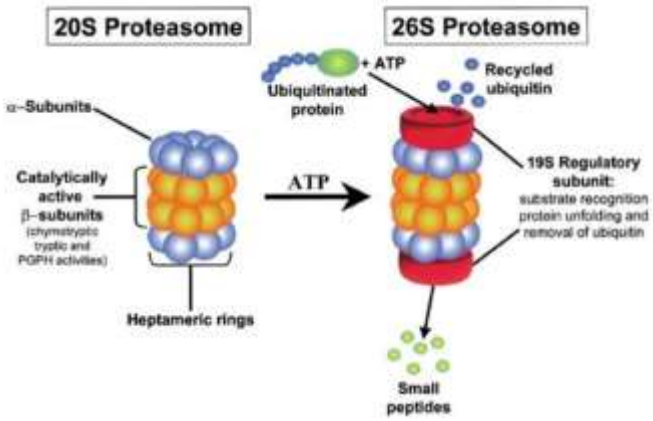

Figure 2: The ubiquitin proteasome pathway. Proteins are conjugated to ubiquitin and rapidly degraded by the 26S proteasome which is an ATP-dependent complex containing the 20S core and two 19S regulatory subunits. Figure from Almond and Cohen (2002) with permission from Springer Nature.

Autophagy

Autophagy is a lysosomal degradation pathway used to maintain internal homeostasis and avoid proteotoxic stress by identifying and recycling unwanted or dysfunctional cellular components. The word autophagy is derived from Greek and translates to "self-eating".

Originally autophagy was characterized by De Duve (1963) who used electron microscopy to study autophagy at the fusion step with the lysosome. Pfeifer and Strauss (1981) discovered that autophagy is regulated by nutrient conditions and Gordon and Seglen (1988) identified the phagophore as a sequestering organelle. Takeshige et al. (1992) found that autophagy in yeast is induced in nutrient-deficient conditions, and Harding et al. (1995) reported the Cytoplasm-to-vacuole transport (*CVT*) pathway in yeast that was morphologically similar to autophagy. Oshumi laboratory identified autophagy genes by studying mutants that

were unable to accumulate autophagic bodies under nitrogen starvation (Tsukada and Ohsumi, 1993). They named the first mutant for an autophagy gene they isolated *apg1*. The Thumm and Klionsky laboratories conducted genetic screenings in yeast for autophagy mutants (*aut* mutants) and *CVT* mutants (Harding et al., 1995; Thumm et al., 1994). A nomenclature for autophagy mutants was established, known as AuTophaGy (ATG) mutants, and several autophagy mutants were revealed in further genetic screening. 16 conserved core Atg proteins involved in autophagy in yeast have been identified (Atg1-Atg14, Atg16 and Atg18). So far a total of 41 putative Atg proteins have been identified in yeast (Yao et al., 2015). The studies in yeast led to a search for conserved genes in eukaryotes and their role in the mammalian autophagy system. The mammalian homologues of the conserved yeast Atg proteins have been characterized in mammals (Ohsumi, 2014).

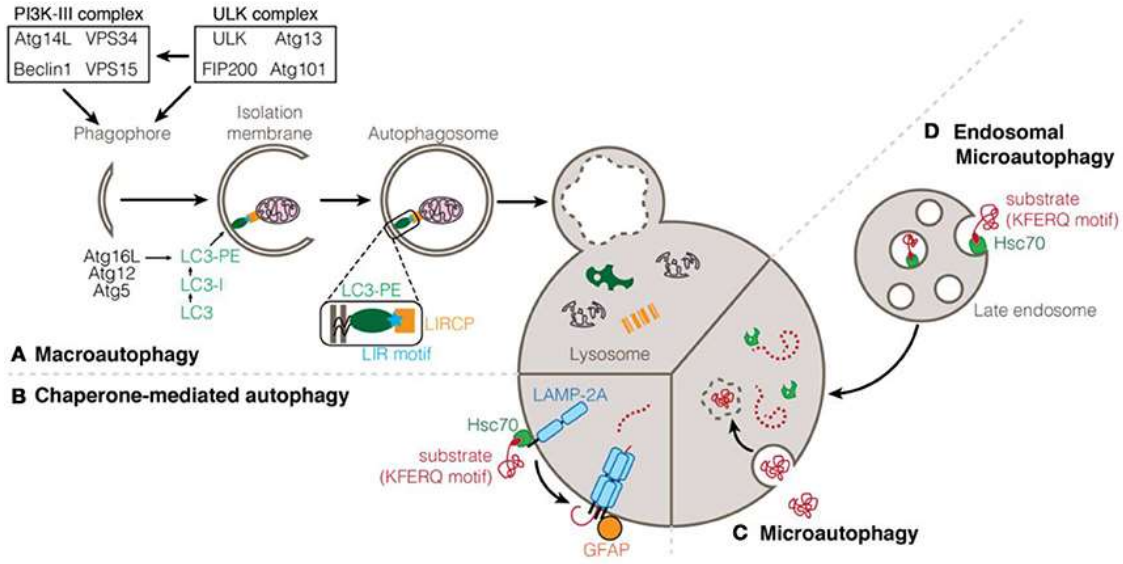

Figure 3: Overview of the main autophagic pathways in eukaryote cells. ***A.*** *Macroautophagy.* ***B.*** *Chaperone-mediated autophagy (CMA) is translocation of proteins with a KFERQ motif into the lysosome through pores formed by LAMP2A.* ***C.*** *Microautophagy is the direct uptake of cytoplasmic material into the lysosome.* ***D.*** *Endosomal microautophagy is uptake of proteins with a KFERQ signal in the late endosome before being addressed to the lysosome. Figure from Jacomin et al. (2018).*

There are three major autophagic pathways and they are distinguished by how the cargo enters the lysosome (Figure 3) (Jokl and Blanco, 2016). In chaperone- mediated autophagy (CMA), proteins with a KFERQ motif are recognized and bound by the chaperone heat shock-cognate protein of 70KDa (Hsc70). The substrate is targeted to the lysosome where it interacts with the lysosomal-associated membrane-2 protein (LAMP2A) receptor on the lysosomal membrane (Dice et al., 1990). For translocation across the lysosomal membrane, the substrate

has to be in an unfolded state, and this is most likely mediated by Hsc70 and co-chaperones (Cuervo and Wong, 2014; Salvador et al., 2000).

Microautophagy was firstly described by De Duve and Wattiaux (1966). It involves direct uptake of cytoplasmic components into the lysosomes or endosomes (Ahlberg et al., 1982; Li et al., 2012; Mortimore et al., 1988). There are at least three morphologically distinct types of microautophagy, two of which takes place at the lysosomes/vacuoles and one at the late endosomes (Oku and Sakai, 2018). Endosomal microautophagy is the best characterized type of microautophagy, and is the uptake of cytosolic proteins to late endosomes. The process is mediated by Hsc70, and the proteins destined for degradation have a KFERQ motif similar to CMA, but unlike CMA substrate unfolding and LAMP2A is not needed (Sahu et al., 2011). The endosomal sorting complex required for transport (ESCRT) is an assembly of protein subcomplexes (ESCRT I-III) and it orchestrates inward budding of the endosomal membrane to form vesicles that are necessary for endosomal microautophagy (Lefebvre et al., 2018). Mitochondria can be degraded by endosomal microautophagy through Parkin- dependent sequestration in Rab5- positive early endosomes in an ESCRT- dependent process (Hammerling et al., 2017). Upon acute amino acid starvation, a number of substrates are rapidly degraded by endosomal microautophagy. This degradation is mediated by ESCRT III-dependent delivery of proteins and mitochondria to late endosomes (Mejlvang et al., 2018).

Macroautophagy, hereafter called autophagy, involves sequestering of cytoplasmic materials into double membrane vesicles termed autophagosomes. Autophagosomes fuse either directly with lysosomes to form autolysosomes or with late endosomes to form amphisomes. The amphisomes then fuses with lysosomes and forms autolysosomes (Figure 4) (Berg et al., 1998; Nakamura and Yoshimori, 2017). The vesicular constituents are degraded in the autolysosomes (Gordon and Seglen, 1988).

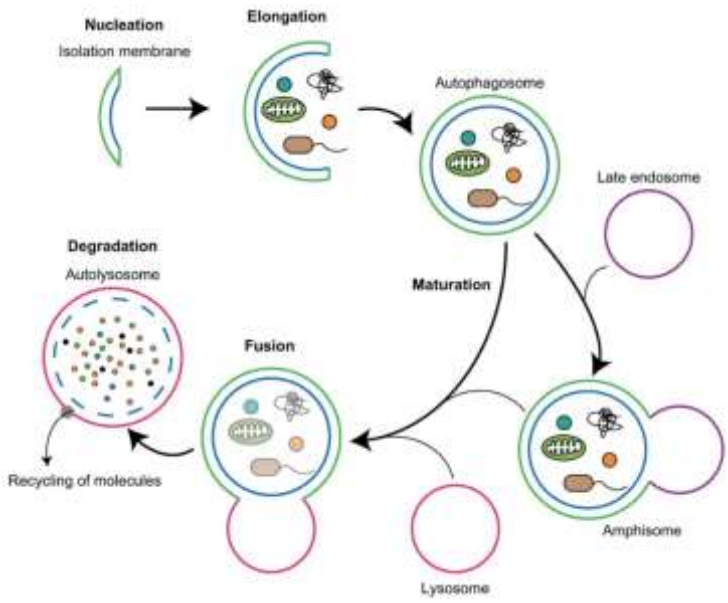

Figure 4: Overview of macroautophagy. The autophagosomes can fuse either directly with the lysosomes to form autolysosomes or first with late endosomes to form amphisomes. The amphisomes then fuses with lysosomes to form autolysosomes, and the sequestered contents are degraded. Figure from Nakamura and Yoshimori (2017) with permission from Company of Biologists.

Early steps of autophagy

The Unc-51-like kinase 1 (ULK1) complex comprised of ULK1 or ULK2, Focal adhesion kinase family-interacting protein of 200kDa (FIP200), ATG13 and ATG101 controls the initiation of autophagy in mammals (Ganley et al., 2009; Hosokawa et al., 2009b; Mercer et al., 2009). ATG13 interacts directly with ATG101, FIP200 and ULK1 and mediates the interaction between FIP200 and ULK1 (Hosokawa et al., 2009b; Jung et al., 2009). Mammalian target of rapamycin (mTOR) regulate the initiation of autophagy by phosphorylating ATG13 and ULK1, and thereby inhibiting ULK1 kinase activity (Figure 5) (Hosokawa et al., 2009a; Jung et al., 2009). In nutrient rich conditions mTOR is active and inhibit autophagy by binding to ULK1 and phosphorylating ULK1 at Ser637 and Ser757 (Kim et al., 2011; Torii et al., 2016), and ATG13 at Ser258 (Ganley et al., 2009; Hosokawa et al., 2009a; Puente et al., 2016). Under starved conditions ULK1 and ATG13 is dephosphorylated and the kinase activity of ULK1 is activated. Furthermore, AMP-activated protein kinase (AMPK) promote autophagy under glucose starvation and activates ULK1 by phosphorylating ULK1 at Ser317 and Ser77 (Kim et al., 2011). Activated ULK1 phosphorylates ATG13 at Ser318 (Joo et al., 2011b) and FIP200 at Ser943, Ser986 and Ser1323 (Egan et al., 2015), and also undergoes autophosphorylation at Thr180, Ser1042 and Thr1046 (Bach et al., 2011; Liu et al., 2016a). These phosphorylations lead to their dislocation (Joo et al., 2011b; Jung et al., 2009). The mTOR binding partner raptor is a direct substrate of AMPK, and raptor phosphorylation is required for suppression of mTOR activity in response to stress (Gwinn et al., 2008). It is worth noting that mTOR and AMPK regulate autophagy in response to nutrient conditions. Under other conditions, such as ER stress (Grotemeier et al., 2010), hypoxia in nucleus pulposus cells (Choi et al., 2016) and other non-

nutritional signals, autophagy can be initiated through signaling by a different set of kinases, such as calcium/calmodulin-dependent protein kinase 1 (CAMK1) and mitogen-activated protein kinase 1 (MAPK1)-cyclin E-BECN1 (Corona Velazquez and Jackson, 2018; Pfisterer et al., 2011; Ugland et al., 2011).

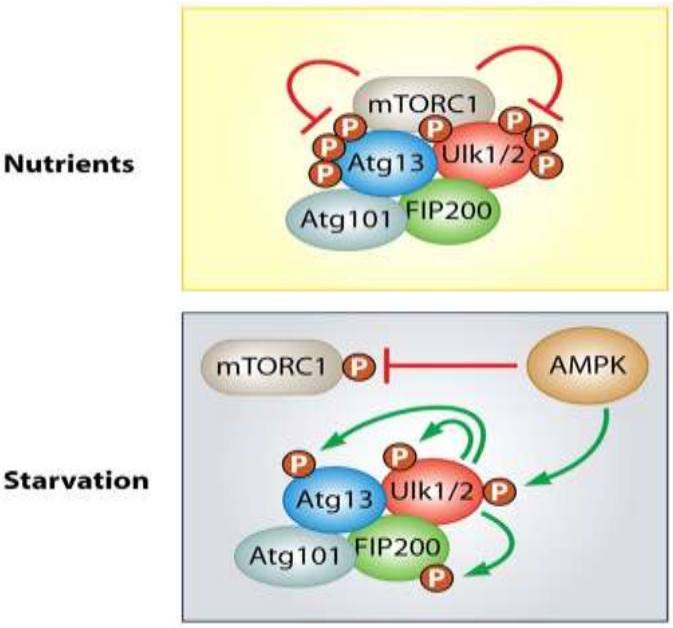

Figure 5: Regulation of autophagy induction by the TOR and AMPK complexes. Under fed conditions mTOR phosphorylates ULK1/2 and ATG13, thereby inhibiting the kinase complex. During starved conditions, mTOR dependent phosphorylation sites in ULK1 are dephosphorylated, and ULK1 autophosphorylates and phosphorylates ATG13 and FIP200. Furthermore, ULK1 is phosphorylated by AMPK and thereby activated. In addition, AMPK inhibit mTOR through phosphorylation of raptor, thereby inducing autophagy. Abbreviations: mTORC1, mammalian target of rapamycin complex 1; ATG, autophagy related; AMPK, AMP- activated protein kinase; ULK1/2, Unc-51-like kinase 1/2; FIP200, Focal adhesion kinase family interaction protein of 200 kDa. Figure is adapted from Alers et al. (2012), originally from Chang and Neufeld (2010), with permission from John Wiley and sons.

Another complex crucial for autophagy is the phosphatidylinositol 3-kinase (PI3K) complex (Kametaka et al., 1998; Seglen and Gordon, 1982). Three types of PI3K have been identified in mammals, Class-I, -II and –III, and they are categorized based on their lipid substrate specificity.

After initiation of autophagy, the ULK1 complex translocates to omegasomes. Omegasomes are membrane platforms connected to the endoplasmic reticulum (ER) (Axe et al., 2008). The ULK1 complex arrives at the autophagy initiation sites and recruits the class III PI3K complex. The complex consists of vacular protein sorting 34 (VPS34), VPS15/p150, Beclin1 and ATG14L. In mammals, there are at least two distinct class III PI3K complexes, the ATG14 complex and the UV radiation resistance associated (UVRAG) complex, each containing specific regulatory subunits that define membrane trafficking pathways (Itakura et al., 2008; Kihara et al., 2001). The ATG14 complex is involved in initiation of autophagy while the UVRAG complex is involved in later stages of the autophagic process. The PI3K class III complex are responsible for generating phosphatidylinositol 3-phosphate (PI3P)-rich

membranes which stabilizes ULK1 at the omegasome and recruitment of PI3P binding proteins to the forming autophagosome (Karanasios et al., 2013). Among the recruited proteins are double FYVE-domain containing protein 1 (DFCP1) and WD-repeat protein interacting with phosphoinositides (WIPI) (Axe et al., 2008; Proikas-Cezanne et al., 2015). There are four WIPI proteins (WIPI1-4) and WIPI2b interacts directly with ATG16L and recruits the light chain 3 (LC3) lipidation machinery, and thereby enable the formation of the autophagosome (Dooley et al., 2014).

The phagophore in mammalian cells are tightly connected to ER, but contacts between phagophore and Golgi, endosomes, mitochondria and lysosomes have also been observed (Biazik et al., 2015; Yla-Anttila et al., 2009). After nucleation, the phagophore keeps growing. The most likely membrane sources are considered to be ER, Golgi, plasma membrane and mitochondria.

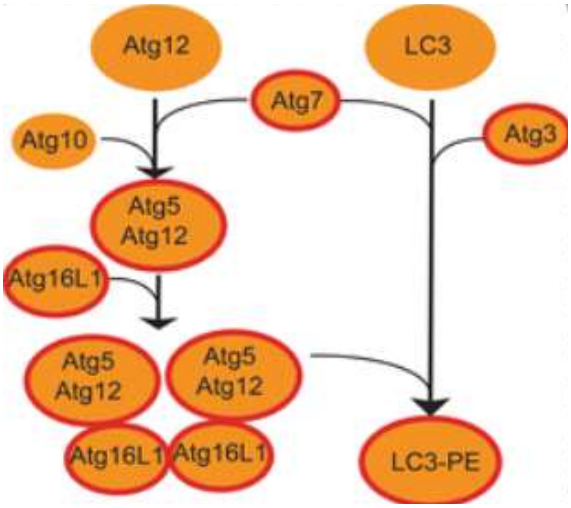

Figure 6: The two conjugation systems, ATG12-ATG5-ATG16L and ATG8/LC3. ATG12-ATG5-ATG16L acts as an E3 ligase and transfers ATG8 from ATG3 to PE. Figure from Dupont and Codogno (2013) with permission from Springer Nature.

The elongation is achieved by two conjugation systems, the ATG12-ATG5-ATG16 and ATG8/LC3 (Figure 6). In the ATG12-ATG5-ATG16 system ATG12 is activated by E1-like ATG7 and transferred to E2-like ATG10 for conjugation to ATG5 (Kim et al., 1999; Shintani et al., 1999). ATG16L homodimerizes and interacts with ATG5 in the ATG12-ATG5 complex (Kuma et al., 2002). The ATG8 family proteins are cleaved at the C-terminal by ATG4, leading to exposure of a glycine residue (Kirisako et al., 2000). ATG7 activates the processed ATG8 and transfers it to the E2-like ATG3 (Ichimura et al., 2000). ATG12-ATG5-ATG16L acts as an E3 ligase and conjugates transfer of ATG8 from ATG3 to phosphatidylethanolamine (PE) (Hanada et al., 2007). PE-ATG8 associates with the inner and outer side of the growing

phagophore. The ATG8-PE on the outside is released to cytoplasm by ATG4 cleavage (Kirisako et al., 2000).

ATG9 is a transmembrane protein that, in yeast, traffics between pre-autophagosomal structures and other punctate structures in the cytoplasm (Reggiori et al., 2004). The mammalian ATG9 is similar, but resides in both trans-Golgi network and late endosomes. ATG9 shuttles around the phagophore and delivers necessary content for elongation (Orsi et al., 2012; Young et al., 2006). ATG9 is able to traffic to omegasomes independent of WIPI2 and ULK1, but its retrieval is dependent on WIPI2, while ULK1 seems to regulate the distribution of ATG9 in areas where new omegasomes can form (Orsi et al., 2012). A recent study found that binding of a positive regulator of autophagy, sorting nexin 18 (SNX18), to Dynamin-2 is important for regulating ATG9A trafficking from recycling endosomes and for formation of ATG16L1 and WIPI2- positive autophagosome precursor membranes (Soreng et al., 2018).

Late steps of autophagy

When the autophagosome is a closed, double membrane structure it, is considered complete. The closure process is not yet fully understood. The ESCRT III complex is recruited to autophagosomes at a late step in autophagosome formation, and the ESCRT machinery mediates phagophore closure (Zhen et al., 2019). Furthermore, the ATG8 conjugation system is believed to be essential for the closure of autophagosomes and degradation of the inner autophagosomal membrane (Sou et al., 2008; Tsuboyama et al., 2016). However, another study using ATG8 KO cells did not see any effect on autophagosome closure, but on the autophagosome fusion with lysosome (Nguyen et al., 2016).

The complete autophagosome is transported to late endosome or lysosome. The complete autophagosome then fuse with the lysosome in a process involving Rab GTPases, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARES) and tethering complexes. Rab7 has a role in the maturation of late autophagic vesicles (Gutierrez et al., 2004; Jager et al., 2004). Rab7 also has a role in recruiting the homotypic fusion and protein sorting (HOPS) complex, SNAREs and the tethering factor Ectopic P granules protein 5 (EPG5) at the autophagosome-lysosome fusion site (Wang et al., 2016; Wijdeven et al., 2016). EPG5 interacts with ATG8s and likely promotes the autophagosome-lysosome fusion process (Wang et al., 2016).

ATG8 and LIR

Among the ATG family proteins is the ubiquitin-like ATG8 family. Mammals have six ATG8 homologues: The microtubule-associated proteins 1A/1B LC3 (MAP1LC3) family (MAP1LC3A, MAP1LC3B and MAP1LC3C) and the γ-aminobutyric acid receptor-associated protein (GABARAP) family (GABARAP, GABARAPL1, GABARAPL2). They share sequence and structure similarity. They have a conserved C-terminal core consisting of four β-strands (β1, β2, β3, β4) and two α-helices (α3, α4) and on the N-terminal end they have two α-helices (α1, α2) (Figure 7) (Noda et al., 2009; Paz et al., 2000; Sugawara et al., 2004).

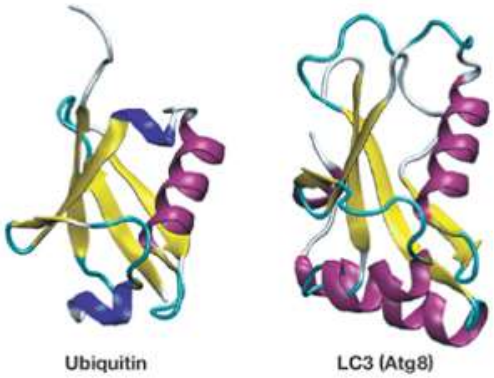

Figure 7: Structural comparison of ubiquitin and LC3. α-helices are shown in purple, β-strands in yellow, β-turns in cyan and unstructured loops in white. Figure is adapted from Geng and Klionsky (2008), originally from Sugawara et al. (2004) and Vijay-Kumar et al. (1987) with permission from Elsevier (Ubiquitin) and John Wiley and sons (LC3).

The ATG8 homologues have β-strands which makes two hydrophopic pockets (HP1 and HP2). HP1 is deep and formed between α2 and β2 and accommodates hydrophopic aromatic residues while HP2 is formed between α3 and β2 and accommodates hydrophobic aliphatic residues. HP1 and HP2 binds to a short linear motif known as LC3 interacting region (LIR), and HP1 and HP2 makes up the LIR docking site (LDS) on the ATG8. The LIR consensus sequences contain [W/F/Y]XX[L/I/V] where X denotes any amino acid. W and F at the aromatic position binding to HP1 is most common, but a few have Y at this position. The hydrophobic residue at position four binds to HP2 (Birgisdottir et al., 2013; Noda et al., 2008; Pankiv et al., 2007). Acidic residues (E, D, S or T) often flank the core motif and they are involved in electrostatic interactions with the N-terminal helices of the ATG8 (Birgisdottir et al., 2013). The first LIR was identified in the autophagy receptor p62/Sequestosome-1 (SQSTM1), hereafter called p62 (Bjorkoy et al., 2005; Pankiv et al., 2007). Autophagy receptors are not the only ones to have LIR domains. Recently it was found that Beclin1,

ATG14 and VPS34 in the PI3K class III complex all have LIR motifs with a clear preference for GABARAP and GABARAPL1 (Birgisdottir et al., 2019).

Recently, Marshall et al. (2019) found a new class of ATG8 interactors in *Arabidopsis thaliana*. They found that ubiquitin-interacting motif (UIM)-like sequences could bind to an alternative site on the ATG8s called UIM docking site (UDS) (Marshall et al., 2019).

Selective autophagy

While UPS is a highly specific and selective route for protein degradation, autophagy was initially believed to be a non-selective, bulk degradation process mainly induced to replenish energy stores upon starvation (Cohen-Kaplan et al., 2016). However, during the last decade evidence have accumulated showing that autophagy can be highly selective. Specific receptors have been found to recognize and deliver proteins and organelles exclusively to autophagosomes, showing that autophagy also can be a selective process (Kirkin, 2019; Rogov et al., 2014).

When cells are stressed by ischemia, hypoxia, starvation or infection, selective autophagy is induced and specifically targets excess or toxic structures, thereby allowing the cell to adapt to the unfavorable conditions (Johansen and Lamark, 2011). Selective autophagy can be further divided based on their particular type of cargo.

For each type of cargo there are selective autophagy receptors (SAR) that bind the cargo and dock on the forming phagophore to enable the degradation of the cargo (Kirkin and Rogov, 2019). The first autophagic receptor to be recognized was p62, which interacts both with LC3B and cargo, and thereby deliver specific cargo to the autophagosomes. p62 itself is degraded with the cargo in the process (Bjorkoy et al., 2005; Pankiv et al., 2007).

Several other autophagy receptors have later been identified, and a group of proteins with similarity to p62 have been named sequestosome-1-like receptors (SLRs) (Figure 8). This group includes p62, Neighbor of BRCA1 gene1 (NBR1), Nuclear Dot protein-52 (NDP52), Optineurin (OPTN) and human T-cell leukemia virus type 1 binding protein (TAX1BP1). The SLRs can be considered as functional homologues. All of them recognize ubiquitylated substrates, interact directly with ATG8 homologues via LIR-LDS interactions, direct the substrate to lysosomal degradation and they get degraded themselves with the substrate (Kirkin et al., 2009; Thurston et al., 2009; Wild et al., 2011). Furthermore, NDP52 and p62 can oligomerize independently or as hetero-oligomers in a three-dimensional helical assembly through a scaffold formed by the Phox and Bem1 (PB1) domain (Ciuffa et al., 2015; Kirkin et

al., 2009). Autophagy-linked FYVE (ALFY) is another SLR which interacts with LC3C and the GABARAPs through a LIR domain (Lystad et al., 2014). It interacts with p62, binds to PI3P and contributes to clearance of aggregated proteins, midbody remnants and viral particles (Clausen et al., 2010; Filimonenko et al., 2010; Isakson et al., 2013; Mandell et al., 2014a; Simonsen et al., 2004).

Another group of autophagy receptors is the membrane-associated autophagy receptors. They include the mitophagy receptors BCL2 and adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), BNIP3-like protein (BNIP3L, also known as NIX), FK506 binding protein 8 (FKBP8) and FUN14 domain-containing protein 1 (FUNDC1) (Bhujabal et al., 2017; Hanna et al., 2012; Liu et al., 2012; Novak et al., 2010). Other membrane-associated autophagy receptors include the ER-phagy receptors cell-cycle progression gene 1 (CCPG1) and Reticulon-3 (RTN3) (Grumati et al., 2018; Grumati et al., 2017; Smith et al., 2018), the glycophagy receptor starch-binding domain containing protein 1 (STBD1) (Jiang et al., 2011) and Atg39 is a membrane-associated receptor for yeast nucleophagy (Mochida et al., 2015; Papandreou and Tavernarakis, 2019). Other autophagy receptors include the ferritinophagy receptor nuclear receptor coactivator 4 (NCOA4) (Mancias et al., 2014), and Tripartite motif (TRIM) proteins, which recognize many different targets, such as virus and aggregates, and assembles effectors and regulators of autophagy (Kimura et al., 2015; Mandell et al., 2014a).

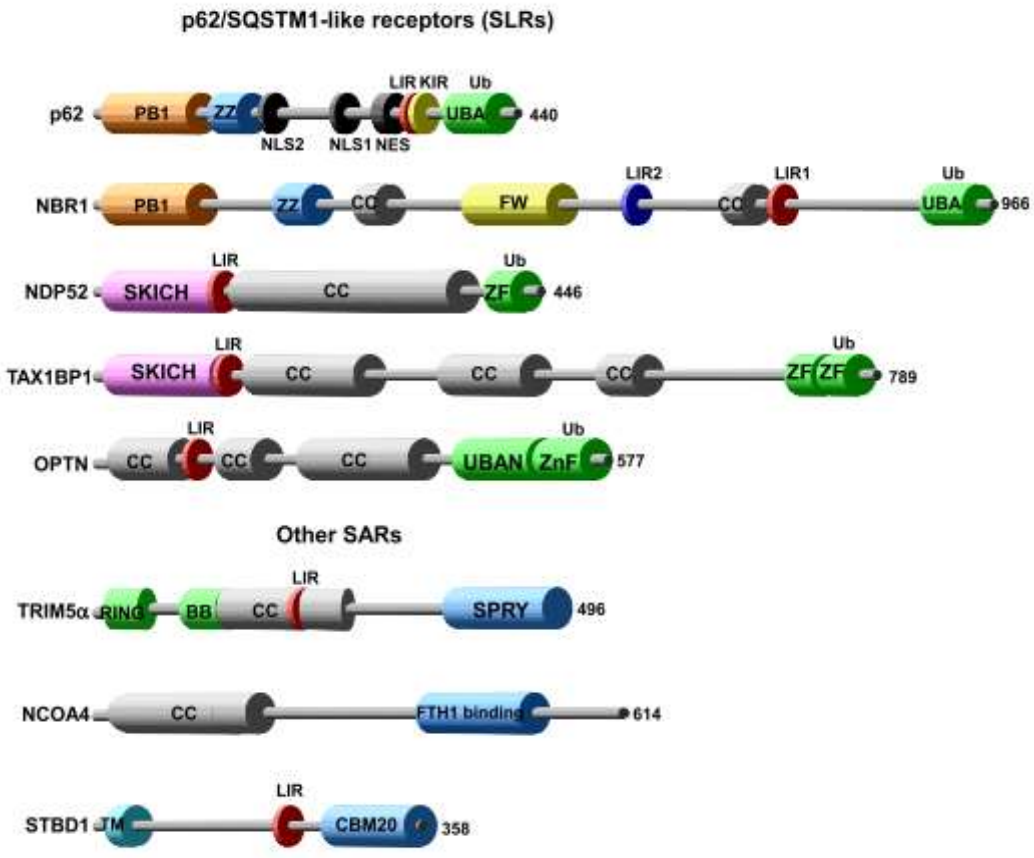

Figure 8: Domain structure of SLRs and other SARs. Indicated motifs and domains: PB1, Phox and Bem1 domain; ZZ, ZZ-type zinc finger domain; CC, coiled coil domain; NLS, nuclear localization signal; NES, nuclear export signal; LIR, LC3-interacting region; KIR, KEAP1 interacting region; UBA, ubiquitin-associated domain; FW, four tryptophan domain; SKICH, SKIP carboxyl homology domain; ZF, zinc finger domain; UBAN, ubiquitin binding in ABIN and NEMO domain; RING, RING finger domain; BB, B-box domain; SPRY, SPRY domain; CBM20, family 20 carbohydrate-binding module domain; TM, transmembrane domain. The green boxes indicate ubiquitin binding domains. Figure is from Johansen and Lamark (2019), with permission from Elsevier. The material is under a Creative Commons License: [CC BY NC ND; https://creativecommons.org/licenses/by-nc-nd/4.0/].

p62

The domain architecture of p62 show an N-terminal self-interacting PB1 domain, followed by ZZ-type zinc finger domain, two nuclear localization signals (NLS), a nuclear export signal (NES), a LIR motif, a KEAP1 interacting region (KIR) and a C-terminal ubiquitin associated (UBA) domain (Figure 8) (Pankiv et al., 2007; Pankiv et al., 2010). In addition to self-interacting, the PB1 domain interacts with atypical protein kinase Cs, mitogen activated protein kinase kinase 5 (MEK5) and NBR1 (Lamark et al., 2003; Sanchez et al., 1998; Wilson et al., 2003). The UBA domain interacts with ubiquitylated cargo, and has affinity towards K63-

linked polyubiquitin chains. Furthermore, p62 docks directly unto Kelch-like ECH-associated protein 1 (KEAP1) via the KIR motif (Jain et al., 2010).

p62 undergoes polymerization with the PB1 domain, and form long polymeric chains. These polymeric chains are mixed with ubiquitin chains and leads to phase separation and structures referred to as droplets (Sun et al., 2018; Zaffagnini et al., 2018). p62 then connects the cargo to the autophagosome through a LIR- mediated interaction with the ATG8s (Lamark et al., 2003). Thus, p62 select specific cargo for degradation by the lysosome. p62 itself is also degraded by the lysosome in the process (Bjorkoy et al., 2005).

In autophagy deficient cells p62 accumulates in aggregates. The accumulation of p62 aggregates have been linked to diseases affecting both the brain and the liver. These diseases include Lewy bodies in dementia, Alzheimer's disease, and huntingtin aggregates in the brain (Komatsu et al., 2007; Kuusisto et al., 2001; Nagaoka et al., 2004; Zatloukal et al., 2002), and Mallory bodies, hyaline bodies and α1 antitrypsin aggregates in the liver (Zatloukal et al., 2002). The p62 aggregates are positive for ubiquitin and they form in response to stress, such as amino acid starvation, oxidative stress, accumulation of defective ribosomal products, or inhibition of autophagy (Komatsu et al., 2007; Pankiv et al., 2007).

NF-E2-related factor 2 (NRF2) is a transcription factor that regulates the transcription of antioxidant genes. During non-stress conditions KEAP1 forms a complex with Cullin3 (Cul3), the KEAP1-Cul3 complex, which polyubiquitylates NRF2 and destines it for degradation (Cullinan et al., 2004; Kobayashi et al., 2004). During oxidative stress, KEAP1 is destabilized and interacts with p62 through the KIR domain on p62 and the NRF2 binding site on KEAP1, sequestering it in aggregates (Jain et al., 2010; Komatsu et al., 2010). This interaction titrates KEAP1 away from NRF2, stabilizing NRF2. Furthermore, p62 induction is mediated by NRF2 and p62-KEAP1 interaction is required for p62 to stabilize NRF2 (Jain et al., 2010). The LIR and KIR domains are localized close to each other and cannot be utilized simultaneously.

Post-translation modifications of p62 have been shown to regulate its function. Phosphorylation of the UBA domain at Ser409 and Ser405 by ULK1 and Ser403 by TANK-binding kinase 1 (TBK1) and Casein kinase 2 (CK2) increase the binding affinity of p62 to ubiquitin (Lim et al., 2015; Matsumoto et al., 2011; Pilli et al., 2012). Furthermore, ubiquitylation of p62 can modulate the p62 function. An E2 interacting region (EIR) was mapped to residues 294-320, which precedes the LIR domain. Two E2 conjugating enzymes, UBE2D2 and UBE2D3, interacts directly with p62 through the EIR domain (Peng et al., 2017). TRIM21 ubiquitylates p62 at Lys7, and this ubiquitylation abrogates p62 oligomerization and

sequestration of proteins (Pan et al., 2016). Ring finger protein 26 (RNF26) ubiquitylates p62 in the UBA domain, thereby enhancing interaction between p62 and other ubiquitin adaptors such as Toll-interacting protein (TOLLIP) and facilitating vesicular cargo sorting (Jongsma et al., 2016). RNF166 ubiquitylates p62 at Lys91 and Lys189, facilitating p62's role in xenophagic degradation of intracellular bacteria (Heath et al., 2016). The KEAP1-Cul3 complex ubiquitylates p62 in the UBA domain at Lys420, and this ubiquitylation may regulate p62 function (Lee et al., 2017).

NDP52

NDP52 has a SKIP carboxyl homology (SKICH) domain, a coiled-coil region, a C-LIR motif and a C-terminal Ubiquitin binding zinc finger (UBZ) domain (Figure 8). NDP52 interacts with nucleasome assembly protein 1 (NAP1) in unique heterotetramers through its SKICH domain and recruit TBK1 (Fu et al., 2018). TBK1 regulate the autophagic functions of NDP52 by phosphorylation, including phosphorylation of positions in the SKICH domain (Heo et al., 2015). The UBZ domain recognizes ubiquitylated proteins (Xie et al., 2015a).

NDP52 recognizes and targets ubiquitin coated *Salmonella* for degradation (Thurston et al., 2009). Furthermore, NDP52 mediates selective autophagic degradation of retrotransposon RNA, DICER and protein argonaute-2 (Ago2) in the miRNA pathway, mitochondrial antiviral signaling protein (MAVS) in immune signaling, the signaling adaptor protein TIR domain-containing adaptor molecule 1 (TRIF) and Myeloid differentiation primary response protein MyD88 (MyD88) (Gibbings et al., 2012; Guo et al., 2014; Inomata et al., 2012; Jin et al., 2017). NDP52 form a trimeric complex with FIP200 and NAP1 and promote progression of anti-bacterial autophagy (xenophagy) (Ravenhill et al., 2019).

NDP52 has a Galectin-8 (Gal8) interacting region between position 371 and 381, the area between the coiled coil and UBZ domains (Thurston et al., 2012). The sequence form a hook-like structure that strategically places side chains for hydrophobic interactions with Gal8 (Li et al., 2013). Two tyrosine residues in NDP52, Tyr376 and Tyr380, specifically interacts with two tyrosine residues in Gal8, Tyr266 and Tyr272 (Kim et al., 2013).

NDP52 has also been reported to play a role in mitophagy. NDP52 is recruited to the mitochondria by the internal mitochondrial protein PTEN-induced kinase 1 (PINK1). Furthermore, NDP52 and OPTN recruit the autophagy initiation machinery and the ATG8s for degradation of mitochondria (Lazarou et al., 2015). NDP52 interacts with ULK1 through FIP200, and the association between NDP52 and the ULK1 complex is enhanced during

mitophagy (Vargas et al., 2019). The capability of NDP52 to induce mitophagy is dependent on its interaction with the ULK1 complex.

Genetic mutations in NDP52 have been linked to Crohn's disease (Ellinghaus et al., 2013).

TRIM proteins

TRIM family proteins have many different roles in the cells, including intracellular signaling, transcription, autophagy, innate immunity, cell cycle progression, DNA repair and carcinogenesis. There are 77 different human TRIM proteins known (Figure 9) (Hatakeyama, 2017). The TRIM family proteins are characterized by a RING domain, one or two B box domains, a coiled coil domain and a variable C-terminal domain. The arrangement is conserved through evolution, supporting that it has a functional relevance (Reymond et al., 2001). The RING domain is a specialized zinc finger and constitutes E3 ligase activity. A number of TRIM proteins have also been shown to self-ubiquitylate through the RING domain (Duan et al., 2008; Huang et al., 2013; Yamauchi et al., 2008). There are some TRIM proteins that lack the RING domain but they are evolutionary closely related, and TRIM16 has been shown to have E3 ubiquitin ligase activity through its B-box domain (Bell et al., 2012; Hatakeyama, 2017). B-box domains are zinc-binding motifs and their function is not fully understood, but they are involved in homo-oligomerization of TRIM5α and in autoinhibition of TRIM21 (Diaz-Griffero et al., 2009; Dickson et al., 2018). Many TRIM proteins homodimerize through the coiled coil domain, and it occurs in an antiparallel fashion positioning the N-terminal RING domains at opposite ends of the dimer (Reymond et al., 2001; Sanchez et al., 2014).

The TRIM proteins are categorized into 11 different subgroups based on their C-terminal domain, which can contain any of 10 different motifs either alone or in combination (Figure 9) (Ozato et al., 2008). The C-terminal domain facilitate substrate recruitment, but can also have additional roles. The C-terminal domain of class VI is a plant homeodomain (PHD) which has small ubiquitin-like modifier (SUMO) E3 ligase activity (Ivanov et al., 2007). Furthermore, TRIM28, which is a class VI TRIM protein, does not harbor E3 ligase activity on its own and is unable to self-associate (Stevens et al., 2019).

Several TRIM proteins are shown to regulate autophagy. TRIM55/MuRF2 was the first TRIM protein shown to interact with p62 and it has a role in the turnover of muscle proteins (Lange et al., 2005; Perera et al., 2011). Mandell et al. (2014a) used a small interfering (si)RNA screen and found that many different TRIMs affect autophagy. Several members of the TRIM

family have been shown to promote autophagy induction by interacting with ULK1 and Beclin1 (Chauhan et al., 2016; Fusco et al., 2018; Mandell et al., 2014a). Furthermore, some TRIM proteins act as selective autophagy receptors by binding cargo and ATG8 family proteins to mediate autophagic degradation (Mandell et al., 2014a). An example is TRIM5α which recognize and targets HIV-1 for autophagic degradation (Mandell et al., 2014b). In contrast to the autophagy- promoting TRIMs, such as TRIM5α, TRIM17 protects a variety of selective autophagy targets from degradation. However, TRIM17 also contribute to the elimination of midbodies, thus, depending on the target, either positively or negatively regulate autophagy (Mandell et al., 2016).

Ubiquitylation of TRIM proteins are shown to implicate their roles in the TRIMs role in autophagy. Imam et al. (2019) show that K63-linked ubiquitylation is required for the association of TRIM5α with autophagosomal membranes and p62. TRIM13 is a transmembrane protein localized in the ER membrane, and it is an ubiquitin dependent ER-phagy receptor (Ji et al., 2019). K63-ubiquitylated TRIM13 recruits p62, and the TRIM13-p62 complex undergoes oligomerization. The oligomerized complexes are separated along with ER compartments and targeted to autophagosomes (Ji et al., 2019). TRIM32 is required for muscle autophagy in atrophic conditions by binding Activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) and ULK1, and stimulate ULK1 activity through K63-linked polyubiquitin (Di Rienzo et al., 2019).

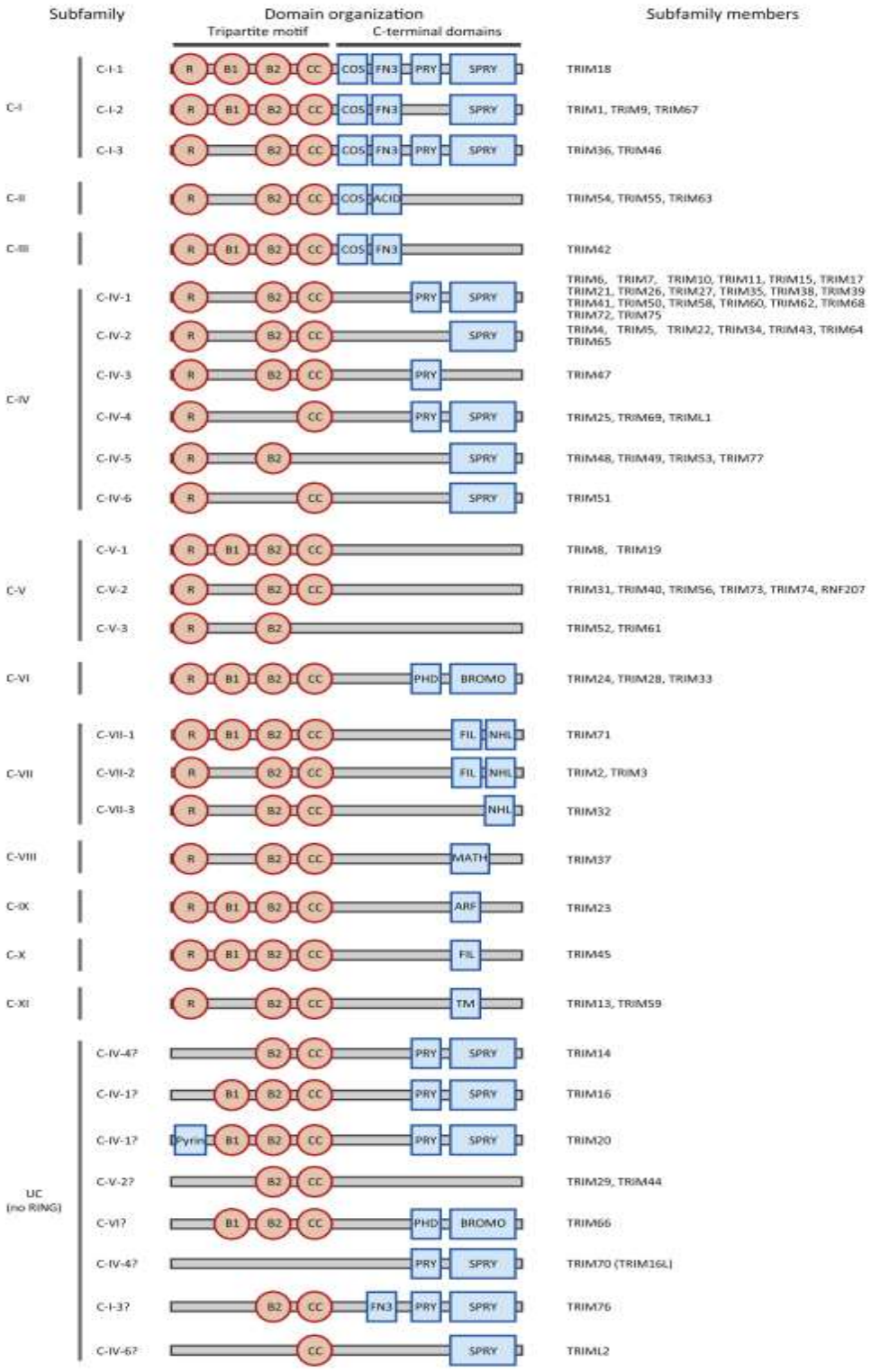

Figure 9: The TRIM family proteins are classified from C-1 to C-XI, and an unclassified group which lacks the RING domain. Abbreviations: R, RING finger domain; B1, B-box domain 1; B2, B-box domain 2; CC, coiled-coil domain; COS, cos box; FN3, fibronectin type III repeat; PRY, PRY domain; SPRY, SPRY domain; ACID, acid-rich region; FIL, Filamin-type IG domain; NHL, NHL domain; PHD, PHD domain; BROMO, bromodomain; MATH, Meprin and TRAF homology domain; ARF, ADP-ribosylation factor family domain; TM, transmembrane region. Figure from Hatakeyama (2017) with permission from Elsevier.

TRIM proteins are associated with several diseases, including cancer, infectious diseases, developmental diseases and neuropsychiatric disorders (Watanabe and Hatakeyama, 2017). Many TRIM proteins have roles in cancers, both as tumor suppressors and as oncogenes. Translocation of TRIM19 is involved in acute promyelocytic leukaemia (APL) (Goddard et al., 1991; Kakizuka et al., 1991). TRIM24 is an oncogenic transcriptional activator that promotes cell growth in prostate cancer cells, and as a proliferation regulator in gastric cancer and hepatocellular carcinoma (Fang et al., 2017b; Groner et al., 2016). TRIM28 have been reported to play a role in regulating DNA damage response (DDR) (White et al., 2012), cell differentiation (Chen et al., 2012a), immune response (Tie et al., 2018) and tumorigenesis (Fitzgerald et al., 2013; Hu et al., 2015). Furthermore, TRIM28 function as a tumor promoter in B-cell non-Hodgkin lymphoma (B-NHL) (Zhang et al., 2018b). TRIM22 and TRIM24 target p53 for degradation (Appikonda et al., 2016; Obad et al., 2004) while TRIM13 overexpression causes stabilization of p53 followed by induction of apoptosis (Joo et al., 2011a). TRIM29 regulates the Wnt/β-catenin signaling pathway and acts as an oncogene in gastric cancer (Qiu et al., 2015), while TRIM8 is downregulated in glioma tissues and interacts with and negatively regulates Protein inhibitors of activated STAT (PIAS3) (Micale et al., 2015; Okumura et al., 2010).

TRIM proteins also play important roles in innate immunity. TRIM52 activates NF-κB signaling, and this activation is dependent on the RING domain. Furthermore, TRIM52 expression is induced by tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) expression (Fan et al., 2017). TRIM38 interacts with TNFR-associated factor 6 (TRAF6) and promotes K48-linked polyubiquitylation and thereby proteasomal degradation of TRAF6 (Zhao et al., 2012). TRIM39 interacts with Cactus interactor (Cactin). Cactin inhibit NF-κB and Toll-like receptor (TLR)-mediated transcriptions, and TRIM39 suppresses the NF-κB signal through stabilization of Cactin (Suzuki et al., 2016). TRIM7 may serve as a positive regulator of TLR4-mediated signaling (Lu et al., 2019), while TRIM9 is a positive regulator of type I interferon (IFN) signaling and a negative regulator of NF-κB activation (Qin et al., 2016; Shi et al., 2014).

Furthermore, TRIM proteins have roles in other diseases. Single nucleotide polymorphisms (SNPs) in TRIM10, TRIM15, TRIM26, TRIM39 and TRIM40 were discovered using a genome wide association (GWA) studies in multiple sclerosis (MS) (Baranzini et al., 2009). TRIM3 is upregulated in schizophrenia (Martins-de-Souza et al., 2009), and a GWA study found a mutation in the RING domain of TRIM19 in schizophrenia patients (Xu et al., 2011). Another GWA study found that two SNP mutations in TRIM76 are associated with schizophrenia (Chen et al., 2011). A mutation in TRIM20 is found to be involved in onset of

Alzheimer's disease (Arra et al., 2007). Three different TRIM proteins are shown to associate with attention deficit hyperactivity disorder (ADHD): a rare copy number variations in TRIM32, a SNP in TRIM31 and a SNP in TRIM33 (Lionel et al., 2011; Rizzi et al., 2011; Xia et al., 2014). TRIM63 mutations are identified in patients with cardiac hypertrophy (Chen et al., 2012b).

TRIM32

TRIM32 is a 653 amino acids long protein and it has six NHL (NCL-1/HT2A/LIN-41, which are the proteins where the domain initially was described) repeats as its C-terminal domain and is therefore part of the NHL-family (Figure 9, 10). The NHL domain is involved in protein dimerization and substrate recognition (Koliopoulos et al., 2016). Furthermore, Loedige et al. (2014) found that all TRIM-NHL proteins have a positively charged top surface, which may suggest that RNA binding is a conserved feature of the NHL-family. However, the RNA targets may vary between the NHL domains and therefore the TRIM proteins in this family may have different roles (Kumari et al., 2018).

Figure 10: Domains of TRIM32 and the localization of the pathogenic mutations. Abbreviations: BBS11, Bardet Biedl syndrome 11; LGMD2H, Limb girdle muscular dystrophy 2H; RING, Really interesting new gene; NHL, NCL-1/HT2A/LIN-41. Figure from Shieh et al. (2011) with permission from Elsevier.

TRIM32 is expressed throughout the body but with highest expression in the heart and brain (Frosk et al., 2002). The B-box cooperate with the RING domain to specify TRIM32 subcellular localization (Lazzari et al., 2019). Furthermore, studies suggests that the B-box is coordinating TRIM interactions, both self-interaction and protein-protein interactions (Li et al., 2011; Tao et al., 2008; Wallenhammar et al., 2017). The B-box contains a zinc-finger motif and it has therefore been suspected that it confer some E3 ligase activity (Bell et al., 2012). However, a recent study showed that this was not the case for TRIM32 (Lazzari et al., 2019).

During cell division, TRIM32 localizes at the spindle poles through a recruitment mediated by Cdk1/cyclin B phosphorylation (Izumi and Kaneko, 2014). At the spindle poles TRIM32 interacts with and poly-ubiquitylates MYCN and destines it for degradation, thereby inducing asymmetric cell division (Izumi and Kaneko, 2014). TRIM32 associates with

myofibrils and ubiquitylates actin, and likely has a role in myofibrillar protein turnover during muscle adaption (Kudryashova et al., 2005). TRIM32 is important for neuronal differentiation of neural progenitor cells and ubiquitylates cMyc, targeting it for degradation (Hillje et al., 2011). In mice, the myogenic differentiation is disrupted in adult muscle stem cells in absence of TRIM32 (Nicklas et al., 2012). Furthermore, TRIM32 interacts with the deubiquitylating enzyme herpesvirus- associated ubiquitin specific protease (USP7) and USP7 deubiquitylates and stabilizes c-Myc (Nicklas et al., 2019). TRIM32 interacts with Argonaute1 (Ago1), but Ago1 is not ubiquitylated by TRIM32. However, TRIM32 activates miRNA Let-7a through the Ago1 interaction (Schwamborn et al., 2009). Cyclic GMP-AMP synthase (cGAS) detect virus infection and produces a second messenger to activate stimulator of IFN genes (STING). TRIM32 has a role in the cGAS-STING pathway by ubiquitylating NEMO, which subsequently activates IKKβ. IKKβ is required for TBK1 and NF-κB activation, which are required for the cGAS-STING activation (Fang et al., 2017a). Furthermore, TRIM32 ubiquitylates STING itself at multiple residues, which promotes the interaction of STING with TBK1 (Zhang et al., 2012). Other targets of TRIM32 includes dysbindin, p53, Oct4, PIAS3 and N-myc down-regulated protein 2 (NDRG2) (Bahnassawy et al., 2015; Liu et al., 2014; Locke et al., 2009; Mokhonova et al., 2015; Qu et al., 2007).

TRIM32 has a role in autophagy by regulating ULK1 activity in muscles under atrophy conditions (Di Rienzo et al., 2019). TRIM32 is transported to ULK1 by AMBRA, an autophagy cofactor, to stimulate its kinase activity through unanchored K63-linked polyubiquitin chains. Notably, in a recent study Di Rienzo et al. (2019) found that mutations in TRIM32 responsible for the LGMD2H disrupts its ability to bind ULK1 and induce autophagy in muscle cells.

TRIM32 undergoes auto-ubiquitylation in the presence of E2 enzymes belonging to the subfamily of D and E enzymes. Furthermore, TRIM32 produce K63- polyubiquitin chains with the E2 UBE2N (Kudryashova et al., 2005; Lazzari et al., 2019; Napolitano et al., 2011).

TRIM32 is associated with limb girdle muscular dystrophy 2H (LGMD2H) through seven mutations in the NHL domain (D487N, R394H, V591M, D588del, T520TfsX13, L535SfsX21 and I590LfsX38) (Frosk et al., 2002; Servian-Morilla et al., 2019), and a completely unrelated disease, a multisystemic disorder called Bardet Biedl Syndrome 11 (BBS11) through a point mutation in the B-box domain (P130S) (Chiang et al., 2006). BBS11 is characterized by obesity, retinal degradation, genito-urinary tract malformations, and cognitive impairment, but not showing muscle alterations (Chiang et al., 2006). Interestingly, the TRIM32 LGMD2H mutants D487N and R394H lost their E3 ligase activity and were unable to ubiquitylate dysbindin (Locke et al., 2009). Kudryashova et al. (2009) created a murine

TRIM32 KO model and found that muscular dystrophy due to TRIM32 mutations involves both neurogenic and myogenic characteristics. Furthermore, studies on the TRIM32 KO mice have shown that TRIM32 plays a key role in muscle regrowth after atrophy, but it is not necessary to trigger muscle atrophy (Kudryashova et al., 2012). The same group generated TRIM32 knock in (KI) mice and found that the LGMD2H mutation destabilizes the protein, leading to its degradation, and results in the same phenotype as the TRIM32 null mice (Kudryashova et al., 2011). The pathological mechanism of LGMD2H is unknown, however there are theories that the muscle dystrophy is due to the loss of E3 ligase activity or the loss of interaction properties of the mutated TRIM32 (Frosk et al., 2002; Locke et al., 2009; Saccone et al., 2008).

TRIM32 has important roles in other diseases than LGMD2H and BBS11, including cancers. TRIM32 is upregulated in breast tumors and promote cell growth through activation of the NFκB signaling pathway (Zhao et al., 2018). It activates RARα-mediated transcription and thereby function as a tumor suppressor (Sato et al., 2011). TRIM32 also acts as an oncogene because it degrades tumor suppressor Abl interactor 2 (ABI2) and thereby promotes cell proliferation, transformation and metastasis in squamous cell carcinoma, papilloma and head and neck carcinoma (Horn et al., 2004; Kano et al., 2008). TRIM32 is upregulated in hepatocellular carcinoma (HCC) cell tissues and cell lines. Furthermore, the overexpression was coupled with inhibition of apoptosis (Cui et al., 2016). A recent study found that TRIM32 interacts with and degrades the transcription factor Gli1, and thereby negatively regulates the sonic hedgehog (SHH) signaling. By this regulation of SHH signaling TRIM32 acts as a tumor suppressor (Wang et al., 2019). TRIM32 has an important role in p53-mediated cellular stress responses. In response to stress, p53 induce expression of TRIM32. In turn, TRIM32 interacts with p53 and promotes p53 degradation. Thus, TRIM32 and p53 form an auto-regulatory negative feedback loop for p53 regulation in cells (Liu et al., 2014). TRIM32 also regulate Parkinson's disease (PD) by positively influencing the PD associated gene alpha-synuclein (snca) (Pavlou et al., 2017).

TRIM27

TRIM27, also called Zinc finger protein and RET finger protein (RFP), is 513 amino acids long, has a PRY/SPRY domain and is a part of the largest subclass of TRIM proteins, C-IV (Figure 9, 11). The PRY/SPRY domain confer target specificity for the E3 ligase activity (Ozato et al., 2008). TRIM27 was originally identified as the fusion partner of RET tyrosine kinase proto-

oncogene (Takahashi et al., 1985). TRIM27 may be localized to cytoplasm, nucleus and plasma membrane depending on the cell type (Tezel et al., 1999).

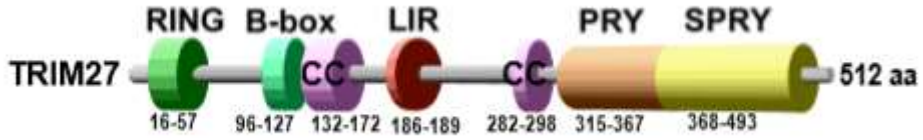

Figure 11: Domains of TRIM27. Abbreviations: RING, Really interesting new gene; LIR, LC3 interacting region; PRY, PRY domain; SPRY, SPRY domain; CC, coiled coil domain.

TRIM27 has roles in cell proliferation, transcriptional repression, negative regulation of NF-κB activation, apoptosis and innate immune response (Ma et al., 2016; Shimono et al., 2000; Zha et al., 2006; Zheng et al., 2015). TRIM27 interacts with several transcription factors, including enhancer of polycomb (EPC1), Mi-2β, retinoblastoma protein (RB1) and basic helix-loop-helix (bHLH) family members (Bloor et al., 2005; Krutzfeldt et al., 2005; Shimono et al., 2000; Shimono et al., 2003). TRIM27 makes a complex with USP7, and the TRIM27-USP7 complex regulate complexes involved in endosomal trafficking, immune responses, autophagy and apoptosis (Cai et al., 2018; Hao et al., 2015; Zaman et al., 2013). Recently it was shown that TRIM27 mediates signal transducer and activator of transcription 3 (STAT3) activation promoting colitis and colitis-associated carcinogenesis (Zhang et al., 2018a). STAT3 is overexpressed and constitutively active in triple-negative breast cancer cells and contributes to cell survival, proliferation, invasion, angiogenesis, immunosuppression and chemoresistance by regulatin expression of its downstream targets (Qin et al., 2019).

TRIM27 is highly expressed in a number of cancers, including breast, endometrian, ovarian, lung and colon cancers (Horio et al., 2012; Iwakoshi et al., 2012; Tezel et al., 2002; Tezel et al., 2009; Tsukamoto et al., 2009). High expression of TRIM27 predicts poor prognosis in breast and endometrial cancer (Zoumpoulidou et al., 2012). TRIM27 interacts with PML nuclear bodies (NB) through the B-box and CC domains of TRIM27. Furthermore, TRIM27 have a role in regulating cellular growth and differentiation in PML (Cao et al., 1998). High expression of TRIM27 correlates with proliferation, migration and chemosensitivity for some tumor cells, and TRIM27 could act as a potential prognostic indicator in some cancers. Tezel et al. (2009) found that TRIM27 is localized in breast carcinomas and not in benign breast tissue.

TRIM27 binds and ubiquitylates nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and NOD2 mutations are linked to susceptibility to Crohn's disease.

TRIM27 was found to have increased expression in Crohn's disease patients (Zurek et al., 2012).

Aim of study

This study cover works on TRIM proteins and their roles in autophagy. At the time this study was initiated, certain TRIM proteins had been recognized to act as autophagy receptors. Our hypothesis was that within the nearly 80 different proteins of the TRIM family, additional ones are degraded by autophagy and act as autophagy receptors.

The following aims were defined to test this hypothesis:

1. Apply a fluorescent double-tagged- screen to identify TRIM proteins degraded by the lysosome
2. Identify if these TRIM proteins have roles as autophagy regulators
3. Identify if these TRIM proteins have characteristics as autophagy receptors

Summary of papers

Paper I

TRIM32, but not its muscular dystrophy-associated mutant, positively regulates and is targeted to autophagic degradation by p62/SQSTM1.

Overå, K.S., Garcia, J.G., Bhujabal, Z., Jain, A., Øvervatn, A., Larsen, K.B., Deretic, V., Johansen, T., Lamark, T., and Sjøttem, E. (2019).

J. Cell Science. PMID 31828304.

In this paper we employed a fluorescence- based double tag screen of 22 TRIM proteins, representing each of the TRIM subfamilies. Interaction studies *in vivo* and *in vitro* revealed co-localization of TRIM32 and p62 and LC3B, and direct binding between TRIM32 and p62. Furthermore, autophagy assays showed that lysosomal degradation of TRIM32 was dependent on ATG7 and the sequestosome-like receptors receptors. p62 was able to mediate degradation of TRIM32, while ubiquitylation assays identified p62 as a substrate of TRIM32. TRIM32 mono-ubiquitylated p62 on lysine residues involved in regulation of p62 activity. The muscle dystrophy LGMD2H mutant of TRIM32 was not able to undergo autophagic degradation, and also failed to ubiquitylate p62. In contrast, the BBS11 mutant strongly facilitated p62 ubiquitylation, sequestration and degradation. This indicates a dual role for TRIM32 in autophagy, both as a p62 cargo and as a positive regulator of p62. Importantly, the functional assays of the LGMD2H disease mutant point towards dysfunctional TRIM32 mediated regulation of p62 as a pathological mechanism in LGMD2H.

Paper II

TRIM32 – a putative regulator of NDP52 mediated selective autophagy

Overå, K.S., Bhujabal, Z., Garcia, J.G., Sjøttem, E.

Manuscript

In paper II we show that TRIM32 downregulates the protein levels of all SLR proteins, without affecting their expression at the RNA level. Autophagic degradation of TRIM32 is dependent on SLRs, and we found that NDP52 are able to direct TRIM32 to degradation in the lysosome. TRIM32 interacts directly with and ubiquitylates NDP52. Furthermore, a mitophagy assay showed that mitophagy is reduced in HEK293 TRIM32 KO cells compared to normal HEK293 cells and TRIM32 KO cells reconstituted with TRIM32. Expression of TRIM32 WT and the BBS11 mutant in the TRIM32 KO cells enhanced ULK1 stability and TBK1

autophosphorylation, while expression of the LGMD2H mutant did not. Thus, this study identify TRIM32 as a potential regulator of NDP52, ULK1 and TBK1, facilitating mitophagy.

Paper III

TRIM27 is an autophagic substrate and putative regulator of LC3B

Garcia, J.G., Overå, K.S., Knutsen, E., Bhujabal, Z., Evjen, G., Lamark, T., Johansen, T., Sjøttem, E.

Manuscript

Here we identify TRIM27 as an autophagic substrate, depending on ATG7 and the SLRs for its lysosomal degradation. We mapped a LIR domain in its coiled coil region with specificity towards LC3C and GABARAP. Establishment of TRIM27 KO cells showed that TRIM27 do not affect the starvation induced autophagy response. However, loss of TRIM27 affected p62 and LC3B protein levels under nucleotide mediated stress, mimicking virus induced activation of the innate immune response. The HEK293 TRIM27 KO cells displayed enhanced proliferation rate and reduced cell migration, suggesting that TRIM27 may have a role in epithelial-mesenchymal transition. Both TRIM27 and LC3B were aberrantly expressed in various breast cancer cell lines, and their expression levels seemed to be inversely correlated. Thus, these cell lines are potent model systems to identify the roles of TRIM27 and autophagy in breast cancer.

Discussion

In this thesis, we started out by doing a screen of 22 different TRIM proteins representing each of the 11 TRIM family subgroups to identify candidates with a potential role in autophagy. Earlier publications had already recognized a number of TRIMs, including TRIM5α, TRIM13, TRIM16, TRIM20, TRIM21 and TRIM63, as regulators and receptors in selective autophagy (Chauhan et al., 2016; Kimura et al., 2017; Kimura et al., 2016; Mandell et al., 2014b). In our screen we further identified TRIM32, TRIM27 and TRIM45 as potential autophagic substrates. In paper I we investigated the role of TRIM32 in autophagy, and found it to be a substrate of p62. Furthermore, we found TRIM32 to be a direct interactor of p62, and to regulate p62 activity by ubiquitylation. This ubiquitylation was abolished in mutants of TRIM32 implicated in the muscular dystrophy disease LGMD2H. In paper II we studied the role of TRIM32 in association with the four other SLRs, and found it to interact with and ubiquitylate NDP52. NDP52 is involved in mitophagy and xenophagy in cooperation with the ULK1 complex and TBK1. Interestingly, we found mitophagy to be reduced in TRIM32 KO cells. In paper III, we identify TRIM27 as an autophagic substrate. However, TRIM27 does not seem to affect autophagy activity under normal or starved conditions. However, under infectious stress mimicked by poly (AT) or plasmid transfections, the p62 and LC3B response was impaired in TRIM27 KO cells. Furthermore, we found TRIM27 mRNA levels to be strongly upregulated in cancer tissue from breast cancer patients, and in various breast cancer cell lines TRIM27 was aberrantly expressed and its expression level seemed to be inversely correlated with LC3B expression.

For a more detailed discussion than provided below, please look at the discussion in each of the papers.

TRIM32 and autophagy proteins

LC3-I is localized in the cytosol, but it is conjugated to PE to form LC3-II, which is localized on the outer and inner membrane of the autophagosome (Kabeya et al., 2000). LC3 is a widely used autophagy marker because the number of LC3-II reflects the number of autophagosomes (Yoshii and Mizushima, 2017). Another marker for autophagy is p62 because it binds to LC3 and is selectively degraded by autophagy (Pankiv et al., 2007). We found a direct interaction between TRIM32 and the SLRs p62 and NDP52, and we found the protein levels of all five SLRs to be modified by $TRIM32^{WT}$ and the BBS11 mutant, but not by the LGMD2H disease mutant. The formation of p62 bodies was reduced in the TRIM32 KO cells while it was strongly

induced in the TRIM32 KO cells reconstituted with $TRIM32^{WT}$ and $TRIM32^{P130S}$. $TRIM32^{D487N}$, however, was not able to rescue the p62 dot formation. Notably, inhibition of autophagosome degradation also increases the amount of LC3-II and p62 (Bjorkoy et al., 2005; Tanida et al., 2005). Furthermore, LC3-II can localize on non-autophagy structures that are not turned over in the lysosome, particularly if overexpressed by transient transfection or if aggregates are formed in the cells (Kuma et al., 2007). Therefore, the amount of LC3-II and p62 at any given time does not necessarily represent the degradation of autophagosomes. To distinguish whether autophagosome accumulation is due to autophagy induction or inhibition of degradation, autophagic flux assays can be performed (Mizushima et al., 2010). We looked at the autophagic flux by looking at the protein levels of the SLRs and LC3B in TRIM32 KO cells and TRIM32 KO cells reconstituted with TRIM32 wild type and disease mutants. The levels of the SLRs are reduced in the cells reconstituted with E3 ligase active TRIM32. Thus, E3 ligase activity of TRIM32 seems to be important for autophagic degradation of SLRs and for p62 dot formation, either through phase separation or through sequestration.

TRIF is an adaptor protein that promotes inflammatory mediators and activation of antimicrobial responses, and it is essential for TLR3- and TLR4- mediated signaling pathways (Ullah et al., 2016; Yamamoto et al., 2003). TRIF leads to activation of IRF3 through TBK1 and it is involved in the NF-κB activation pathway through recruitment of receptor-interacting protein 1 (RIP1) (Liu et al., 2015; Meylan et al., 2004). TRIM32 is involved in the autophagic degradation of TRIF, and knockdown of LC3B inhibited TRIM32-mediated degradation of TRIF. Furthermore, overexpression of TRIM32 enhanced the interaction of LC3B with TRIF (Yang et al., 2017). These results suggest that TRIM32 mediates degradation by the autophagy-lysosome pathway, further supporting our results that TRIM32 facilitates selective autophagy. However, we could not detect a strong and reproducible effect on LC3B lipidation in the TRIM32 KO and reconstituted cells, suggesting that TRIM32 has a role in SLR mediated selective autophagy, and not global autophagy as such.

We found that the disease mutant $TRIM32^{D487N}$ is not an autophagy substrate, suggesting dependence on it oligomerization and/or auto-mono-ubiquitylation abilities to be recognized as an autophagic substrate. This is in contrast to a recent report showing BafA1 mediated stabilization of a TRIM32 LGMD2H mutant in primary myoblasts isolated from a patient (Servian-Morilla et al., 2019). This discrepancy may be explained by the different cell systems, as we used HEK 293 cells, HeLa cells and C2C12 cells, while Servian-Morilla et al. (2019) used primary myoblasts.

TRIM32 is a regulator of ULK1 activity in atrophic muscle cells, and the regulation is dependent on the E3 ligase activity of TRIM32 and on AMBRA1 (Di Rienzo et al., 2019). AMBRA1, interacting with the E3 ligase TRAF6, ubiquitylates ULK1 with K63-linked ubiquitin chains (Nazio et al., 2013). We immunoprecipitated ULK1 with $TRIM32^{WT}$ and mutants to detect whether TRIM32 regulate ULK1 through ubiquitylation. However, we could not detect any ubiquitylation of ULK1. This is in line with the work of Di Rienzo et al. (2019), where they found that ULK1 is not a direct substrate of TRIM32. We found that ULK1 is stabilized in cells expressing E3 ligase active TRIM32, but not in cells expressing the LGMD2H disease mutant. The stabilization of ULK1 is required for starvation-induced autophagy. Stabilization of ULK1 is achieved through post- translational modifications, such as ubiquitination, phosphorylation and dephosphorylation of specific sites (Xie et al., 2015b). Thus, TRIM32 may have a role in the post- translational modifications of ULK1, such as by regulating ULK1 modifiers.

Ubiquitylation of p62

The PB1 domain of p62 oligomerize in a front to back fashion, driving the formation of helical filaments (Ciuffa et al., 2015). Dimerization and ubiquitylation of p62 is incompatible with each other because the dimer occludes the ubiquitin binding sites (Isogai et al., 2011; Long et al., 2010). Peng et al. (2017) identified two E2 conjugation enzymes, UBE2D2 and UBE2D3, which interacts directly with p62 via the EIR region and mediates ubiquitylation at several lysine residues, among them K157, K295 and K420. This polyubiquitylation is thought to relieve the inhibition of the UBA domain in the p62 UBA dimer, leading to enhanced binding and tethering of ubiquitylated cargo to the ATG8s conjugated to the phagophore (Conway and Kirkin, 2017). In our studies we identified by MS that $TRIM32^{WT}$ and the BBS11 disease mutant $TRIM32^{P130S}$ ubiquitylate p62 at K157 and K295. Ubiquitylation assays showed that TRIM32 may mono-ubiquitylate p62 on several residues, enhancing p62 body formation and p62- mediated autophagic activity. We were not able to map the exact lysine ubiquitylated by TRIM32, which indicates that multiple lysine residues may be potential targets of TRIM32. Another possibility is that another lysine than those we looked at is modified by TRIM32. Furthermore, we found that the LGMD2H disease mutants were unable to mono-ubiquitylate p62. Interestingly, the interaction with p62 was not inhibited. Other studies have found that LGMD2H mutations impair the interaction between TRIM32 and Piasy and ULK1 (Albor et al., 2006; Di Rienzo et al., 2019). Thus, the binding site for p62 has to be somewhere else than

the binding sites for Piasy and ULK1. Furthermore, the interaction between TRIM32 and ULK1 and Piasy may depend on the E3 ligase activity of TRIM32, while the interaction with p62 does not.

In our studies we see mono-ubiquitylation of p62. Most studies find that TRIM32 poly-ubiquitylates its substrates (Di Rienzo et al., 2019; Nicklas et al., 2012; Streich et al., 2013). However, one study report TRIM32 to both mono- and poly-ubiquitylate Piasy (Albor et al., 2006). Hence, while it is not common for TRIM32 to mono-ubiquitylate its substrates it has been reported previously. Further studies should look at the differences between the mono- and poly-ubiquitylation conducted by TRIM32. Peng et al. (2017) looked at the ubiquitylation of p62 and found that p62 is ubiquitylated upon autophagy activation. They found that heat-shock treatment led to elevated levels of polyubiquitylated conjugates. It would be interesting to study at the difference between mono- and poly-ubiquitylated p62 and their role in autophagy.

There is also the possibility that ubiquitylated p62 creates high-complexity scaffolds (Conway and Kirkin, 2017). K63- polyubiquitin chains, but not monoubiquitin, can induce p62 phase separation. Furthermore, p62 polymerization and the interaction between p62 and polyubiquitin chains through the UBA domain are important for the p62 phase separation (Sun et al., 2018). Even though Sun et al. (2018) found that mono-ubiquitylation of p62 does not induce phase separation of p62, the combinations of different post-translational modifications will likely have impact on p62 functions, among them autophagy.

The p62 K157 site is evolutionary conserved and localized in the zinc finger domain. Vault RNA1-1 bind to the ZZ domain and modulate its dimerization and autophagic activity (Horos et al., 2019). RIP, a TNFα signaling adaptor, also binds to the ZZ domain and this interaction serves to link the atypical protein kinase C (aPKC) to activation of NF-κB (Sanz et al., 1999). Hence, post-translational modification of the ZZ domain could regulate such interactions, and this would be interesting to study in further works.

The p62 K295 site lies at the border of the EIR domain and a previously predicted PEST domain, and adjacent to the LIR domain. The PEST sequence serves as a proteolytic signal for rapid degradation, leading to short intracellular half-lives (Liu et al., 2016b). Thus, disruption of these sites may possibly impact the ability of p62 to bind to the ATG8 proteins, it could regulate the interaction with E2 enzymes and it could prevent degradation of p62 and thereby have impact on p62 stability.

TRIM32 and mitophagy

We found that TRIM32 directly interacted with and colocalized with NDP52. Studies have demonstrated that NDP52 plays an important role in selective degradation of depolarized mitochondria (Heo et al., 2015; Lazarou et al., 2015; Vargas et al., 2019). Tethering of NDP52 to the mitochondria recruited the ULK1 complex via FIP200. We induced mitophagy by overexpression of FKBP8 and LC3A, and mitophagy was measured using the double- tagged mCherry-GFP-OMP25TM (Bhujabal et al., 2017). We observed a reduction in mitophagy in the TRIM32 KO cells compared to the normal HEK293 cells. Importantly, reintroduction of TRIM32 to the TRIM32 KO cells restored the mitophagy activity.

TBK1 recruits NDP52 to the mitochondria and facilitates the association of NDP52 with the ULK1 complex (Heo et al., 2015; Vargas et al., 2019). Enrichment of TBK1 on the mitochondrial membrane induces TBK1 S172 autophosphorylation (Kishore et al., 2002; Ma et al., 2012). When we assessed the TBK1 S172 phosphorylation in TRIM32 KO and reconstituted cell lines we found that the amount of S172 phosphorylated TBK1 is highly upregulated in the TRIM32 KO cell lines reconstituted with TRIM32, compared to the TRIM32 KO cells. This suggest that TRIM32 may facilitate TBK1 recruitment to the mitochondrial membrane, and thereby activation which is important for efficient mitophagy. TRIM32 has been reported to be localized at the mitochondria and ER, which further strengthen the possibility that TRIM32 has a role in mitophagy (Zhang et al., 2012). Furthermore, TRIM32 interacts with AMBRA1, and AMBRA1 is known to promote mitophagy with PARKIN and HUWE1 (Di Rienzo et al., 2019; Di Rita et al., 2018; Van Humbeeck et al., 2011).

An important question for further studies is whether the TRIM32 mediated ubiquitylation of NDP52 directly facilitates its role as a receptor in selective autophagy, such as mitophagy.

Pathological mechanisms of LGMD2H

Muscle growth occurs during development and in response to mechanical overload and anabolic hormonal stimulation. During development, the muscle growth depends on protein and cellular development. Ubiquitin signaling and the autophagy-lysosome pathway are important quality control mechanisms for muscle proteins. Muscle atrophy involves the shrinkage of myofibrils due to a net loss of proteins, organelles and cytoplasm, and the protein degradation exceeds the protein synthesis. UPS and autophagy are activated during muscle atrophy (Sandri, 2013). Vici syndrome is a muscle dystrophy that is associated with defective autophagy through a mutation in the autophagic gene EPG5 (Cullup et al., 2013).

Four of the TRIM32 LGMD2H mutations, R394H, D487N, D588del and T520TfsX13, are shown to be unable to self-interact (Saccone et al., 2008). The remaining LGMD2H mutations were not identified yet at the time of the study conducted by Saccone et al. (2008), and were therefore not included. In our study, we show that the LGMD2H mutants, but not the BBS11 mutant, are unable to self-ubiquitylate as well. This is supported by a study showing that the TRIM32 LGMD2H mutants D487N and R394H have impaired E3 ligase activity and is unable to ubiquitylate dysbindin (Locke et al., 2009). Interestingly, TRIM32 dimerize through the RING domain, and this dimerization is crucial for the catalytic activity (Koliopoulos et al., 2016). Thus, the impaired oligomerization and ubiquitylation properties may be important for the development of LGMD2H.

The molecular mechanisms of the TRIM32 mutants causing LGMD2H and BBS11 are far from understood. We show that the LGMD2H disease mutant of TRIM32 is unable to mono-ubiquitylate p62. The mutations responsible for LGMD2H is localized in the NHL domain, and they do not inhibit the binding of TRIM32 to p62. Previous studies have reported that the LGMD2H mutants display a more diffuse cytoplasmic localization than $TRIM32^{WT}$ and $TRIM32^{P130S}$, which are enriched in puncta in most cells (Locke et al., 2009). Furthermore, knock in mice carrying the mutation in murine TRIM32 corresponding to the human D487N mutation had normal mRNA levels of TRIM32 but a severe reduction in mutant TRIM32 at the protein level (Kudryashova et al., 2011). Supporting this, the LGMD2H mutations seems to lead to reduced TRIM32 protein levels in muscle samples from LGMD2H patients (Servian-Morilla et al., 2019). It is known that the self-ubiquitylation of TRIM32 is important for its activation. We show that this mono-ubiquitylation may be important for degradation of TRIM32 through selective autophagy. TRIM32 has been shown in several reports to ubiquitylate proteins whose deregulation might influence the onset and progression of muscular dystrophy, including actin, tropomycin, troponins, α-actinin, dysbindin and c-Myc (Tocchini and Ciosk, 2015). Interestingly, p62 is strongly implicated in Chaperone-assisted selective autophagy (CASA), which is essential for muscle maintenance (Arndt et al., 2010). We found that the LGMD2H mutations results in an accumulation of the SLRs and reduced autophagic activity of p62 and NDP52. Reduced autophagic activity may lead to accumulation of aggregates. Interestingly, a study looking at biopsies from LGMD2H patients found an increase of cytoplasmic bodies in the muscle cells (Servian-Morilla et al., 2019), which may be caused by decreased autophagic activity. Hence, LGMD2H may be due to dysfunctional selective autophagy. These findings contribute to the understanding of the pathological mechanisms of LGMD2H.

TRIM27 as a regulator of xenophagy

TRIM27 belongs to the same TRIM family subclass as TRIM5α, which has been shown to act as a selective autophagic receptor, targeting a restricted virus to the autophagosomes for degradation (Mandell et al., 2014a). They demonstrated that TRIM5α interacts directly with ATG8s via a LIR motif. Here, we identified a putative LIR in the coiled coil region of TRIM27. However, TRIM27 does not undergo autophagic degradation in the absence of the SLRs and is therefore not an autophagic receptor, but rather an autophagic substrate.

Xenophagy is the cells defense mechanism against invading pathogens. Damage to the phagophore containing pathogens expose glycans to cytosol. These glycans attract Gal8, which is recognized by NDP52 (Thurston et al., 2012). NDP52 then recruits NAP1, similar to NAP1 TBK1 adaptor (SINTBAD), TBK1 and LC3C (Shpilka and Elazar, 2012; von Muhlinen et al., 2012). All of the SLRs have a LIR domain and interacts with ATG8 proteins, but the bindings are selective. LC3C is the only ATG8 that interacts with NDP52 and has a specific role in xenophagy (von Muhlinen et al., 2012). In absence of LC3C, the remaining ATG8 orthologues are unable to support efficient antibacterial autophagy. We found that TRIM27 interacts with LC3C, GABARAP and GABARAPL1, but not to the other ATG8 proteins. When the LIR region was mutated, we got a strong reduction in the binding to LC3C, and some reduction in the binding to GABARAP and GABARAPL1. Furthermore, TRIM27 regulates innate immune response by interacting with and mediating K48-linked ubiquitylation of NOD2 (Zurek et al., 2012). Together these results propose that TRIM27 could have a role in xenophagy, representing a link between innate immunity and selective autophagy.

By looking at the TRIM27 KO cells we found that the starvation-induced degradation of p62 and LC3B is abolished when the cells are stressed by plasmid transfection. TRIM27 interacts with USP7, which has been shown to inhibit the RNA virus mimic poly I:C- mediated IFN-stimulated response element (ISRE) and IFN-β activation (Cai et al., 2018). We infected the TRIM27 KO cells with poly AT that mimics DNA virus infection and observed inhibition of the starvation-induced degradation of p62. These results further supports that TRIM27 may have a regulatory effect on autophagy induction under immune stress conditions.

TRIM27, a potential prognostic marker linked to autophagy

Initially, TRIM27 was identified as a gene involved in oncogenic rearrangement with the RET proto-oncogene (Takahashi and Cooper, 1987). In our study, we found HEK293 TRIM27 KO

cells to display enhanced cell proliferation, suggesting that TRIM27 is an inhibitor of cell proliferation. However, it was recently found that knock down of TRIM27 in colorectal cancer cells significantly inhibits proliferation (Zhang et al., 2018c). The observed discrepancy may be due to the different cell lines used. We also found that TRIM27 facilitate cell migration, which is in agreement with previous studies (Lv et al., 2016; Zhang et al., 2018c).

Epithelial-to-mesenchymal transition (EMT) occur naturally during development and tissue repair, but it is also recognized as the event where cancer cells acquire an invasive phenotype by promoting invasion and metastasis (Colella et al., 2019; Li and Li, 2015). EMT involves multiple mechanisms, and among them is the activation of the phosphorylated serine/threonine kinase AKT (p-AKT). There is a complex cross-talk between autophagy and EMT (Colella et al., 2019). Autophagy can modulate EMT through selective degradation of EMT players (Lv et al., 2012). The effect of autophagy on EMT seems to depend on the cell type and the stimulus employed for activating or inhibiting autophagy (Colella et al., 2019). Our proliferation and migration studies suggest that TRIM27 may be implicated in EMT. This is in line with a recent study which found that TRIM27 promotes EMT and activation of p-AKT (Zhang et al., 2018c).

TRIM27 is upregulated in cancer tissue from various cancers, including breast (Tezel et al., 2009), lung (Iwakoshi et al., 2012), ovarian (Horio et al., 2012; Ma et al., 2016) and endometrial (Tsukamoto et al., 2009), compared to normal tissue. Upregulated TRIM27 in breast cancer has been linked to its nuclear role implicated in estrogen receptor transcription complexes, and associated with human epidermal growth factor receptor 2 (HER2) status (Tezel et al., 2009; Townson et al., 2006). We used bioinformatics tools to analyze TRIM27 mRNA expression in cancer tissue and normal tissue from breast cancer patients, and found that TRIM27 mRNA was significantly upregulated in cancer tissue compared to normal tissue. Interestingly, TRIM27 expression varies substantially within the various breast cancer subtypes, and so does LC3B. Notably, the expression of TRIM27 and LC3B seemed somehow inversely correlated in these cell lines. This is in line with our observation of LC3B in the TRIM27 KO cells, where LC3B seemed to be stabilized. Thus, TRIM27 may impact autophagy in breast cancer cells via regulation of the LC3B expression levels. This is an important observation to analyze in further studies.

Methodological considerations

We have used the double- tagged mCherry-EYFP system for identification of TRIM proteins that are substrates for autophagic degradation. This is an overexpression assay, thus we cannot be certain that the endogenous proteins behave in the same manner. Furthermore, we only used an N-terminal mCherry-EYFP tag, and therefore there is a possibility that the tag may have influenced the ability of some proteins to be degraded by autophagy. However, many of the TRIM proteins that were identified as autophagic substrates in our screen are previously shown to have a role in autophagy (Chauhan et al., 2016; Kimura et al., 2015; Mandell et al., 2014a). This indicates that the double-tag assay function well as a screening method for identifying TRIM proteins implicated with autophagy.

We have used the CRISPR/Cas9 technology to generate knock out cell lines (Ran et al., 2013). We made specific guide RNA sequences targeting the gene of interest early in exon 1 using the bioinformatics tools made by the Feng Zhang group (Ran et al., 2013), and cloned them into a plasmid expressing Cas9. The Cas9 makes double-stranded DNA cut after recognition of the protospacer adjacent motif (PAM) sequence and binding of guide RNA with its specific target gene sequence. The guide RNA is normally 21 nucleotides long and can potentially bind to non-target genes leading to off-target effects (Fu et al., 2013). Off-target mutations may be due to non-specific nuclease activity, leading to mutations in regions other than the target. Fu et al. (2013) detected off-target alterations induced by four out of six CRISPR RNA guided endonucleases. Many of the off-target sites are mutagenized with frequencies comparable to the intended on-target site (Fu et al., 2013). Off- target mutations may cause genomic instability and disrupt the functionality of otherwise normal genes (Gupta et al., 2019; Zhang et al., 2015). To reduce the off-target effects, a mutant form of Cas9 have been developed. This mutant Cas9 makes single-stranded DNA nicks and requires binding of two guide RNA target sequences for generating a staggered double-stranded DNA cut (Ran et al., 2013). This increases the specificity of the CRISPR/Cas9 method. Initially we tried making KO cells by this method, however, we did not get any surviving clones. Therefore, our KO cells are made by the double-stranded DNA cut, and thus we cannot rule out the possibility for off-target effects. There is continuous ongoing work to improve the CRISPR/Cas9 method and reduce the off-target effects. Methods to identify if the phenotype in the KO is due to knock out of the gene of interest, or an off target effect, are i) to validate the phenotype in more than one KO clone, ii) to re-introduce the KO gene into the KO clone and verify that the phenotype is reversed to wild type, iii) to sequence the genome of the KO cells, either complete genome

sequencing, or sequence the regions of the genome that have closest similarity to the guideRNA and therefore is most exposed to off-target effects. We verified the KO cell lines by i and ii of the aforementioned methods.

CRISPR/Cas9 cell lines are established from only one cell. However, the cells within a cell line differs in rate of protein expression and metabolic activity. One should therefore be aware of that the differences observed in protein expression may be due to clonal effects and not the KO phenotype. This can be verified by analyzing more than one KO clone, and to compare the phenotypes of the KO clones with the phenotype obtained when the gene of interest is knocked down by siRNA in the mother cell line.

The FlpIn T-REX system was used for generating cell lines with stably expressing TRIM32 and TRIM32 mutants. This system is based on the tetracycline-inducible expression of the gene of interest. An expression level similar to wild type is hard to obtain, therefore the expression level of the proteins in this system is commonly not at the same level as endogenous. The gene expression may be regulated by titrating tetracycline, but it is difficult to achieve the exogenous protein level. Our cell lines stably expressing TRIM32 and TRIM32 mutants have a background of endogenous protein, which may influence the phenotypes of the mutants as TRIM proteins have a tendency to dimerize. Furthermore, the gene of interest is not under control of the endogenous promoter, but a CMV promoter in the FlpIn system. Hence, a main drawback of the stable cell lines is that their expression is not at the same level as the endogenous protein and that they are not regulated in the same way as the endogenous protein. However, we got similar results by using the reconstituted KO cells as the stable cell lines. Notably, the reintroduced TRIM32 proteins in the KO cells were also over-expressed compared to endogenous protein level. Thus, our results obtained in the KO and reconstituted KO cells do not necessarily reflect the endogenous situation, but we believe that they gave some information on the roles of these TRIMs in autophagy.

In the ubiquitylation assay we over-expressed TRIM32 and p62/NDP52 in cells. Hence, we cannot rule out that the ubiquitylation is only due to over-expression of TRIM32. In our initial ubiquitylation experiments we also co-overexpressed ubiquitin. However, we noticed that over-expression of ubiquitin alone induced ubiquitylation of p62. This is in accordance with previous studies, which have found that overexpression of ubiquitin activates autophagy in a p62-dependent manner (Peng et al., 2017). Therefore, we have analyzed TRIM32- mediated ubiquitylation of the SLRs without ubiquitin overexpression, which we believe reflects more the endogenous role of TRIM32.

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Paper I

Paper II

Paper III