

# ER stress: Autophagy induction, inhibition and selection

Harun-Or Rashid,<sup>1</sup> Raj Kumar Yadav,<sup>1</sup> Hyung-Ryong Kim,<sup>2</sup> and Han-Jung Chae<sup>1,\*</sup>

<sup>1</sup>Department of Pharmacology; Medical School; Chonbuk National University; <sup>2</sup>Department of Dental Pharmacology; College of Dentistry; Wonkwang University

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**Abbreviations:** AKT1/AKT, v-akt murine thymoma viral oncogene homolog 1; ALS, familial amyotrophic lateral sclerosis; AMPK, AMP-activated protein kinase; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; ATG, autophagy related; BAX, BCL2-associated X protein; BCL2/Bcl-2, B-cell CLL/lymphoma 2; ERAD, ER-associated degradation; BECN1, Beclin 1, autophagy related; CASP4, caspase 4, apoptosis-related cysteine peptidase; CASP12, caspase 12 (gene/pseudogene); DDIT3/CHOP, DNA-damage-inducible transcript 3; DDIT4/REDD1, DNA-damage-inducible transcript 4; DAPK1, death-associated protein kinase 1; ECM, extracellular matrix; EIF2AK3/PERK, eukaryotic translation initiation factor 2- $\alpha$  kinase 3; EIF2S1/eIF2 $\alpha$ , eukaryotic translation initiation factor 2, subunit 1  $\alpha$ , 35kDa; ER, endoplasmic reticulum; ERN1/IRE1, endoplasmic reticulum to nucleus signaling 1; FOXO, forkhead box O; HD, Huntington disease; HSPA5/GRP78, heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa); Hyp-PDT, hypericin-mediated photodynamic therapy; ITPR/IP3R, Inositol 1,4,5-triphosphate receptor; LIR, LC3-interacting region; MAM, mitochondria-associated membrane; MAP3K5, mitogen-activated protein kinase kinase kinase 5; MAPK8/JNK, mitogen-activated protein kinase 8; mtHTT, mutant HTT (huntingtin); MTOR/mTOR, mechanistic target of rapamycin (serine/threonine kinase); NBR1, neighbor of BRCA1 gene 1; NFE2L2/NRF2, nuclear factor, erythroid 2-like 2; PRKAA1, protein kinase, AMP-activated,  $\alpha$  1 catalytic subunit; PRKCCQ, protein kinase C, theta; TRAF2, TNF receptor-associated factor 2; PI3K, phosphoinositide 3-kinase; PtdIns3K, class III phosphatidylinositol 3kinase complex; RAB7A/RAB7, RAB7A, member RAS oncogene family; RIDD, regulated ERN1 dependent decay; RPS6KA3/RSK2, ribosomal protein S6 kinase, 90kDa, polypeptide 3; SESN2, sestrin-2; SQSTM1/p62, sequestosome 1; TRAF2, TNF receptor-associated factor 2; TRIB3/TRB3, tribbles pseudokinase 3; TSC1/TSC, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; UBL, ubiquitin-like; ULK1, unc-51 like autophagy activating kinase 1; UPR, unfolded protein response; XBP1, X-box binding protein 1.

An accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) leads to stress conditions. To mitigate such circumstances, stressed cells activate a homeostatic intracellular signaling network cumulatively called the unfolded protein response (UPR), which orchestrates the recuperation of ER function. Macroautophagy (hereafter autophagy), an intracellular lysosome-mediated bulk degradation pathway for recycling and eliminating wornout proteins, protein aggregates, and damaged organelles, has also emerged as an essential protective mechanism during ER stress. These 2 systems are dynamically interconnected, and recent investigations have revealed that ER stress can either stimulate or inhibit autophagy. However, the stress-associated molecular cues that control the changeover switch between induction and inhibition of autophagy are largely obscure. This review summarizes the crosstalk between ER stress and autophagy and their signaling networks mainly in mammalian-based systems. Additionally, we highlight current knowledge on selective autophagy and its connection to ER stress.

## Introduction

The ER is a vast membranous network and the major assembly site for almost all secretory and integral membrane proteins. Through translocation mechanisms, nascent proteins enter secretory pathways in the ER,<sup>1</sup> where they are subsequently folded and assembled into higher order complexes through covalent modifications. The availability of molecular chaperones in the packed molecular domain of the ER makes it an ideal and unique milieu for proper protein folding as well as for identifying and marking improperly folded proteins for destruction. Indeed, in this way, only accurately folded proteins that pass ER quality control checkpoints are allowed to exit. In addition, co-translational and post-translational modifications of proteins in the ER finalize their 3-dimensional native structures,<sup>2</sup> facilitating their movement across the ER to endocytotic and exocytotic pathways. However, those quality control systems can be influenced by various extracellular and intracellular stimuli.

Because protein folding is a complex and error-prone process, the protein-folding capacity of the ER can easily be saturated under a number of physiological and pathological insults such as glucose deprivation, aberrant Ca<sup>2+</sup> regulation, viral infection, environmental toxins, hypoxia, oxidative injury, hypoglycemia, mutant protein expression, aging, and simple increases in secretory protein synthesis. To buffer ER stress and orchestrate the

\*Correspondence to: Han-Jung Chae; Email: hjchae@jbnu.ac.kr; Hyung-Ryong Kim; hrkimdp@wonkwang.ac.kr  
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recovery of ER function, cells use 4 different strategies. First, cells focus on translation attenuation for a few hours, thereby lessening the freshly prepared protein load into the ER until mRNAs encoding unfolded protein response (UPR) proteins are processed.<sup>3</sup> In a second attempt, the UPR upregulates the folding machinery by inducing ER chaperone genes. Third, the ER compartment proliferates to accommodate the high protein load and then begins ER-associated degradation (ERAD) of unfolded or misfolded proteins. ERAD mainly consists of 2 mechanisms: ubiquitin-proteasome-dependent ERAD (type I) and autophagy-lysosome dependent ERAD (type II). Type II ERAD represents an autophagic pathway in which both soluble and insoluble forms of misfolded proteins are targeted, whereas type I ERAD targets only soluble misfolded proteins.<sup>4</sup> Initially, it was thought that ER stress initiates autophagy only when aggregated proteins become excessive enough to overwhelm the canonical ubiquitin-proteasome-dependent ERAD. Current findings suggest, however, that ERAD-mediated partially processed proteins are also a target of autophagy, which includes all other unfolded proteins.<sup>5</sup> Finally, programmed cell execution is initiated when stress exceeds a given threshold and the ER is so severely impaired that compensatory mechanisms are no longer able to sustain its function.<sup>6,7</sup>

Although ER stress and autophagy can function independently, they share a number of common features including protecting cells by relieving stress and inducing cell death under extreme conditions. Furthermore, altering the functions of one of these systems can influence the other. Therefore, ongoing work has attempted to integrate the signaling pathways responsible for the induction of autophagy upon ER stress and the cellular consequences in different cell types. Nevertheless, the relationship between autophagy and ER stress is not yet fully understood. This review provides a comprehensive overview of autophagy and its relationship to UPR signaling, and emphasizes recent advances in identifying the underlying mechanisms involved in ER-regulated autophagy that control several unanticipated pathways to cell fate regulation. In addition, we also review the role of ER stress in selective autophagy to provide a better understanding of the broad pathways by which ER stress can regulate autophagy.

## UPR Signal Transduction Mechanism

In a perfectly functioning and “stress-free” ER, HSPA5/GRP78 (heat shock 70kDa protein 5 [glucose-regulated protein, 78kDa]), a critical member of the HSP70 family, binds to and inhibits the 3 transmembrane ER stress sensors: ERN1/IRE1 (endoplasmic reticulum to nucleus signaling 1), EIF2AK3/PERK (eukaryotic translation initiation factor 2- $\alpha$  kinase 3) and ATF6 (activating transcription factor 6).<sup>8</sup> These 3 sensors transduce information regarding ER protein folding status to the nucleus via the cytosol to reestablish protein-folding capacity. Accumulated unfolded proteins in the ER bind to and recruit HSPA5 away from those complexes, thereby activating the sensors.<sup>9</sup> However, it has also been proposed that dissociation of HSPA5 alone is not always sufficient to activate ER stress sensors. Thus,

direct interaction of the unfolded proteins with the sensors or other additional mechanisms might also regulate their activation.<sup>10,11</sup>

### ERN1/IRE1

ERN1/IRE1 is the most evolutionarily conserved bifunctional type I transmembrane protein of the UPR. It possesses both an endoribonuclease and a kinase domain that regulates XBP1 (X-box binding protein 1) processing and MAPK8/JNK1 (mitogen-activated protein kinase 8) activation, respectively.<sup>12-14</sup> Mammalian ERN1/IRE1 has 2 homologs, ERN1/IRE1 $\alpha$  and ERN2/IRE1 $\beta$ , which differ with respect to their expression patterns and together compose a substantial part of the UPR signaling network (Table 1).<sup>12</sup>

### EIF2AK3/PERK

Following ER stress, EIF2AK3, a type-I transmembrane protein with a kinase domain, causes phosphorylation of EIF2S1/EIF2 $\alpha$  (eukaryotic translation initiation factor 2, subunit 1  $\alpha$ , 35kDa).<sup>15</sup> and NFE2L2/NRF2 (nuclear factor, erythroid 2-like 2).<sup>16</sup> and stimulates various downstream signaling pathways. Furthermore, transcription factor ATF4 is selectively translated under EIF2AK3 activation, which in turn activates the DDIT3/CHOP transcription factor and regulates stress-mediated cell death (Table 1).<sup>9</sup>

### ATF6

ATF6, a type II transmembrane-activating transcription factor, has 2 homologs, ATF6/ATF6 $\alpha$  and ATF6B/ATF6 $\beta$ . Under ER stress conditions, both of them move to the Golgi for processing and subsequent nuclear translocation. The ER luminal domain of ATF6 is essential for stress sensing as it stably interacts with HSPA5 in unstressed cells and dissociates specifically during stress. However, the cytoplasmic domain of ATF6 is not necessary for its translocation to the Golgi apparatus (Table 1).<sup>17</sup>

## ER Stress and Cell Fate Determination

Mild to moderate ER stress-induced UPR signaling is seen as a compensatory mechanism, whereas severe and chronically prolonged ER stress deteriorates cellular functions and switches from an adaptation program to apoptosis to remove irreversibly injured cells.<sup>18,19</sup> Decreasing the ER protein load is the first attempt the ER makes to prevent such cell execution. ERN1 participates in the reduction of protein translation by degrading mRNAs in a process called regulated ERN1 dependent decay (RIDD), which is independent of the endoribonuclease function of ERN1. Specifically, RIDD reduces the production of membrane and secreted proteins to decrease the number of new proteins entering the ER.<sup>20</sup> In addition to RIDD, mRNAs that encode for proteins prone to accumulate in the ER undergo rapid degradation mediated by the ribonuclease domain of activated ERN1.<sup>21</sup> Similarly, ERN1 upregulation caused by XBP1 deficiency in pancreatic  $\beta$ cells enhances RIDD of cytosolic mRNAs through a negative feedback mechanism.<sup>22</sup> EIF2AK3 activation

**Table 1.** Molecular mechanisms of UPR events and their connection to ER homeostasis and autophagy

UPR branch	Target protein	Activation mechanism	Physiological outcomes	Role in autophagy
ERN1	XBP1	Unfolded protein-mediated dissociation of HSPA5 leads to the homodimerization and autophosphorylation of IRE1. Activated IRE1 uses endoribonuclease (RNase) for nonconventional mRNA splicing and transmission of UPR signals from the ER to the cytosol and nucleus. The RNase function of ERN1 involves cleavage of a 26-base fragment intron from the <i>XBP1</i> mRNA and subsequent religation by an uncharacterized RNA ligase and formation of a frame shift that encodes the spliced transcript isoform <i>XBP1s</i> . <sup>9</sup>	The encoded XBP1 protein subsequently translocates to the nucleus where it acts as a potent transcription factor by binding to UPR elements and activating a broad spectrum of UPR-related genes involved in ER membrane biogenesis, ER protein folding, physical expansion of the ER, ERAD, protein entry into the ER, protein quality control, protein secretion from the cell, and autophagy. <sup>87,226,227</sup>	Induction and inhibition. <sup>54,186,187</sup>
	MAPK8	Cytoplasmic domain of activated ERN1 recruits and forms a complex with TRAF2 and MAP3K5, which in turn phosphorylates and activates MAPK8. <sup>205</sup>	Activated MAPK8 controls both cell survival and cell death by regulating autophagy and apoptosis accordingly. The latter is converged into NFKB signaling and CASP4-CASP12 induction. <sup>228</sup>	Induction and inhibition. <sup>93,95</sup>
EIF2AK3	NFE2L2	EIF2AK3 substrate NFE2L2 is phosphorylated and translocated to the nucleus under ER stress conditions. In unstressed cells NFE2L2 is sequestered in the cytoplasm via association with KEAP1. <sup>16</sup>	In the nucleus, NFE2L2 binds with the antioxidant response element and upregulates expression of genes involved in the synthesis of antioxidant proteins such as glutathione, drug transport, xenobiotic metabolism, and elimination of ROS and autophagy. <sup>229,230</sup>	Induction. <sup>203,229</sup>
	EIF2S1	ER stress causes dimerization of the cytosolic kinase domain of EIF2AK3 resulting in autophosphorylation, which in turn phosphorylates EIF2S1. <sup>15</sup>	p-EIF2S1 blocks global protein translation, thereby lessening the ER protein burden. <sup>231</sup> In addition, autophagy-mediated cell protection under various stress conditions also substantially depends on EIF2S1 activation.	Induction. <sup>103</sup>
	ATF4	<i>ATF4</i> mRNA is selectively translated following EIF2S1 phosphorylation because it contains upstream open reading frames that allow it to escape from the p-EIF2S1-mediated translational blockage resulting in synthesis of a transcription factor. <sup>9</sup>	Transcription factor ATF4 translocates to the nucleus and activates a set of UPR target genes involved in amino acid metabolism, antioxidant responses, autophagy, and apoptosis. <sup>133,232</sup>	Induction. <sup>103</sup>
	DDIT3	DDIT3, a potent transcription factor, is upregulated via the transcription activity of ATF4.	DDIT3 potentiates autophagy and apoptosis through various molecular signals. An apoptosis signal is implicated in enhanced protein synthesis, which in turn leads to increased oxidative stress and subsequent cell death. <sup>133,232</sup>	Induction and inhibition. <sup>133,215</sup>
ATF6	ATF6	ATF6 activation includes monomer formation by reduction of ATF6 luminal intramolecular and intermolecular disulfide bonds and exposition of a Golgi-localization sequence (GLS). Those monomers then translocate to the Golgi through interactions with coat protein II (COPII). In the Golgi, ATF6 is processed into an active form of transcription factor ATF6 via the sequential activity of 2 Golgi-resident proteases, MBTPS1/S1P and MBTPS2/S2P. <sup>233</sup>	In the nucleus, ATF6 binds to ER stress response elements and stimulates transcription of a subset of UPR target genes, including <i>HSPA5</i> and <i>DDIT3</i> . ATF6 also upregulates <i>XBP1</i> mRNA levels. XBP1 is then further processed by ERN1 and translocates to the nucleus to bind with the unfolded protein response elements therein. Thus, ATF6 gives positive feedback to the UPR. <sup>13</sup>	Induction. <sup>113</sup>

and EIF2S1 phosphorylation could also selectively inhibit the translation of proteins prone to aggregation, which has been attributed to induction of RIDD.<sup>23</sup> Furthermore, accumulation

of proteins on the ER membrane leads to activation of the transcription factor NFKB, which initially stimulates the expression of cytoprotective genes, and this activity peaks when the kinase

**Table 2.** Involvement of the 3 axes of the UPR and ER Ca<sup>2+</sup> and their molecular mechanisms in control of autophagy

ER stress sensors	Core autophagic pathway	Molecular mechanism of autophagy regulation
ERN1 and its downstream regulator	MTOR AMPK	ND* Activated ERN1 regulates AMPK by MAPK8 and RPS6KA3 activation. MAPK8 stimulation converges into calcium-dependent AMPK activation by regulating BCL2-mediated Ca <sup>2+</sup> release from the ER, whereas RPS6KA3 can directly activate AMPK.
	BECN1 and ATG genes	ERN1-dependent MAPK8 activation controls <i>BECN1</i> expression at the transcriptional level by activating transcription factor JUN. At the posttranslational stage, MAPK8 directly phosphorylates BCL2, resulting in dissociation of the BECN1 essential for PtdIns3K formation. XBP1 directly binds with the DNA of <i>BECN1</i> and transcriptionally upregulates its expression.
EIF2AK3 and its downstream regulator	MTOR	EIF2AK3-dependent nuclear translocation of ATF4 results in upregulation of several genes involved in MTORC1 inhibition, including SESN2, DDIT4, and DDIT3. SESN2 and DDIT4 act directly on MTORC1, whereas DDIT3 upregulates TRIB3, which minimizes MTORC1 activity via AKT1 inhibition.
	AMPK BECN1 and ATG genes	DDIT3 can activate AMPK by lowering cellular ATP under prolonged ER stress. EIF2AK3-mediated activation of transcription factors ATF4 and DDIT3 upregulates the expression of genes involved in PtdIns3K formation ( <i>BECN1</i> ), autophagosome elongation ( <i>ATG5</i> , <i>ATG7</i> , <i>ATG8</i> , <i>ATG10</i> , <i>ATG12</i> , <i>ATG16L1</i> ), cargo selection and incorporation into forming autophagosome ( <i>SQSTM1</i> , <i>NBR1</i> , <i>BNIP3L</i> ), and autophagosome maturation ( <i>ATG8</i> , <i>GABARAP</i> , <i>GABARAPL2</i> ).
ATF6	MTOR	ATF6-mediated MTORC1 inhibition is implicated in HSPA5-dependent AKT1 downregulation.
	AMPK BECN1 and ATG genes	ND ATF6 stimulates DAPK expression by interacting with transcription factor CEBPB. Stimulated DAPK phosphorylates BECN1 and releases it from the autophagy inhibitory BECN1-BCL2 complex.
Ca <sup>2+</sup> regulated autophagy activators	MTOR AMPK	ND Increased cytoplasmic Ca <sup>2+</sup> results in AMPK phosphorylation through activation of CAMKK2B.
	BECN1 and ATG genes	DAPK is associated with BECN1 phosphorylation under ER stress-mediated elevated cytosolic Ca <sup>2+</sup> conditions.
	Unknown	Following Ca <sup>2+</sup> release from the ER to the cytoplasm, PRKQC gets activated and localized with LC3 into the elongating phagophore, thereby enhancing autophagosome formation.

\*ND, Not determined

activity of ERN1 and the translation inhibitory activity of EIF2AK3 combine.<sup>24,25</sup>

The exact mechanism controlling the transition of ER stress from “cytoprotective” to “cytotoxic” is not yet fully understood. In cases in which mild ER stress activates all UPR sensors, survival is favored as a consequence of increased instability of the mRNAs and proteins that promote apoptosis compared to those that facilitate protein folding and adaptation. In particular, prolonged ER stress attenuates ERN1 and ATF6 activity in mammalian cells.<sup>26</sup> Conversely, EIF2AK3 signaling for translational attenuation and pro-apoptotic transcription regulation remains active under the same conditions.<sup>26</sup>

ER stress can stimulate at least 3 apoptotic signals, including both intrinsic and extrinsic pathways.<sup>27</sup> The first and most vital mechanism of ER stress-induced apoptosis is mediated through DDIT3, which regulates cell death through diverse mechanisms. Different branches of the UPR regulate DDIT3 at both the transcriptional and post-translational levels. Genes encoding DDIT3

contain promoter-binding sites for transcription factors from all 3 branches of the UPR, ATF4, ATF6, and XBP1.<sup>28,29</sup> However, the EIF2AK3-EIF2S1-ATF4 branch is dominant over the other branches. ER stress-induced death is absent in *DDIT3*<sup>-/-</sup> cells, possibly due to a decreased ER protein load. In addition, increasing biosynthesis of ER client proteins is caused mainly by dephosphorylation of EIF2S1 proteins by PPP1 (protein phosphatase 1), and DDIT3 regulates that pathway by activating PPP1R15A/GADD34.<sup>30</sup> Furthermore, DDIT3 promotes transcriptional activation of a myriad of pro-apoptotic factors: TNFRSF10B/DR5 (tumor necrosis factor receptor superfamily, member 10b), TRIB3/TRB3 (tribbles pseudokinase 3), CA6 (carbonic anhydrase VI), ERO1A (endoplasmic reticulum oxidoreductase  $\alpha$ ), and members of the BCL2 family of proteins.<sup>31</sup>

ERN1 activation can also induce cell death, and that activation could partly converge into DDIT3-mediated pathways. Specifically, activated ERN1 recruits the adaptor protein TRAF2 (TNF receptor-associated factor 2), which in turn forms a complex with MAP3K5/



**Table 3.** List of ER stress-regulated proteins and their role in autophagy inhibition

ER stress sensors	Downstream regulator	Target stage of autophagy	Mode of action
ERN1	XBP1	Initiation	XBP1 interacts with the autophagy gene regulator FOXO1, marking it for proteasome-mediated degradation, thereby minimizing autophagy induction. Knockdown of XBP1 in ALS and HD models upregulates autophagy genes by facilitating the translocation of FOXO to the nucleus.
	MAPK8 TRAF2- MAP3K5	Initiation Fusion (autophagosome and lysosome)	MAPK8 prevents autophagy by restraining FOXO in the cytoplasm. ERN1-mediated TRAF2 and MAP3K5 association leads to impaired autophagy flux by inhibiting autophagosome-lysosome fusion.
Unknown	SCAMP5	Fusion (autophagosome and lysosome)	ER stress-mediated SCAMP5 expression impairs autolysosome formation by disrupting lysosome function.
Unknown	RAB7A	Fusion (autophagosome and lysosome)	Following thapsigargin-mediated ATP2A/SERCA inhibition, elevated cytosolic Ca <sup>2+</sup> prevents recruitment of RAB7A to autophagosomes, which is essential for the formation of autolysosomes.
Unknown	VMA21	Fusion (autophagosome and lysosome)	ER stress increases lysosomal pH by decreasing VMA21 expression, which is essential for the assembly of lysosomal proton pumps (vacuolar-type ATPases).

ASK1 (mitogen-activated protein kinase kinase kinase 5).<sup>32</sup> The ERN1-TRAF2-MAP3K5 complex then goes on to activate MAPK/p38 (mitogen-activated protein kinase) and MAPK8. Among those targets, MAPK8-mediated phosphorylation of BCL2 (B-cell CLL/lymphoma 2) family of proteins such as pro-apoptotic BCL2 and BCL2L11/BIM promote cell death, whereas MAPK/p38 activation converges on a common cell death pathway induced by DDIT3.<sup>32</sup> Post-translational regulation of DDIT3 in the form of phosphorylation by MAPK/p38 results in the activation of DDIT3-mediated apoptosis.<sup>33</sup> In this way, DDIT3-induced cell death pathways can intersect both the ERN1 and EIF2AK3 corridors upon ER stress. Further downstream, caspases play a substantial role in cell demise caused by ER stress. Specifically, ER stress-inducing agents can activate the catalytic properties of ER membrane-localized human CASP4/caspase-4 and murine CASP12/caspase-12.<sup>34</sup> Interestingly, CASP4- and CASP12-mediated cell death is cytosolic Ca<sup>2+</sup> dependent, evidenced by the inhibition of CASP4- and CASP12-dependent cell execution under concomitant treatment with the Ca<sup>2+</sup> chelator BAPTA-AM and ER stress inducers.<sup>35</sup> Moreover, cell death signaling stimulated by causes other than ER stress cannot activate CASP4 and CASP12, suggesting that CASP4- and CASP12-dependent cell death is ER-stress specific.<sup>34</sup> However, Obeng et al. demonstrated that CASP4 and CASP12 are not necessarily important for apoptosis induction under ER stress conditions.<sup>36</sup> In addition to CASP4 and CASP12, other caspases such as CASP2 and CASP8 can trigger BID cleavage leading to loss of mitochondrial membrane potential and thereby connecting the signaling events from ER stress to the mitochondrial death machinery.<sup>37</sup>

### Salient Features of Cells and Animals with Knocked Out Upr Components

A compromised UPR caused by genetic manipulation in vivo and in vitro shows various specialized physiological and pathological outcomes in distinct organs.

#### Deletion of the EIF2AK3 axes of the UPR

Murine *Eif2ak3* knockdown is not embryonic lethal; however, postnatal death is observed, probably due to severe cell degeneration, hyperglycemia, exocrine pancreatic impairment, and diabetes mellitus.<sup>17</sup> Strikingly, *Eif2ak3* knockdown results in aberrant ER stress by stimulating global ER stress markers. In addition, severe osteopenia and spinal curvature, skeletal dysplasia, and compromised locomotor activity are also consequences of in vivo *Eif2ak3* deletion.<sup>38</sup> Conditional mammary tissue-specific knockdown of *Eif2ak3* can lead to a dramatic decrease in autophagy level.<sup>39</sup> Similarly, in vitro knockdown in cardiomyocytes causes compromised autophagy (further details in the following section).<sup>40</sup>

A homozygous EIF2S1 S51A mutant results in postnatal death within 24 h of birth, which could be caused, at least in part, by severe hypoglycemia due to impaired gluconeogenesis and glycogen synthesis.<sup>29</sup> Although it remains to be determined whether in vivo *Eif2s1* deficiency is associated with autophagy regulation, conditional nonphosphorylated in vitro knockin (S51A) is associated with compromised autophagy.<sup>41</sup> Mice that lack *Atf4* are neither embryonic lethal nor vulnerable to postnatal death. However, various diseases such as microphthalmia,<sup>42,43</sup> growth retardation,<sup>44</sup> pancreatic hypotrophy,<sup>45</sup> and hematological defects, including severe anemia,<sup>46</sup> are observed during their life spans. DDIT3 ablation is not associated with embryonic death or developmental defects; rather it protects essential organs such as the lungs and kidneys from stress-associated injury. However, conditional knockdown results in liver injury and compromised autophagy, similar to the results seen in the in vitro knockdown model.

#### Deletion of the ATF6 axes of the UPR

Although no embryonic lethality or postnatal death results, genetic deficiency of *Atf6* in mice results in intolerance to ER stress. Acute liver injury, kidney damage, and  $\beta$ -cell degeneration-associated diseases are aggravated in *atf6*<sup>-/-</sup> animals

challenged with ER stress inducers compared with their wild-type counterparts.<sup>47</sup> Furthermore, combined knockdown of *Atf6* and *Atf6b* is embryonic lethal.<sup>48</sup> Compromised autophagy in *atf6*<sup>-/-</sup> MEF cells suggests its involvement in the autophagy process.<sup>49</sup>

### Deletion of the ERN1 axes of the UPR

In terms of embryonic death, *ern1*<sup>-/-</sup> and *xbp1*<sup>-/-</sup> mice display distinguishing phenotypes compared to other UPR branches. Both types of animals die at the embryonic stage.<sup>50-52</sup> *Ern1* knockdown can cause improper functioning of the placental blood vessels and reduce production of VEGF (vascular endothelial growth factor).<sup>50</sup> Similarly, embryonic lethality in *xbp1*<sup>-/-</sup> mice is caused by hypoplastic fetal livers and depleted hematopoiesis that leads to severe anemia.<sup>52</sup> More interestingly, embryonic lethality can be reversed by reconstituting XBP1 and ERN1 expression.<sup>50,53</sup> Conditional adult knockdown of both of these proteins can lead to enhanced autophagy (further details in the following section). However, in vitro knockdown of *ERN1* and *XBP1* can jeopardize ER stress and starvation-induced autophagy.<sup>54</sup>

## Autophagy

Autophagy is a genetically programmed ancient catabolic system first described by Christian de Duve in the late 1950s after observing cytosolic vacuoles in mammalian cells under an electron microscope.<sup>55</sup> During the past 3 decades of autophagy research and discovery, comprehensive studies in yeast have shed significant light on its core molecular mechanism.<sup>56-58</sup> To date, numerous studies have been performed to understand the status of autophagy in cells, and scientists have found that cells maintain optimum activity by sustaining a minimum basal level of autophagy.<sup>59</sup> In this particular context, basal autophagy can be stimulated to play a crucial role in cellular adaptation to starvation and other cellular stress by endolysosomal degradation and elimination of long-lived and misfolded proteins, potentially detrimental cellular substances, defective organelles, and invading pathogens.<sup>60,61</sup> In addition, autophagy acts as a source of energy and building blocks for the biosynthesis of new macromolecules by recycling metabolites produced by lysosomal proteolysis. Likewise, autophagy can regulate the energy balance of not only single cells, but also entire organisms through the enhancement of metabolic activity. Furthermore, autophagy is crucial for cell growth and differentiation, tumor suppression, innate and adaptive immunity, life-span extension, and cell death.<sup>62</sup> In this way, autophagy plays a substantial role throughout the entire life span of an organism. For example, during pre-implantation processes, cytoplasmic components of the oocytes need to be cleared, whereas post-fertilization requires removal of paternal mitochondria; both processes are mediated by autophagy.<sup>63,64</sup> Furthermore, energy production in newborn mice substantially depends on autophagic processes. Specifically, prior to birth, the fetus uses maternal nutrients as an energy source, but that becomes unavailable just after birth due to placental cessation. Autophagy

acts as an energy source at this critical stage by recycling metabolites.<sup>65</sup>

At least 3 subtypes of autophagy have been documented in mammalian cells. They differ according to their physiological function and the mechanism of cargo transport for proteolytic degradation at common destination lysosomes. These subtypes are formally specified as chaperone-mediated autophagy, macroautophagy, and microautophagy.<sup>61,66</sup> Among those mechanisms, macroautophagy, the most widely studied mechanism, uses cytosolic double-membrane vesicles called autophagosomes to deliver cytosolic content to lysosomes.<sup>67</sup> In contrast, microautophagy, the least characterized mechanism, results in the isolation of cytoplasmic proteins and organelles by inward invagination or septation of lysosomal membranes.<sup>68</sup> In chaperone-mediated autophagy, targeted proteins reach the proteases of the lysosomal matrix by direct translocation across the lysosomal membrane with the help of LAMP2A (lysosomal-associated membrane protein 2A) and lysosomal HSPA8/hsc73/lys-HSC70.<sup>69</sup>

### Molecular mechanism of autophagy

Mechanistically, autophagy is a complex process that can be categorized into several sequential steps at the molecular level. Numerous cellular and external cues can initiate autophagy, which is followed by cargo selection and packaging, expansion of the phagophore membrane, closure to generate the completed autophagosome, fusion of the matured autophagosomes with lysosomes, degradation of the autophagosomal content by lysosomal hydrolases, and finally efflux of the breakdown products.<sup>70-72</sup> Molecularly, autophagy initiation is controlled by the ULK1/2 complex, which includes ULK1 (the mammalian homolog of yeast Atg1), ATG13, ATG101 (a novel autophagic factor), and RB1CC1/FIP200 (a functional ortholog of yeast Atg17),<sup>73</sup> which remains inhibited by MTOR (mechanistic target of rapamycin [serine/threonine kinase]). Starvation and other stress conditions inhibit MTOR and activate the ULK1/2 complex, which in turn facilitates the formation of the phagophore.<sup>74</sup> Phagophore formation involves a class III phosphatidylinositol 3kinase complex (PtdIns3K), composed of BECN1/BECLIN 1, PIK3C3/VPS34, PIK3R4/p150, ATG14, and UVRAG.<sup>75-77</sup> Anti-apoptotic BCL2 or BCL2L1/BCL-XL holds BECN1 at bay by directly binding to BECN1s BH3 domain, and in doing so restrains it from constructing an autophagy-inducing PtdIns3K (Fig. 1C).<sup>78</sup>

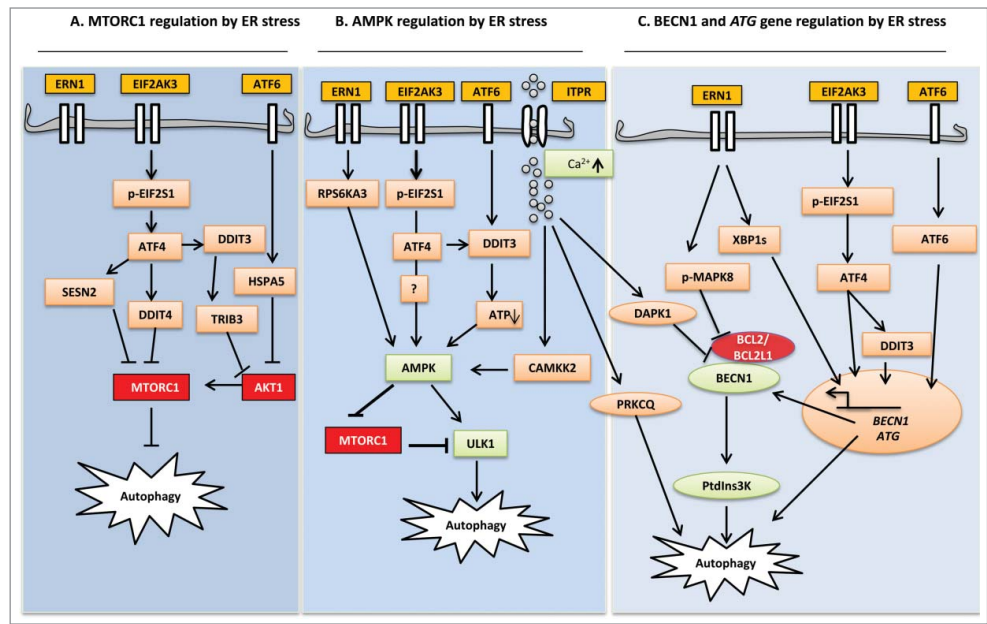
Phagophore expansion and closure require several ubiquitin-like (UBL) proteins that take part in 2 conjugation reactions.<sup>79,80</sup> ATG12-ATG5-ATG16L1, a product formed in the first conjugation reaction, stimulates the recruitment and conversion of proteolytically processed cytosolic MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3), LC3-I, to the membrane-bound, lipidated form, LC3-II.<sup>81-83</sup> The nascent LC3 is cleaved by the cysteine protease ATG4 in the second reaction and this is followed by conjugation with membrane-bound phosphatidylethanolamine and subsequent incorporation into the phagophore membrane with the help of the ATG7 and ATG3 enzymes.<sup>79,84</sup> ATG4 can cause delipidation (deconjugation) and recycling of LC3-II on the outer surface of the autophagosome,<sup>85</sup>

whereas inner autophagosomal membrane-bound LC3-II reflects the abundance of autophagosomes and their ability to reach autolysosomes for degradation. Hence, LC3-II is a marker protein for autophagy detection.<sup>86</sup>

Cytosolic autophagosomes need to access lysosomes at the perinuclear region of the cells. Various cytosolic and lysosomal proteins are involved in this complex and incompletely understood process. Cytoskeletal microtubules and the motor protein dynein seem to play major roles in autophagosomes trafficking to lysosomes.<sup>87</sup> Similarly, autophagosomal transport and fusion is substantially mediated by the small GTPase RAB7A/RAB7 and its association with FYCO1 (FYVE and coiled-coil domain containing 1) and lysosomal membrane proteins LAMP1/2 accelerate the fusion machinery.<sup>88,89</sup> ATG9, an integral membrane protein, is also thought to be involved in the fusion machinery by transporting vesicle fusion proteins, SNAREs, and autophagic membranes to growing phagophores.<sup>90</sup> Furthermore, the class C vacuolar protein sorting-HOPS complex regulates autophagosome tethering with lysosomes by activating RAB7A.<sup>91</sup> Finally, autophagosomal-sequestered cargo is degraded by lysosomal/vacuolar acid hydrolases such as CTSB, CTSD, and CTSL immediately after accessing the lysosomal lumen.<sup>92</sup>

### Autophagy Induced by 3 UPR Axes

The 3 canonical branches of the UPR regulate autophagy in different ways during ER stress. Correlation between autophagosome formation and ER expansion caused by ER stress was first described in 2006.<sup>93</sup> Specifically, electron microscopy studies in yeast revealed that ER volume increases 5-fold under ER stress and that cells undergoing such stress exhibit concomitant increases in autophagosome abundance.<sup>94</sup> According to Ogata et al., tumor cells activate autophagy as a survival mechanism to escape ER stress-induced toxicity. ERN1-mediated MAPK8 phosphorylation appears to be a major regulator in this pathway. MAPK8 is considered a “stress-regulated protein kinase,” whereby stress-induced autophagy and apoptosis depend substantially on MAPK8 activation.<sup>93,95</sup> Specifically, ERN1 activation leads to MAPK8 phosphorylation, and that might initiate



**Figure 1.** Schematic diagram highlighting ER stress signaling in the control of autophagy pathways. **(A)** MTORC1 regulation by ER stress: Autophagy initiation begins with MTOR inhibition. Following EIF2AK3 activation, transcription factor ATF4 leads to the upregulation of *SESN2* and *DDIT4*. Further downstream, DDIT3 upregulates *TRIB3*. *SESN2* and *DDIT4* directly inhibit the kinase activity of MTORC1, whereas *TRIB3* decreases AKT1 phosphorylation, which in turn inhibits MTORC1. **(B)** AMPK regulation by ER stress: ER stress integrates all UPR branches at AMPK by elevating cytoplasmic  $Ca^{2+}$ , upregulating RPS6KA3, and depleting cellular ATP. ERN1 activation results in stimulation of RPS6KA3, which in turn activates AMPK and subsequently activates the ULK1 complex. EIF2AK3 and ATF6 activate AMPK via DDIT3-mediated ATP depletion. Elevated cytosolic  $Ca^{2+}$  activates CAMKK2, causing phosphorylation of AMPK and subsequent ULK1 activation. **(C)** BECN1 and ATG gene regulation by ER stress: MAPK8 (ERN1 dependent) and DAPK1 ( $Ca^{2+}$  dependent) activation have been implicated in the formation of PtdIns3K through the phosphorylation and dissociation of BCL2 and BECN1, respectively. Transcription factors JUN, XBP1, ATF4, DDIT3, and ATF6 from all UPR sensors induce expression of *BECN1* and other ATG genes essential for autophagy.  $Ca^{2+}$ -dependent phosphorylation of PRKCQ leads to its colocalization with LC3 on the elongating phagophore and facilitates autophagosome formation.

autophagy and allow cells to adapt to stress.<sup>93</sup> The autophagy-related gene *BECN1* is the leading downstream regulator of MAPK8, and its activation is followed by direct phosphorylation of BCL2, which in turn disrupts the interaction between BECN1 and BCL2 and induces autophagy in tumor cells (Fig. 1A).<sup>96,97</sup> Interestingly, *BECN1* is transcriptionally upregulated by JUN/c-Jun, a MAPK8-dependent transcription factor, in a human cancer cell model treated with the ER stress inducers ceramide and topotecan.(Fig. 1A).<sup>98</sup> In addition to kinase activity, the endoribonuclease activity of ERN1 also participates in the induction of autophagy. Spliced XBP1 binds directly to the *BECN1* promoter in the nucleus and promotes an autophagic response via transcriptional upregulation of *BECN1* (Fig. 1C).<sup>54</sup> Notably, MAPK8-mediated autophagy in rat aortic smooth muscle cells under oxidative stress is ERN1 independent,<sup>99</sup> suggesting an alternative pathway for activation of MAPK8. The ubiquitin proteasome system and autophagy degrade intracellular content independently. However, proteasome inhibition in a colorectal carcinoma model triggers autophagy via ER stress-mediated MAPK8 phosphorylation and is independent of ERN1s endoribonuclease activity.<sup>100</sup> That study suggests a functional linkage



between 2 independent protein degradation pathways under which autophagy can be compensatory by enabling cells to cope with stress conditions that compromise the proteasome machinery.

Following ER stress, MAPK8 regulates both autophagy and apoptosis. Scientists have done kinetic studies to uncover the molecular switch that regulates the transition of MAPK8-dependent autophagy to apoptosis under stress conditions and discovered that the BH3 domain-containing proteins such as BECN1 and BAX are the main regulatory components.<sup>101</sup> Transient activation of MAPK8 activates autophagy by dissociating small amounts of BCL2 from BECN1 while leaving the interaction between BCL2 and BAX unchanged. In contrast, prolonged activation of MAPK8 induces apoptosis by fully dissociating BAX from BCL2, leading to BAX activation.<sup>101</sup>

The main events of autophagy, such as phagophore formation and maturation, are substantially maintained by LC3-II and the ATG12-ATG5 conjugate.<sup>102</sup> To sustain autophagy flux, transcriptional upregulation of the corresponding autophagy genes is crucial. Evidence suggests that under ER stress conditions, the EIF2AK3 branch of the UPR helps to regulate those genes.<sup>103</sup> The association of EIF2AK3 in ER stress-mediated autophagy was first documented by Kouroku et al.<sup>104</sup> Specifically, they showed that aggregated polyglutamine (polyQ) proteins in the cytosol reduce proteasome activity, leading to the induction of autophagy through activation of the EIF2AK3 branch of the UPR. Under hypoxic conditions, transcriptional upregulation of *LC3* and *ATG5* depends on EIF2AK3-dependent ATF4 and DDIT3 induction (Fig. 1C).<sup>105</sup> Similarly, *ATG12* mRNA and protein levels are also enhanced by phosphorylated EIF2S1, a downstream regulator of EIF2AK3.<sup>104</sup> EIF2AK3-mediated activation of ATF4 is sufficient for upregulation of a dozen autophagy genes, including *MAP1LC3B*, *BECN1*, *ATG3*, *ATG12*, and *ATG16L1* (Fig. 1C), whereas the interaction of ATF4 and DDIT3 causes transcriptional upregulation of *SQSTM1/p62*, *NBR1*, and *ATG7*. In addition, *ATG10*, *GABARAP*, and *ATG5* transcription is followed by activation of the DDIT3 transcription factor alone.<sup>103</sup> Furthermore, DDIT3 can stimulate autophagosome formation through downregulation of BCL2 expression.<sup>106</sup> Therefore, EIF2AK3 appears to be the most efficient UPR branch with respect to the regulation of autophagy genes under ER stress. EIF2AK3 maintains its autophagy regulatory activity in different pathological models, as well. For example, cardiomyocytes undergo an autophagy process as a protective mechanism upon ER stress triggered by lipopolysaccharides, and genetic ablation of EIF2AK3 leads to autophagy inhibition and the death of cardiomyocytes upon treatment with lipopolysaccharides.<sup>40</sup> Several mechanisms enable cancer cells to induce autophagy for their survival. Koumenis et al. demonstrated that in mouse and human lymphomas the proto-oncogene product MYC triggers ER stress by activating the EIF2AK3-EIF2S1-ATF4 branch and thereby inducing cytoprotective autophagy.<sup>107</sup> Similarly, autophagy enables ovarian cancer cells to survive under metformin-induced ER stress, and its inhibition results in cell death.<sup>108</sup> Alternatively, autophagic cell death can be a consequence of EIF2AK3 activation. For instance,

nelfinavir and bortezomib, which are ER stress-inducing anticancer drugs, trigger autophagy by upregulating ATF4-mediated SESN2 (sestrin 2), an endogenous MTOR inhibitor (Fig. 1A).<sup>109</sup> Through this process, both nelfinavir and bortezomib lead to autophagy-dependent growth arrest and the radiosensitization of cancer cells.<sup>109</sup> In summary, EIF2AK3-EIF2S1-ATF4 pathway-mediated autophagy can have both a cytotoxic and cytoprotective capacity; however, cytoprotective autophagy is predominant under conditions of ER stress.<sup>107</sup> The duration/intensity of the stress and cell types might be a vital and deciding factor for the contradictory function of the EIF2AK3-EIF2S1-ATF4 pathway.

Notably, it has been observed that proteasome inhibition stimulates ATF4-dependent LC3 conversion in breast cancer cells, which connects ER stress to autophagy in an EIF2AK3-independent manner.<sup>110</sup> Interestingly, the role of EIF2S1 in autophagy regulation extends beyond ER stress. For example, viral infections and amino acid starvation can cause EIF2S1 phosphorylation-dependent autophagy, regardless of ER stress.<sup>41,111,112</sup> Collectively, those findings indicate that in particular settings, EIF2S1-ATF4 signaling is enough to trigger autophagy, and the role of EIF2AK3 phosphorylation is negligible. It is also clear that downstream EIF2AK3 regulators are mainly associated with the enhancement of autophagy. Thus, it could be important for future work to focus on the threshold of the EIF2AK3-EIF2S1 branch required to switch between compensation and cell execution.

The ATF6 branch of the UPR is the least characterized branch in the context of ER stress and autophagy. Nevertheless, transcription activity of ATF6 is involved in autophagy induction through upregulation of HSPA5 expression and subsequent downregulation of AKT1/AKT (further details in the following section).<sup>113</sup> In addition, the ATF6-associated transcription factor CEBPB (CCAAT/enhancer binding protein [C/EBP],  $\beta$ ) has been linked to IFNG-dependent autophagy through the expression of DAPK1 (death-associated protein kinase 1) (Fig. 1C).<sup>114</sup> The subsequent phosphorylation of BECN1 results in diminished affinity for BCL2 and leads to the formation of a complex between the autophagy initiator BECN1 and PIK3C3.<sup>115,116</sup> Interestingly, genetic ablation of *ATF6* causes repression of DAPK1 expression and ultimately failure of autophagy induction during treatment with IFNG.<sup>114</sup>

### Upr Branches Induce Autophagy by Regulating Akt1-Mtor Signaling

In mammals, growth factors, nutrients, ER stress inducers, and other stressors act as on-off switches for MTOR-regulated cell growth, survival, and energy balance. Among the 2 different forms of MTOR (MTOR complex 1 [MTORC1] and MTORC2), MTORC1 is known as the master regulator of nutrient signaling and autophagy.<sup>117</sup> The serine/threonine kinase AKT1 is a positive upstream regulator of MTOR, and initiation of autophagy depends substantially on the AKT1-MTOR pathway. Negative regulation of this pathway leads to the release of



ULK1 from inactive MTOR and the subsequent formation of autophagosomes. One common consequence of ER stress is downregulation of AKT1, which in turn contributes to the induction of autophagy by decreasing MTOR activity.<sup>118</sup> Furthermore, it has been well established that the transcriptional activity of ATF6 upregulates the ER chaperone HSPA5 (Fig. 1A), which triggers autophagy in placental choriocarcinoma cells by limiting AKT1 phosphorylation activity.<sup>113</sup> However, although ER stress could contribute to AKT1 inhibition, a more complicated scenario is likely that moderate and recoverable ER stress can activate 2 major cytoprotective signaling cascades, involving AKT1 and MAPK.<sup>119</sup> Indeed, rapid activation of phosphoinositide 3-kinase (PI3K)-AKT1-MTORC1 signaling and cell survival upon pharmacological induction of the UPR appears to be regulated by the ATF6 branch.<sup>120</sup> It is therefore plausible that autophagy can be negatively regulated under such conditions. Although the factors associated with ER stress-mediated switching of AKT1 activation to inhibition are not fully understood, stress intensity and duration are thought to be crucial. Recent observations have also implicated an additional level of complexity: activated AKT1-dependent cell survival is due at least in part to upregulation of HSPA5.<sup>121</sup> Specifically, the failure of AKT1 activation in a *HSPA5* knockout model could depend on upregulated PPP2/PP2A (protein phosphatase 2) activity that results in dephosphorylated AKT1.<sup>122</sup> Thus, it is surprising that although HSPA5 can downregulate AKT1, it can also promote cell survival in some situations.<sup>122</sup> This finding suggests that stimulation of HSPA5 signaling could be a novel strategy to induce autophagy and simultaneously promote AKT1 activation. Thus, the role of HSPA5 signaling in autophagy requires more comprehensive study.

Both the upstream and downstream signaling associated with MTOR can be regulated by ER stress, which can either stimulate or inhibit the anabolic activity of MTOR.<sup>123</sup> PI3K-AKT1 stimulates MTORC1 as a cell growth mechanism via phosphorylation and inhibition of TSC1 (tuberous sclerosis 1) and TSC2, which are negative regulators of MTOR,<sup>124</sup> whereas ER stress limits MTOR activity by stimulating TSC1/2.<sup>125</sup> Likewise, TSC deficiency impedes ER stress-mediated autophagy induction via constitutive activation of MTOR.<sup>118</sup> Moreover, cells with hyperactive MTORC1 due to TSC1/2 knockdown exhibit increased basal levels of ER stress that can be further augmented by treatment with stress inducers,<sup>126</sup> indicating that TSC2 is crucial for canonical feedback of ER stress. Conversely, MTOR deactivation under stress conditions is associated with downregulation of the AKT1-MTOR pathway in part via TSC1/2.<sup>118</sup> According to those studies, TSC1/2 is the common target for AKT1 and ER stress, and their effects are inversely related with respect to autophagy.

Following ER stress, ATF4 and CEBPB regulate the dephosphorylation of MTORC1 via DDIT4/REDD1 (DNA-damage-inducible transcript 4) expression,<sup>127</sup> which mainly acts on the TSC1/2 complex. DDIT4<sup>-/-</sup> breast cancer cells are unable to dephosphorylate MTOR under ATP depletion conditions or direct activation of AMPK.<sup>128</sup> Furthermore, EIF2AK3-EIF2S1-ATF4-dependent DDIT4 induction and subsequent inhibition

of MTORC1 is also evident in in vivo models.<sup>129</sup> These data indicate that TSC-dependent MTOR regulation is primarily mediated by the activated EIF2AK3-EIF2S1-ATF4 branch of the UPR.

The ERN1-MAPK8 pathway appears to be the most abundant UPR branch downstream of activated MTORC1. Chronic activation of MTORC1, following genetic or pharmacological inhibition of TSC1/2, downregulates AKT1, which in turn selectively activates ERN1-MAPK8.<sup>130</sup> An apoptotic signal is initiated, probably caused by increased protein synthesis and high protein load in the ER, along with depleted cell survival-signaling AKT1 following activation of ER stress. It has also been suggested that constitutive activation of MTORC1 triggers an incomplete induction of the UPR, whereby all branches of the UPR other than the EIF2S1 branch are compromised.<sup>131</sup> Thus, it is intriguing to consider the possibility that existing studies describe shared pathological conditions whereby long-term activation of MTORC1 is linked to cellular demise caused by ER stress. This highlights a peculiar anomaly in that MTOR, a positive regulator of cell growth, can also stimulate cell death signaling in particular contexts.

TRIB3, an ER stress-associated protein, negatively regulates the AKT1-MTOR axis by directly binding to AKT1.<sup>132</sup> Various forms of ER stress can stimulate TRIB3 expression. The inability of ATF4-DDIT3-deficient malignant gliomas to induce TRIB3 under conditions of ER stress indicates that *TRIB3* transcriptional activation is ATF4-DDIT3 dependent.<sup>133</sup>  $\Delta^9$ -tetrahydrocannabinol, an active component of marijuana, can induce TRIB3-dependent autophagy in a human glioblastoma multiforme model as well.<sup>132</sup> However, a negative feedback mechanism of hyperactivated TRIB3 degrades the transcriptional activity of ATF4 and DDIT3.<sup>132,134</sup> Thus, TRIB3-mediated stimulation of autophagy appears to be unnecessary for cell survival. Moreover, autophagy induction that relies on the transcriptional activity of ATF4 and DDIT3 reflects a cytotoxic condition, and that might happen only during the end stage of ER stress.

ER-localized MTORC2 is stress sensitive and interacts with the ER proteins PDIA3/GRP58 and HSP70.<sup>135</sup> AKT1 is also found at the ER and acts as a direct substrate of MTORC2. However, it is unclear how MTORC2 is regulated. ER stress-mediated phosphorylation of GSK3B/GSK-3 $\beta$  (glycogen synthase kinase 3  $\beta$ ), an AKT1 substrate, inhibits AKT1 signaling through RICTOR phosphorylation.<sup>136</sup> This finding supports the possibility that in addition to MTORC1-dependent AKT1 inhibition, MTORC2 could also regulate ER stress-mediated AKT1 activity, either by stimulating autophagy pathways or by triggering apoptotic signaling. Further studies are required to clarify this issue. However, the relationship between AKT1 signaling and GSK3B regulation appears to be complex. Specifically, in a model of AKT1 hyperactivation, apoptosis and ER stress are decreased by AKT1-mediated GSK3B inhibition.<sup>137</sup> It is therefore likely that under ER stress conditions, AKT1 and GSK3B act in contrasting fashion and dominate each other's roles in the cell. Taken together, multiple lines of evidence now indicate that prolonged ER stress activates apoptotic pathways by downregulating AKT1. However, at the early stage of ER stress, stimulated

AKT1 acts as a survival kinase via MTORC1 activation. Thus, it is plausible to suggest that ER stress-regulated MTOR-dependent autophagy could be crucial for cell survival when AKT1 is inhibited (Table 2).

### Upr Branches Induce Autophagy by Regulating Ampk Signaling

AMPK is an energy sensor that is mainly activated when the energy state of a cell is compromised, such as with increased levels of AMP and ADP. A wide array of metabolic stress stimuli can stimulate AMPK. Autophagy induction via AMPK stimulation is thought to be separated into 2 regulated pathways. Previously, it was shown that AMPK-dependent autophagy induction is based only on MTORC1 inactivation through TSC regulation, followed by dissociation and activation of ULK1.<sup>138</sup> However, elegant work from Kim et al. and Egan et al. revealed that AMPK induces autophagy by directly phosphorylating ULK1 at 6 different sites (S467, S555, T574, S637, S777, S317).<sup>139,140</sup> In addition, feedback signaling from active AMPK minimizes MTORC1 activation, resulting in an increase in activated ULK1 levels.

A key function of AMPK is to defend against ER stress-mediated cytotoxicity, primarily by inducing autophagy. The resulting bulk degradation of cytosolic cargo is beneficial for stressed cells. Supporting this notion, recent work from Lee et al. demonstrated that AMPK activation has suppressive effects on ER stress-mediated cellular toxicity upon albumin treatment.<sup>141</sup> In addition, pharmacological stimulation of AMPK reduces ER stress-stimulated DDIT3 and caspase activation, and genetic ablation of *PRKAA1* renders cells susceptible to stress-mediated demise.<sup>142</sup> More recently, Cheng et al. demonstrated that RPS6KA3/RSK2 (ribosomal protein S6 kinase, 90kDa, polypeptide 3) (Fig. 1B), a serine/threonine protein kinase, links ER stress and autophagy by activating AMPK. Specifically, silencing *RPS6KA3* expression suppresses autophagy induction and aggravates ER stress in a human breast cancer model. It is plausible that an AMPK-mediated protective role might be deregulated, which in turn exacerbates the severity of stress. In support of this, ERN1 depletion blocks RPS6KA3 activation, and inhibition of *RPS6KA3* expression results in reduced levels of phospho-AMPK. Thus, it is possible that aggravated ER stress results from *PRKAA1* depletion through a process controlled by the upstream regulator RPS6KA3. Taken together, these data suggest that the ERN1-RPS6KA3-AMPK axis is a potential regulator of ER-stress-mediated autophagy.<sup>143</sup> ATP depletion under ER stress is regulated primarily by the EIF2S1-ATF4-DDIT3 branch of the UPR, which can cause rapid stimulation of AMPK (Fig. 1B).<sup>144,145</sup> For instance, bufalin, an active component of *Bufo gargarizan* venom and a treatment for malignant glioma, causes ER stress-mediated ATP depletion and upregulation of AMPK activity with increased autophagy and overall survival of glioma cells.<sup>146</sup> EIF2AK3 involvement in AMPK-mediated cytoprotective autophagy has also been described in metabolic stress conditions caused by the extracellular matrix (ECM) detachment

of mammary epithelial cells.<sup>147</sup> Adhesion to the ECM is essential for the proper function and homeostasis of epithelial tissues. ECM detached cells undergo rapid activation of the EIF2AK3 branch of the UPR, which in turn induces autophagy by activating AMPK and subsequently downregulating MTORC1.<sup>147</sup> This mechanism of autophagy induction is essential for the re-attachment and survival of the detached cells. It is well known that AMPK downregulates protein synthesis by inhibiting the cell growth regulator MTOR and subsequently phosphorylating EIF4EBP1/4E-BP1 and RPS6KB/p70S6K. However, the transcriptional activity of ATF4 and DDIT3 increases protein synthesis and cell death,<sup>145</sup> which has resulted in a controversy regarding the role of AMPK under ER stress. Further mechanistic insight is required to clarify how ATF4 and DDIT3 induce protein synthesis in parallel with AMPK activation. Based on all of these studies, it is our opinion that AMPK activation under ER stress conditions could represent a therapeutic target to protect against unwanted cellular demise (Table 2).

### ER Ca<sup>2+</sup> Changes Regulate Autophagy

An abundance of Ca<sup>2+</sup> in the ER is essential for its proper functioning, including nascent protein folding and ER homeostasis. Interestingly, the ER is an intracellular Ca<sup>2+</sup> reservoir that ensures regular communication with other organelles and effector proteins. Loss of luminal Ca<sup>2+</sup> is frequently accompanied by ER stress, which can activate various Ca<sup>2+</sup>-regulated pathways based on the degree and duration of stress. Alternatively, ER stress can cause loss of ER luminal Ca<sup>2+</sup>. The influx and release of Ca<sup>2+</sup> from the ER are tightly controlled by various regulatory systems. Cytosolic Ca<sup>2+</sup> enters the ER through a Ca<sup>2+</sup> pump called ATP2A/SERCA (ATPase, Ca<sup>2+</sup> transporting) that is expressed on the ER surface.<sup>148</sup> Ca<sup>2+</sup> storage in the ER is facilitated primarily by a number of intra-ER Ca<sup>2+</sup> binding proteins and Ca<sup>2+</sup>-dependent chaperones, including CASQ/calsequestrin, CALR (calreticulin), CANX (calnexin), HSPA5, HSP90B1/GRP94, and PDI (protein disulfide isomerase).<sup>149</sup> Together, they ensure the primary quality control of proteins destined for Ca<sup>2+</sup>-dependent modification in the ER. Release of stored Ca<sup>2+</sup> from the ER is mediated by 2 different receptor channels that reside on the membrane of the ER called RYR (ryanodine receptor) and ITPR/IP3R (inositol 1,4,5-trisphosphate receptor).<sup>149</sup>

Optimum Ca<sup>2+</sup> movement across the ER membrane ensures the proper functioning of various kinases and proteases. Indeed, perturbation of this dynamic Ca<sup>2+</sup> movement can lead to activation of various Ca<sup>2+</sup>-regulated pathways, including autophagy. In a breast tumor model, the cytosolic Ca<sup>2+</sup> mobilizing agents thapsigargin, ionomycin, and vitamin D activate CAMKK2/CAMKK $\beta$  (calcium/calmodulin-dependent protein kinase kinase 2,  $\beta$ ), which in turn triggers autophagy via AMPK-dependent MTORC1 inhibition.<sup>150</sup> Those data are supported through sophisticated work by Jia et al.<sup>151</sup> They demonstrated that autophagy-deficient T lymphocytes have an expanded ER compartment due to excessive Ca<sup>2+</sup> in the ER. Defective Ca<sup>2+</sup> influx results in extensive Ca<sup>2+</sup> stores in the ER, which could be caused

by the inability of the  $\text{Ca}^{2+}$  stores to be depleted. This defect in  $\text{Ca}^{2+}$  influx can be repaired by treatment with the sarcoplasmic reticulum/ER  $\text{Ca}^{2+}$ -ATPase pump inhibitor thapsigargin,<sup>151</sup> which indicates that autophagy can control  $\text{Ca}^{2+}$  movement across the ER. Taken together, these data suggest a relationship between  $\text{Ca}^{2+}$  mobilization and autophagy and indicate they can influence each other. ER stress can also be a consequence of extracellular  $\text{Ca}^{2+}$  influx; however, no direct evidence suggests that extracellular  $\text{Ca}^{2+}$  induction can cause ER stress-mediated autophagy. Furthermore, increasing cytosolic  $\text{Ca}^{2+}$  with exogenously introduced  $\text{Ca}^{2+}$  phosphate precipitates can induce autophagy at early time points without altering the ER condition.<sup>152,153</sup> Therefore, it is likely that ER  $\text{Ca}^{2+}$  is the main regulator of autophagy mediated by the UPR, whereas  $\text{Ca}^{2+}$  from other sources can induce autophagy that does not involve the UPR.

ITPR, a second messenger, makes the scenario more complex by playing a negative role in autophagy induction. Emerging evidence from various experimental systems suggests that pharmacological and genetic inhibition of ITPRs trigger autophagy independent of  $\text{Ca}^{2+}$ .<sup>154,155</sup> This appears to contradict the role of ER- $\text{Ca}^{2+}$  depleting agents (the ATP2A/SERCA antagonist thapsigargin) and luminal ER  $\text{Ca}^{2+}$  store stimulators (the ITPR antagonist xestospongins B), both of which can induce autophagy. One possible explanation is that perturbation of typical ER  $\text{Ca}^{2+}$  levels can lead to autophagy. Conversely, autophagy signaling caused by ITPR inhibition might be mechanistically distinct from ER stress-induced autophagy. Moreover, blocking ITPRs and RYRs can attenuate thapsigargin-induced ER stress,<sup>156</sup> which might provide a better explanation for the negative role of ITPRs in autophagy induction. At basal levels, hyperactivation of autophagy is observed in cells in which all 3 ITPR isoforms are deleted.<sup>157</sup> In addition, the kinase activity of MTORC1 is significantly disrupted in such cells. Thus, it can be assumed that ITPR-dependent  $\text{Ca}^{2+}$  signaling ensures an elevated level of MTORC1 activity, which in turn negatively regulates basal levels of autophagy.<sup>157</sup> This notion is consistent with the fact that inhibition of new protein production and autophagy induction are mediated by the AMPK-induced  $\text{Ca}^{2+}$ -calmodulin-dependent enzyme EEF2K and also suggests that basal levels of autophagy vary with changes in cytosolic  $\text{Ca}^{2+}$  levels and AMPK status.

$\text{Ca}^{2+}$ -mediated autophagy under ER stress conditions is regulated by the known tumor suppressor DAPK1 (Fig. 1C).<sup>7,115</sup> Disruption of BECN1 and BCL2L1 interaction is mediated by activated DAPK1 through direct phosphorylation of BECN1 on Thr119, which allows autophagy to proceed.<sup>116</sup> Hypoxia stimulates extensive downregulation of protein synthesis through activation of the EIF2AK3-EIF2S1-ATF4 and AMPK-MTORC1 pathways.<sup>158,159</sup> Likewise, autophagy can also be triggered under hypoxic conditions, which can be attributed to hypoxia-induced  $\text{Ca}^{2+}$  influx and activation of CAMK1 (calcium/calmodulin-dependent kinase I) and CAMK4, which in turn can contribute to WIPI1 stimulation and autophagosome formation.<sup>160,161</sup>

Sufficient evidence shows that activation of PRKCCQ/PKC $\theta$  (protein kinase C, theta), a member of the novel-type PKC family, is required for ER stress-induced autophagy.<sup>162-164</sup>

Interestingly,  $\text{Ca}^{2+}$  is an essential component of PRKCCQ activation under ER stress conditions, which is supported by the observation of PRKCCQ deactivation and the subsequent blockage of autophagy in cells treated with BAPTA-AM, an intracellular  $\text{Ca}^{2+}$  chelating agent.<sup>163</sup> This scenario mirrors the observation that cytosolic  $\text{Ca}^{2+}$  mobilization upon ER stress is mandatory for autophagy induction. Moreover, it has been reported that PRKCCQ acts as a sensor for ER stress in skeletal muscle. Activation of PRKCCQ alone can stimulate autophagy in the absence of ER stress, but it is augmented under ER stress conditions.

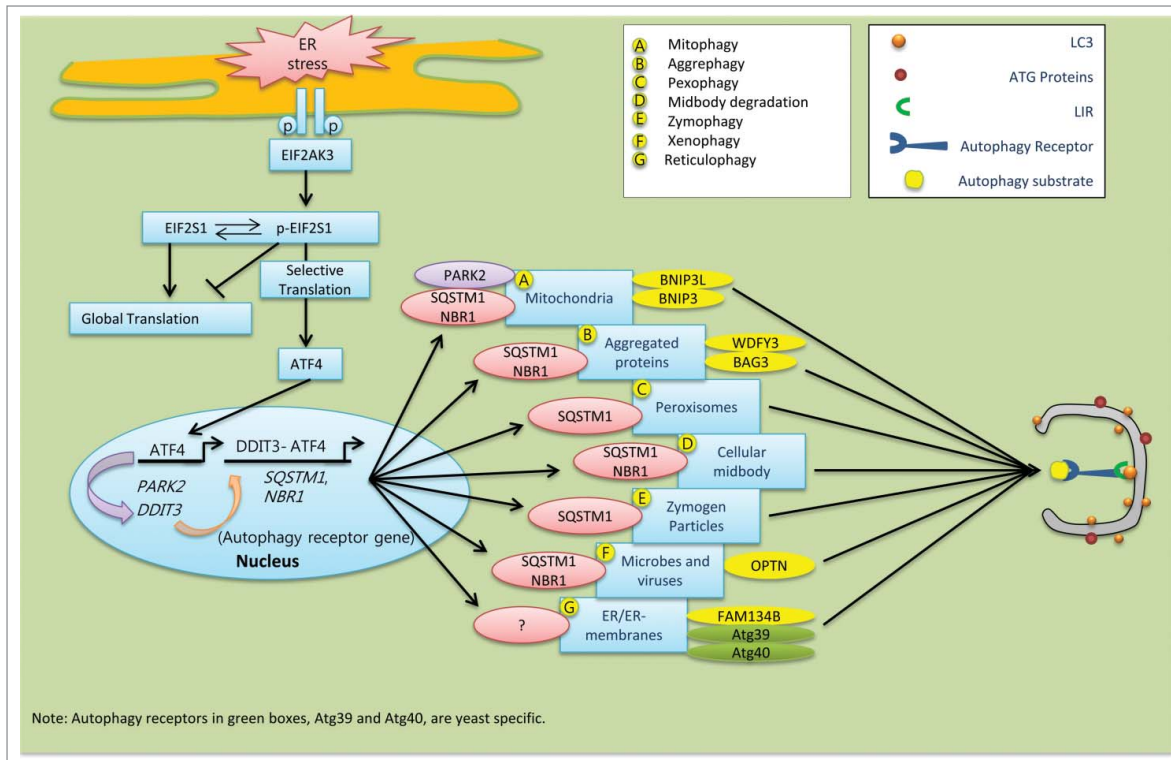
BAPTA-AM can also block autophagy flux following ER stress induced by proteasome inhibitors.<sup>165</sup> Specifically, BAPTA-AM-mediated  $\text{Ca}^{2+}$  chelation disrupts lysosomal function and subsequently inhibits autophagosome degradation.<sup>165</sup> Accordingly, it can be suggested that depletion of cytosolic  $\text{Ca}^{2+}$  might interrupt the lysosomal function essential for complete autophagy flux. In the same vein, other studies have shown that intracellular  $\text{Ca}^{2+}$  mobilization caused by plasma membrane damage results in increased interaction between lysosomes and SNAREs, which are crucial for membrane fusion.<sup>166</sup> Such elevated levels of  $\text{Ca}^{2+}$  could facilitate autophagosome and lysosomal fusion and thus enhance autophagy flux.

In addition to regulation of BECN1, it has been reported that ER-localized BCL2 is also liable for depleting ER  $\text{Ca}^{2+}$  and reduces agonist-induced  $\text{Ca}^{2+}$  leakage from the ER, possibly via an interaction through ITPR, in an attempt to negatively regulate autophagy.<sup>150</sup> Opposing the latter mechanism, Reed and colleagues demonstrated that TMBIM6/BI-1 promotes autophagy by reducing steady-state levels of ER  $\text{Ca}^{2+}$  through ITPRs.<sup>167</sup> Reduced ER  $\text{Ca}^{2+}$  is attributed to the depletion of mitochondrial  $\text{Ca}^{2+}$  uptake and subsequent ATP depletion, which in turn stimulates AMPK-dependent autophagy. Mechanistically, the ER and mitochondria are associated through the mitochondria-associated membranes (MAMs), and the efficiency of ITPRs in the MAM might be reduced in cells overexpressing TMBIM6, thereby decreasing  $\text{Ca}^{2+}$  transfer to the mitochondria through MAM-associated ITPRs. In the context of TMBIM6, it can be assumed that BCL2 modulates ER  $\text{Ca}^{2+}$  by regulating ITPRs outside of MAMs. Furthermore,  $\text{Ca}^{2+}$  leakage via ITPRs at distinct positions on the ER membrane could be followed by distinct outcomes (Table 2).

## ER Stress Regulates Selective Autophagy

Autophagy was previously considered an essential pathway for random nonselective lysosomal bulk degradation of cytosolic components. However, accumulating evidence suggests that cells might consume part of their cytoplasm for self-nourishment and targeted removal of undesirable components, such as superfluous or damaged mitochondria through a process called mitophagy. Likewise, lipophagy, reticulophagy, ribophagy, xenophagy, pexophagy, zymophagy, and aggrephagy refer to autophagic removal of faulty lipid droplets, surplus endoplasmic reticulum, ribosomes, pathogens, peroxisomes, zymogen particles, and protein aggregates,





**Figure 2.** Schematic model of the speculative roles of ER stress components involved in selective autophagy in mammalian cells. During ER stress, EIF2AK3-dependent ATF4 translation and its interaction with DDIT3 in the nucleus stimulate expression of the autophagy receptor genes *SQSTM1* and *NBR1*, which are ubiquitous. (A) ER-stress-mediated activation of ATF4 sustains the expression of PARK2. Damaged mitochondria recruit PARK2 as a degradation signal recognized by various autophagy receptors, including SQSTM1 and NBR1. (B) Following ER stress, upregulated SQSTM1 and NBR1 interact with ubiquitin aggregates and enhance its selective clearance through aggrephagy. Autophagy receptor WDFY3 and BAG3 are also involved in aggrephagy.<sup>185</sup> (C) During pexophagy, SQSTM1 causes superfluous or damaged peroxisomes to cluster and subsequent be delivered into the growing selective phagophore.<sup>220</sup> (D) After cell division, midbody remnants are recognized by the autophagy receptors SQSTM1 and NBR1, which deliver them to the phagophore for degradation.<sup>221</sup> (E) The ubiquitin-binding protein SQSTM1 interacts with and traffics ubiquitinated zymogen granules to the nucleating phagophores.<sup>222</sup> (F) Ubiquitinated proteins interact with OPTN. The resulting autophagy receptor complex accelerates the selective autophagy flux of viruses, microbes, and other non-host entities.<sup>223</sup> (G) In mammals, the autophagy receptor FAM134B interacts with LC3 and GABARAP, and facilitates ER turnover by reticulophagy.<sup>175</sup> Similarly, yeast specific reticulophagy is Atg39 and Atg40 dependent.<sup>224,225</sup> However, the role of SQSTM1 and NBR1 in reticulophagy has not been clarified yet.

respectively (Fig. 2; reviewed in reference<sup>168</sup>). It is also evident that selective autophagy peaks during development, pathogenic infections, and alteration of nutrient sources.<sup>169</sup> Interestingly, selective autophagy was first described in yeast, which is mainly based on the autophagy-like process called the cytoplasm to vacuole targeting (Cvt) pathway for delivering prApe1 (precursor aminopeptidase I), the precursor form of a vacuole-resident enzyme, from the cytosol to the vacuole.<sup>170</sup> Moreover, genetic screens in yeast have identified proteins essential for various forms of selective autophagy, and in mammalian cells a systematic procedure to identify the molecular determinants of selective autophagy is now emerging.

What differentiates selective autophagy from bulk autophagic degradation? To achieve selectivity, specific molecular markers are required for recognition of the autophagy cargos destined for degradation. Accumulating evidence suggests that Ub chains represent a general recognition signal.<sup>171-173</sup> Ubiquitinated cargos are then

identified by special types of proteins called autophagy receptors, which can simultaneously interact with small UBL modifiers such as Atg8/LC3/GABARAP. Many of the autophagy receptor proteins possess an LC3-interacting region (LIR)<sup>171</sup> that enables them to bind with, and subsequently deliver, the autophagy cargo to the nucleating phagophore. There, UBLs facilitate their docking, which is followed by formation of the selective autophagosome.<sup>174</sup> Among the 2 dozen LIR-containing autophagy receptors, several in mammals can bridge cargo and phagophore membranes, including FAM134B (a functional homolog of yeast Atg40),<sup>175</sup> SQSTM1, NBR1, SMURF1, FUNDC1, STBD1, OPTN (optineurin), CALCOCO2/NDP52, BNIP3, BNIP3L/NIX, BAG3, WDFY3/ALFY and CBL/c-Cbl. (reviewed in reference.<sup>171</sup>) Mounting evidence suggests that SQSTM1 and NBR1 can act as universal receptors for almost all types of ubiquitinated cargo.<sup>172,174,176,177</sup> Unfolded and ubiquitinated protein sequestration during selective autophagy is mainly mediated by the cooperative activity of both SQSTM1 and NBR1. These 2 proteins are identical in terms of LIR motif, an



N-terminal PB1 domain, and a ubiquitin-interacting C-terminal UBA domain.<sup>174</sup> Notably, degradation and clearance of SQSTM1 and NBR1 proteins are mainly autophagy-specific and proteasome-independent. Manipulation of autophagy results in SQSTM1 and NBR1 aggregation under conditions of normal proteasomal activity. However, these 2 proteins differ in their size and primary sequence.<sup>174</sup> Conversely, the rest of the selective autophagy receptors in mammals are cargo specific. For example, CALCOCO2, SMURF1, and OPTN are required for invasive pathogens, BNIP3 and BNIP3L act as mitophagy-specific receptors,<sup>178</sup> and reticulophagy is FAM134B dependent.<sup>175</sup> Similarly, WDFY3/ALFY and BAG3 orchestrate aggrephagy,<sup>179,180</sup> and OPTN is specific for xenophagy, along with both SQSTM1 and NBR1 (Fig. 2).

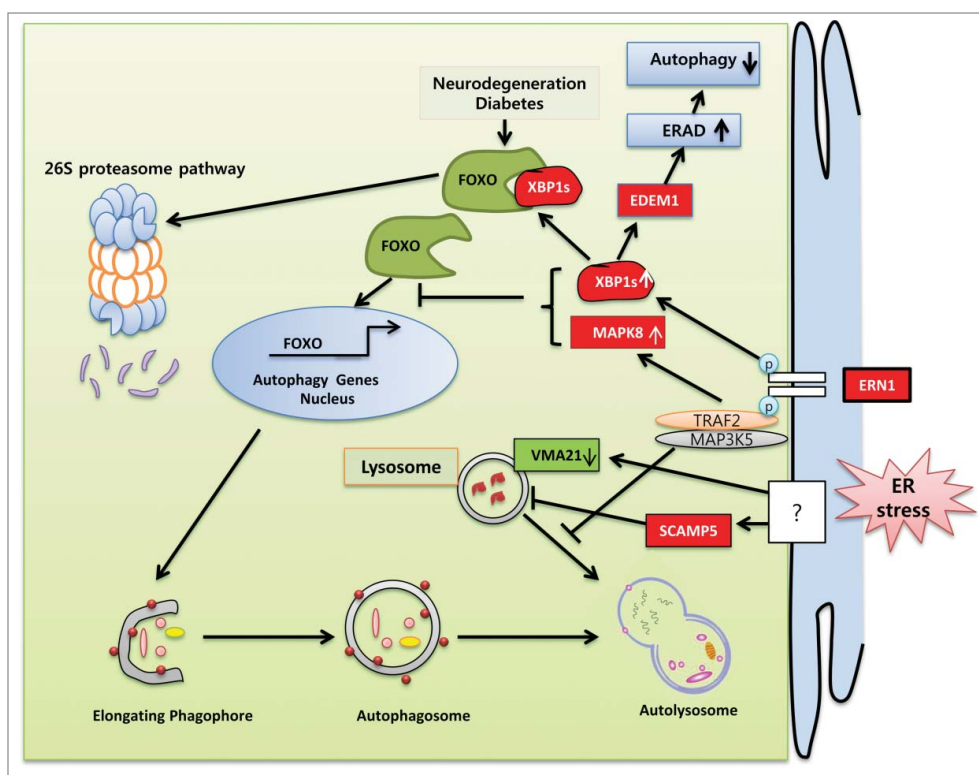
Does ER stress also participate in selective autophagy? Limited evidence supports this idea, especially for mammalian cells. However, the ER is connected with autophagy in various ways, including ER stress-mediated autophagy activation and the formation of autophagosomes at the ER. In addition, the ER itself can be subject to autophagy, and thus it is possible that ER stress can regulate selective autophagy. A recent study in yeast by Schuck et al. demonstrated that ER stress can selectively degrade excess ER membranes by triggering a special type of autophagy called reticulophagy, in which the core autophagy machinery is not implicated.<sup>181</sup> Alternatively, ER stress leads to the formation of large ER whorls that can be subjected to autophagy by selective uptake into lysosomes. Instead of forming autophagosomes, ER whorls are sequestered by direct invagination by the vacuolar membrane, suggesting a topological similarity between microautophagy and reticulophagy. However, the proteins involved in microautophagy are not required for reticulophagy.<sup>181</sup> These observations raise the question of the function of selective autophagy under ER stress. Reticulophagy regulates cell homeostasis by degrading excess ER membrane formed due to protein overload under stress.<sup>181</sup> Conversely, the UPR transcription factor XBP1s is responsible for the ER expansion necessary to accommodate more folding proteins during ER stress;<sup>182</sup> thus ERN1-XBP1 might serve as an upstream regulator of the reticulophagy machinery. Strikingly, accumulation of ER-containing autophagosomes under UPR activation suggests that the ER in mammalian cells can be subjected to reticulophagy.<sup>93</sup> Upon ERN1 deletion, compromised autophagosome formation and cell demise strengthens the speculation that the ERN1-XBP1 axis could serve as a potential mediator of ER stress-mediated reticulophagy.<sup>93</sup>

With respect to cell homeostasis, following ER stress, elimination of damaged mitochondria is crucial, and it remains possible that mitophagy can be initiated to mitigate overall cellular stress. This notion is supported by mitophagy-mediated cell protection in a transient neuronal ischemia model under pharmacologically induced ER stress.<sup>183</sup> In that context, ER stress acts as a mitophagy-dependent neuroprotector by sustaining ATF4-mediated PARK2-expression. PARK2 forms a complex with PINK1 at the outer membrane of damaged mitochondria that causes recruitment of the autophagy receptor SQSTM1, which in turn may trigger mitophagy.<sup>184</sup> Furthermore, activated DDIT3 can interact with ATF4 in the nucleus to trigger stress-induced expression of the autophagy receptor genes *SQSTM1* and *NBR1* (Fig. 2A-B). Thus, selective autophagy might be a potential outcome of

ER stress. Together, these findings describe a complete autophagy pathway after EIF2AK3-EIF2S1 activation including expression of the genes necessary for phagophore formation, and elongation, and the autophagy receptors (SQSTM1 and NBR1) specific for selective degradation of intracellular components. In agreement with this model, Rubio et al. described and characterized the formation of SQSTM1 bodies and NBR1 in both normal cells and cancer cells following hypericin-mediated photodynamic therapy (Hyp-PDT).<sup>185</sup> Hyp-PDT triggers ER stress by generating ER-localized reactive oxygen species that mediates ATP2A2/SERCA2 loss of function and disrupts ER-Ca<sup>2+</sup> homeostasis. Hyp-PDT treatment leads to the aggregation and sequestration of ubiquitinated, unfolded proteins that are then followed by SQSTM1- and NBR1-associated autophagic degradation.<sup>185</sup> This finding strongly supports the notion of selective autophagy-mediated degradation of aggregated proteins under stress conditions. Because the critical role of the UPR is to process aggregated proteins and re-establish cellular homeostasis, selective autophagy-mediated removal of misfolded proteins, protein aggregates, and protein complexes might also be part of ER stress-mediated autophagy. Indeed, it is likely that SQSTM1 and NBR1 constitute a complex under conditions of ER stress that is responsible for selective autophagic degradation of ubiquitinated proteins and other organelles. Studies in yeast and mammalian-based systems strongly suggest that reticulophagy can be enhanced during ER stress to minimize cell demise. However, to fully establish that scenario, further studies are required.

### Negative Role of ER Stress in Autophagy

The UPR might not always support autophagic processes. In some pathological conditions, ER stress is aberrant and results in impaired autophagy. Particularly in neurodegenerative disease conditions, autophagy goes awry, and ER stress response regulators appear to be at the center of such compromised autophagy machinery. Indeed, a growing body of evidence has begun to shed light on the mechanisms that underlie ER stress-mediated inhibition of autophagy. For example, in Huntington disease (HD) and familial amyotrophic lateral sclerosis (ALS) disease models, knockdown of the ERN1-XBP1 axis elevates autophagy and heightens pathological conditions (Fig. 3), suggesting that UPR branches are molecular determinants of such compromised conditions.<sup>186-188</sup> Similarly, pharmacological induction of ER stress via thapsigargin or tunicamycin causes aggregation of mutant (mt) HTT (huntingtin) proteins and deteriorates the pathological condition of HD patients through ERN1-mediated autophagy inhibition.<sup>189</sup> Likewise, in HD, overexpression of USP14, a deubiquitinating enzyme, enhances autophagic clearance of mtHTT protein through proteasome-mediated degradation of nonphosphorylated ERN1.<sup>176</sup> USP14 assists in proteasome processing by deubiquitinating proteins destined for proteasomal degradation.<sup>190</sup> Phosphorylated ERN1 has less ability to interact with USP14, thus sustaining its level and accentuating mtHTT protein aggregation by inhibiting autophagy. Furthermore, in an ALS disease model, *Xbp1* deficiency leads to



**Figure 3.** Schematic representation of the molecular mechanisms specifically involved in ER stress-regulated compromised autophagy. UPR components are implicated in the pathogenesis of various diseases associated with inadequate protein clearance. The ERN1 axes of the UPR diminish autophagy flux in several neurodegenerative and inflammatory muscle diseases. In HD and ALS, the endoribonuclease activity of ERN1 undermines autophagy by decreasing FOXO levels by trafficking it to the 26S proteasomal pathway for degradation. XBP1 depletion reduces EDEM1 expression and ERAD, which in turn induces autophagy by causing the accumulation of unfolded protein. MAPK8 knockdown enhances several autophagy genes in neurons by translocating FOXO to the nucleus. Following ERN1 activation, the association of TRAF2 and MAP3K5 in HD reduces autophagy flux by preventing autophagosome and lysosomal fusion. ER stress upregulates the expression of SCAMP5 and disrupts lysosomes, which in turn downregulate autophagy flux. ER stress blocks lysosomal pH-dependent autophagy flux by depleting the VMA21 level essential for V-ATPase-mediated proton entry into the lysosome.

the clearance of mutant SOD1 proteins by upregulating basal autophagy in the absence of any additional stimuli.<sup>187</sup> Mechanistically, *XBP1* deficiency promotes high expression of FOXO (forkhead box O), which in turn increases the expression of several genes that positively regulate autophagy (Fig. 3).<sup>147</sup>

FOXO is one of the major intracellular hubs for the regulation of a variety of biological processes important for cell proliferation, development, metabolism, survival, and stress resistance.<sup>191-194</sup> Lately, FOXO has been identified as a critical regulator of autophagy that upregulates the transcription of several autophagy-related genes, such as *ATG12*, *BECN1*, *BNIP3*, *GABARAPL1*, and *LC3*. (reviewed in reference.<sup>195</sup>) There are 4 FOXO family members in mammals: FOXO1/FKHR, FOXO3/FKHRL1, FOXO4/AFX, and FOXO6. Various intracellular signaling pathways, such as the cell proliferative PI3K-AKT pathway, the stress-dependent MAPK8 and MST1 (macrophage stimulating 1) pathways, and the AMPK and SIRT2 pathways, converge onto the FOXO signaling network.<sup>196</sup> These pathways regulate the subcellular localization, protein level, DNA

binding, and transcriptional properties of FOXO.<sup>196</sup> The C-terminal DNA binding domain of FOXO proteins possesses both the nuclear localization and nuclear export sequences. Post-translational modifications such as phosphorylation and monoubiquitination regulate the effectiveness of the nuclear localization and export sequences, thereby providing the molecular basis of FOXO movement in and out of the nucleus. However, protein kinases AKT1 and serum and SGK (serum/glucocorticoid regulated kinase)-mediated phosphorylation lead to the association of FOXO with a chaperone protein named YWHA/14-3-3, which in turn retains FOXO in the cytosol.<sup>197-200</sup> Preferential binding of YWHA/14-3-3 proteins with phosphorylated FOXO also prevents its nuclear re-entry by blocking the nuclear localization signal.<sup>201,202</sup> Conversely, AMPK, MAPK8, MAPK/p38, MST1, and CDK1 (cyclin-dependent kinase 1) proteins can promote FOXO nuclear localization and transcriptional activity by disrupting the interaction between FOXO and YWHA/14-3-3.<sup>200</sup> Nuclear translocation of FOXO and subsequent induction of cytoprotective autophagy is observed in MAPK8 knockout neurons. In that condition, MAPK8 acts as a dominant negative regulator of FOXO. However, mutant fibroblasts with depleted MAPK8 expression exhibit compromised autophagy under serum withdrawal conditions (Fig. 3),<sup>203</sup> indicating that MAPK8 is essential for autophagy induction in cells other than neurons. From the existing information, it is clear that both upregulation and downregulation of the ERN1 branch of the UPR can modulate autophagy. Indeed, a recent study of pancreatic  $\beta$  cells demonstrated that overexpression of *XBP1s* downregulates FOXO expression through direct XBP1 binding and transferring of FOXO toward the 26S proteasome-mediated degradation pathway.<sup>204</sup> This finding supports the mouse model of HD in which *Xbp1* deficiency in mouse brains leads to enhanced autophagy via FOXO accumulation (Fig. 3). Hence, it can be assumed that, among other regulatory components, FOXO remains at the core of autophagy when the UPR signaling network is compromised.

A pertinent question to be asked, in the context of neurodegenerative diseases, is how ER stress blocks autophagy flux. Lee et al. provided a partial explanation in their demonstration that ER stress impedes autophagy flux through the ERN1-TRAF2 pathway in

HD, in which a mutant form of TRAF2 facilitates the clearance of aggregated proteins.<sup>172</sup> The results of that study suggest that the kinase activity of ERN1 leads to an accumulation of mtHTT protein by impairing autophagy flux, which in turn regulates neuronal cell death. Conversely, *XBP1* splicing caused by endoribonuclease activity also inhibits autophagy by FOXO downregulation.<sup>204</sup> Given its importance to TRAF2 and ERN1 association, MAP3K5 might also be involved in autophagy regulation in neurodegenerative disorders. Indeed, MAP3K5 depletion prevents neuronal cells from stress-induced apoptosis by MAPK8 downregulation,<sup>205</sup> which is similar to MAPK8 knockdown models.<sup>203</sup> Whether such depletion of MAP3K5 preferentially activates autophagy remains to be explored.<sup>206</sup> However, the cumulative data regarding the ERN1 branch and its downstream regulation of the UPR are in conflict. Considering all the evidence, 2 possibilities are suggested. First, the ERN1 branch of the UPR is predominant during apoptosis, which might result from impaired autophagy. Second, ERN1-mediated autophagy manipulation is only conserved during pathological conditions. Mechanistically, prolonged ER stress-induced cell death pathways could converge into ERN1-mediated autophagy flux inhibition.<sup>189</sup> It is interesting that, despite having a potential role in autophagy induction, ERN1-MAPK8 inhibits autophagy in neuronal ER stress conditions. Thus, it remains to be determined which factors distinguish the effects of ERN1 in neurons from other types of cells with respect to autophagy. In this context, the duration of ER stress has been shown to be an important factor for the transition of complete autophagy flux to inhibition. In nonalcoholic fatty liver disease, mild ER stress leads to complete autophagy, and chronic stress causes cell death through the blockage of autophagic flux.<sup>207</sup> Interestingly, rapamycin can re-establish regular autophagy flux under chronic ER stress, suggesting that endocytosis pathways remain active and that AKT1-MTOR might be the main regulator of such autophagy blockage. Furthermore, under ER stress conditions, nuclear translocation of FOXO proteins seems to be absent in non-neuronal cells.<sup>164</sup> Notably, the induction of autophagy is a more predominant consequence of ER stress than inhibition; thus a negative interplay between the EIF2AK3-EIF2S1 and ERN1 pathways and chronic stress might activate a feedback loop that further aggravates protein accumulation by changing ER protein-folding capacity.<sup>203</sup>

Autophagy flux is inhibited by imperfect signaling through autophagosomes and lysosomes. Various proteins integrate those signaling pathways and facilitate autophagosome and lysosomal fusion. Activated ERN1 does not inhibit the formation of autophagosomes, but rather leads to their accumulation, which might be caused by disruption of the endocytotic traffic machinery. In agreement with that possibility, Noh et al. showed that ER stress can impair endocytosis pathways by upregulating the expression of SCAMP5 (secretory carrier membrane protein 5) (Fig. 3).<sup>208</sup> Furthermore, SCAMP5 expression is significantly upregulated in the striatum of HD patients, and siRNA-mediated depletion of *SCAMP5* increases endocytosis and autophagic clearance. However, the identity of the specific UPR branch that regulates SCAMP5 activity has not yet been clearly established. ERN1-mediated aggregation of mtHTT and autophagy flux inhibition suggest that SCAMP5 might be a potential target of the

ERN1 branch. Further studies focusing on ERN1 and SCAMP5 in HD patients could represent a good opportunity to identify how to improve the pathological conditions associated with HD. Conversely; MAPK/p38 activation following ER stress can stimulate the RAB5 cycle and endocytosis.<sup>209</sup> Although it remains unclear whether endocytosis and autophagy follow the same pathway, it is well established that the 2 processes are closely related and converge upon common degradation through terminal lysosomes.

Thapsigargin undermines autophagy at multiple stages. It causes decreased autophagosome biosynthesis by activating calpains at an early stage of the autophagy mechanism and blocks autophagy flux at the end stage by inhibiting the fusion of matured autophagosomes with lysosomes.<sup>210-212</sup> The latter is independent of the endocytosis-mediated degradation machinery and might partially explain the distinction between autophagy and endocytosis. Mechanistically, thapsigargin blocks recruitment of RAB7A to autophagosomes while leaving endosomal RAB7A recruitment active.<sup>212</sup> These findings indicate that autophagy uses different machinery than endocytosis to recruit RAB7A to autophagosomes as well as different tethering complexes to bind to lysosomes. Although thapsigargin has proved to be a useful inhibitor of autophagy flux, the overall controversy remains obscure. Grotomeier et al., along with many other researchers, clearly showed that complete autophagy flux remains active under thapsigargin treatment.<sup>213</sup> Moreover, thapsigargin has recently been implicated in the inhibition of autophagy at the autophagosome maturation stage by preventing the closure of elongating phagophores.<sup>214</sup> According to that study, thapsigargin inhibits neither autophagy induction nor complete autophagy flux. Indeed, it causes immature autophagosome formation, as detected by the inability of prostate adenocarcinoma cells to sequester cytosolic LDH/lactate dehydrogenase.<sup>214</sup> Cells treated with the Ca<sup>2+</sup> ionophore A23187 also exhibit a similar effect.<sup>214</sup> Strikingly, the role of ER stress in thapsigargin-mediated autophagy inhibition is not well established; rather ER-Ca<sup>2+</sup> signaling has drawn the major focus. Together, these findings strongly suggest that autophagy modulation is predominantly a consequence of ER-Ca<sup>2+</sup> perturbation rather than ER stress.

*DDIT3* knockdown can accentuate autophagy in AR113Q knockin male mice with spinal and bulbar muscular atrophy. Interestingly, aberrant ER stress in the spinal and bulbar muscular atrophy model remains unchanged in the wild-type and knockout model, confirmed by expression of identical phosphorylated (p)-EIF2AK3 and p-EIF2S1 levels, suggesting a negative role for *DDIT3* in autophagy regulation.<sup>215</sup> Furthermore, a negative interplay between ER stress and autophagy has also been identified after fatty liver graft preservation. Elevated levels of ER stress and impaired autophagy are usually found in steatotic livers in response to cold ischemia/reperfusion injury. Indeed, prolonged cold storage of steatotic livers can lead to the induction of graft injury. Steatotic liver storage in a solution of Institut Georges Lopez, melatonin and trimetazidine, instead of an Institut Georges Lopez solution alone, upregulates autophagy by reducing ER stress through high AMPK phosphorylation. Furthermore, a sharp increase in ER stress with a significant decrease in autophagy is observed when AMPK is inhibited.<sup>216</sup> Together,



these data suggest that ER stress leads to impaired autophagy in steatotic livers in response to prolonged cold storage (Table 3).

## Concluding Remarks

ER stress serves dual roles by favoring mechanisms of both autophagy induction and inhibition. In both mammalian and yeast cells, ER stress-mediated autophagy has been well characterized and is highly consistent. However, this is not the case for several disease models. During physiological ER stress, autophagy serves as an adaptive stress response that helps to sustain cell survival, whereas pathological ER stress can lead to the inhibition of autophagy. Surprisingly, genetic modulation of UPR branches appears to be effective in manipulating autophagy. Notably, it has been uncovered that under pathological ER stress (especially neurodegeneration), autophagy is substantially regulated by the ERN1 branch of the UPR and its downstream regulators. *EDEM1* (ER degradation enhancer, mannosidase  $\alpha$ -like 1), a target gene of XBP1 encoding an essential component of ERAD substrate signaling and recognition, might also play important undermining roles in ER stress-mediated autophagy. RNAi screening shows an elevated level of basal autophagy in an unstressed *xbp1*<sup>-/-</sup> fly model.<sup>217</sup> At the molecular level, *Xbp1* deficiency results in depleted EDEM1 expression followed by impaired ERAD machinery and a subsequent aggregation of misfolded proteins. An autophagy signal might be triggered by those accumulated proteins.<sup>217</sup> XBP1 could suppress autophagy by clearing the aggregated protein through the EDEM1-dependent proteasome degradation pathway. Sporadic inclusion-body myositis has phenotypic similarities to Alzheimer and Parkinson diseases in terms of protein aggregation.<sup>218</sup> In sporadic inclusion-body myositis muscle fibers, ER stress enhances protein aggregation by significantly decreasing the proteolytic activity of CTSB and CTSD. This could be due to the decreased expression of an essential lysosomal V-ATPase assembly chaperone, VMA21.<sup>218</sup> Compromised autophagy is the consequence of decreased VMA21 because of increased lysosomal pH (Fig. 3; Table 3).

Although it remains to be confirmed to what extent autophagy can remove misfolded proteins and damaged organelles, our review of the existing literature demonstrates the exciting possibility that ER stress can lead to the selective degradation/clearance of aggregated proteins and damaged organelles. However, the contribution of ER stress in selective autophagy has been reported very recently. The autophagy field is rapidly growing, and current research is converging into selective autophagy. It is therefore likely that future research focusing on signaling pathways regulating ER stress-induced selective autophagy will be of great importance. To elucidate such signaling pathways, many burning questions need to be answered. Here, in this review, we have related that the autophagy receptors SQSTM1 and NBR1 are ubiquitous in nature and are essential components for all

types of selective autophagy. Furthermore, ER stress can stimulate both of those proteins significantly. However, those 2 cargo receptors work along with others that are organelle specific. Thus, identifying and characterizing more ER stress-specific cargo receptors will be an important focus in the near future. It has already been established that SQSTM1 and NBR1 together can sequester aggregated proteins and form SQSTM1/p62 bodies to deliver them into the degradation pathway and protect cells via sequestration, preventing aggregated proteins from affecting fresh proteins or normally functioning organelles. Thus it needs to be determined whether the UPR under mild or acute stress can produce SQSTM1/p62 bodies as a cell protective mechanism. Because cationic concentration and movement inside the subcellular organelles contribute to their self digestion and indirectly amplify degradation signaling, studies focusing on intracellular organelles' physiological environments will shed new light on stress-mediated selective autophagy.

Thapsigargin-induced autophagy remains an important unresolved issue, with numerous independent studies describing 3 different outcomes. Specifically, while some studies support the inhibition of autophagy at the early,<sup>214</sup> and mid stages,<sup>219</sup> others have observed complete autophagy flux.<sup>213</sup> following thapsigargin treatment. Experimental work from our lab (unpublished data) suggests that the protein levels of the autophagy markers LC3-II and SQSTM1 were significantly increased after thapsigargin treatment for up to 24 h, whereas bafilomycin A<sub>1</sub> treatment did not increase the LC3-II level further. Importantly, these data strongly support the notion of incomplete autophagy flux following thapsigargin treatment. Although several studies have been done to identify the role of cytoplasmic or ER Ca<sup>2+</sup> in autophagy regulation, it remains unclear which compartment's Ca<sup>2+</sup> plays the pivotal role in the autophagy process following ER stress.

The concurrence between ER stress and autophagy is common in several human pathologies, including respiratory diseases, inflammatory diseases, cardiovascular diseases, neurodegenerative disorders, cancer, and diabetes. Hence, it is important to understand how autophagy can be manipulated via ER stress regulators to favor prosurvival or prodeath signaling. Development of new drugs that target the linkages between the UPR branches and autophagy will have significant therapeutic benefits in the treatment of such diseases.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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