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DEGRADATION AND SEQUESTRATION: CELLULAR STRATEGIES TO COUNTERACT PROTEOTOXIC STRESS

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Degradation and Sequestration:

Cellular Strategies to Counteract Proteotoxic Stress

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Shanshan Xu

The public defense of this thesis will take place at **Atrium**, Nobels väg 12b, Solna,
On **Friday 28th October 2022,** at **9:30**.

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To see a world in a grain of sand

须弥藏芥子

To my dad, and the plant taxonomy books we read together.

谨以此文，献给我的父亲,

以及童年科学启蒙的夏日午后。

POPULAR SCIENCE SUMMARY

Proteins may be the most important and busiest executor molecules in cells. They exert their functions to maintain cellular fundamental functions.

If we magnify these molecules and compare them to something we are familiar with, proteins are like the blocks of LEGO. When you want to build up a Hogwarts school with LEGO bricks, you need to assemble certain number of blocks at a local area firstly, and further integrate these local units to build up the entire building. The numbers and positions of these blocks are under precise control according to the handbook. If the school was attacked by the death eaters - heavily stressed, the damaged local units must be removed and disassembled to bricks to reassemble. Otherwise, the accumulation of damaged units is harmful. To make sure Hogwarts school will not collapse, you need to keep the balance of assembly and clearance carefully.

Same is true for cells. To maintain proper cellular functions, cells produce native proteins and degrade damaged proteins. The ubiquitin-proteasome system and autophagy are responsible for protein degradation. If there are too many damaged proteins that fails to be cleared, the damaged proteins will cluster together and form toxic aggregates. When cells are under stress, a protective structure forms to temporary store damaged proteins (like the Room of Requirement in Hogwarts). The main question in this thesis is how important these storage structures are. I also studied how important the coordination of protein production and degradation is. Briefly, how cells deal with various proteotoxic stress.

In paper I, we found that lack of stress granules, which is a transient storage place for damaged proteins, causes a problem for protein degradation. In paper II, we found that too much protein production during stress blocks protein degradation as well. In paper III, we found that a protein fragment derived from spider silk could prevent the formation of aggregate. In paper IV, we found the novel drug-like molecule CBK79 inhibits protein degradation, induces a stress response and kills cancer cells. Overall, the work present in this thesis is to answer how is the precise control system works in cells.

ABSTRACT

Maintenance of proteome homeostasis (proteostasis) is essential to preserve cellular function in response to intrinsic and extrinsic stress conditions. This is regulated by the proteostasis network, which is comprised of machineries for protein synthesis, folding, sequestering, and degradation. The collaboration of these machineries is to ensure the functionality, subcellular localization and appropriate protein abundance, thereby preventing proteotoxic stress.

Disturbances in proteostasis can be caused by gene mutations, temperature fluctuations, alterations in synthesis or degradation, chemical insult, etc. If a disbalance in proteostasis is not addressed in a timely and correctly manner, aberrant proteins can accumulate and form insoluble aggregates, which are the hallmarks of the majority of neurodegenerative diseases and other so-called proteinopathies. Therefore, extending our knowledge and developing techniques to modulate the proteostasis network are important for the development of new therapeutic strategies for these disorders.

The work presented in this thesis describes how the proteostasis network responds to different proteotoxic stress conditions, and how its modulation preserves proteome integrity.

In **paper I**, we describe that the sequestration of aberrant, newly synthesized proteins in cellular stress granules prevent impairment of the ubiquitin-proteasome system in the nuclear compartment in response to thermal stress. In stress granule-deficient cells, these newly synthesized proteins passively diffuse into the nucleus instead of being sequestered in cytoplasmic granules. The newly synthesized proteins translocate to nucleoli in an HSP70-dependent manner. Under stress, HSP70 interacts with newly synthesized proteins to maintain their conformation. Our data suggest that heat shock factor 1 is released from HSP70, thereby prematurely activating the heat shock response while recovering from thermal stress. In line with a premature heat shock response, we found enhanced SUMO2/3-dependent degradation of aggregation-prone proteins, and impairs proteasomal degradation in the nuclear compartment.

In **paper II**, we characterize the effect of the integrated stress response inhibitor ISRIB on the ubiquitin-proteasome system in response to thermal stress. During thermal stress, the integrated stress response is activated to inhibit protein translation, thereby preventing overloading of the proteostasis network with misfolded, newly synthesized proteins. However, ISRIB restores protein translation during stress, resulting in an increased amount of newly synthesized proteins. Part of the newly synthesized proteins under stress are dysfunctional and therefore polyubiquitinated targeted as substrates for proteasomal degradation. Meanwhile, we show that a large fraction of polyubiquitinated proteasome substrates converts to a detergent insoluble state. We propose that a limitation of ubiquitin availability results in the attenuation of ubiquitin proteasome system.

In **paper III**, we studied the effect of a protein aggregation-preventing tag, the NT* domain, on an aggregation-prone protein. The NT* domain is a solubility tag derived from a spider silk protein. The fusion of this solubility tag with an aggregation-prone reporter protein prevented protein aggregation in mammalian cells in the cytosolic and nuclear compartments. This finding provides the possibility to reduce the burden if aggregation-prone proteins on proteostasis with natural anti-aggregation domains.

In **paper IV**, we characterized CBK79 as a novel proteostasis inhibitor that impairs both proteolytic pathways: the ubiquitin-proteasome system and autophagy. As a consequence of the proteostasis collapse caused by CBK79, the compound activates the heat shock response and induces aggresome formation. Intriguingly, preconditioning of cells by thermal stress relieves the negative effect of CBK79 on ubiquitin-proteasome system but not on autophagy.

LIST OF SCIENTIFIC PAPERS

I. **Xu SS**, Gierisch MG, Schellhaus AK, Poser I, Alberti S, Salomons FA, and Dantuma NP. **Cytosolic stress granules relieve the ubiquitin-proteasome system in the nuclear compartment**. Manuscript.

II. **Xu SS**, Gierisch MG, Poser I, Alberti S, Salomons FA, and Dantuma NP. **Chemical inhibition of the integrated stress response impairs the ubiquitin proteasome system.** Manuscript.

III. Schellhaus AK, **Xu SS**, Gierisch MG, Vornberger J, Johansson J and Dantuma NP. **A spider silk-derived solubility domain inhibits nuclear and cytosolic protein aggregation in human cells.** *Communications Biology*, 2022, 5:505.

IV. Giovannucci TA, Salomons FA, Stoy H, Herzog LK, **Xu SS**, Qian WX, Merino LG, Gierisch MG, Haraldsson M, Lystad AH, Uvell H, Simonsen A, Gustavsson, Vallin M and Dantuma NP. **Identification of a novel compound that simultaneously impairs the ubiquitin-proteasome system and autophagy.** *Autophagy*, 2022, 18: 1486-1502.

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LIST OF ABBREVIATIONS

AMPK	5' AMP-activated protein kinase
ATG	Autophagy related gene
ATF6	activating transcription factor 6
CUAAC	coper catalysed azide-alkyne cycloaddition
DUBs	deubiquitinating enzymes
DRiPs	defective ribosome proteins
DYRK3	dual specificity tyrosine-phosphorylation regulated kinase 3
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
FUS	fused in sarcoma
GCN2	general amino acid control non-depressible 2
GDP	Guanosine diphosphate
GTP	Guanosine-5’-triphosphate
GFP	green fluorescent protein
G3BP	Ras GTPase-activating protein-binding protein
HDAC6	Histone deacetylase 6
HPG	L-homopropagylglycine
HRI	heme-regulated inhibitor
HSE	heat shock element
HSF	heat shock transcription factor
HSP	heat shock protein
HSR	heat shock response
IDR	intrinsically disorder region
IRE1	inositol requiring kinase 1
ISR	integrated stress response
ISRB	integrated stress response inhibitor
JNK	c-Jun N-terminal kinase
LC	low complexity
LLPS	liquid liquid phase seperation
LSPS	liquid solid phase seperation
MTOC	microtubule organizing center
mTORC1	mammalian target of rapamycin complex 1
NEF	nucleotide exchange factor

NLS	nuclear localization signal
NES	nucleus export signal
ODC	ornithine decarboxylase
PE	phospholipid phosphatidylethanolamines
PRAS	Proline-rich AKT1 substrate
PERK	PKR-like ER kinase
PKR	protein kinase R
PI3K	class III phosphatidylinositol 3-kinase
RACK1	Receptor for activated C kinase 1
RNF4	RING finger protein 4
Rpt	regulatory particle triple-ATPase
Rpn	regulatory particle non-ATPase
RQC	ribosome quality control
SCA-1	spinocerebellar ataxia type 1
sfGFP	super fold green fluorescent protein
SG	stress granule
STUbL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin modifier
TDP43	TAR DNA binding protein of 43 kDa
TIA1	T cell internal antigen-1
TUBE	tandem ubiquitin binding entities
Ub	Ubiquitin
ULK1	uncoordinated-5 like kinase 1
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
XBP1	X box binding protein 1
YFP	yellow fluorescent protein

INTRODUCTION

1. The proteostasis network

Proteins are the most versatile macro-molecules and the main executors of cellular functions. Therefore, it is essential that their abundance, subcellular localization, and quality are constantly being monitored and regulated. The maintenance of the entire collection of proteins, the proteome, is referred to as protein homeostasis (proteostasis)[1]. Proteostasis is executed by a coordinated network of multiple cellular modulators including protein synthesis, molecular chaperones, proteolytic systems and corresponding regulators (**Figure 1**). Failure in maintaining proper proteostasis can have profound adverse consequences such as cell death or tumorigenesis.

Proteostasis can be challenged by intrinsic stress conditions (including gene mutations, defects in protein translation) and extrinsic stress conditions (such as thermal or oxidative insults). Cells are equipped with several stress response pathways that can counteract such disturbances in proteostasis. The stress response needs to be precisely controlled as well. Although adaption of the proteostasis network to these challenges is robust, it can be insufficient during chronic stress conditions or after severe acute stress, resulting in accumulation of misfolded proteins that cause proteotoxicity.

The capacity of the proteostasis network declines during aging[2], increasing the sensitivity of cells to diverse stress conditions. This is exemplified by the occurrence of a number of age-related neurodegenerative disorders that are characterized by the accumulation and aggregation of misfolded proteins. Overall, the proteostasis network functions to safeguard the delicate balance between protein production and disposal, involving multiple modulators in a temporal and spatial manner.

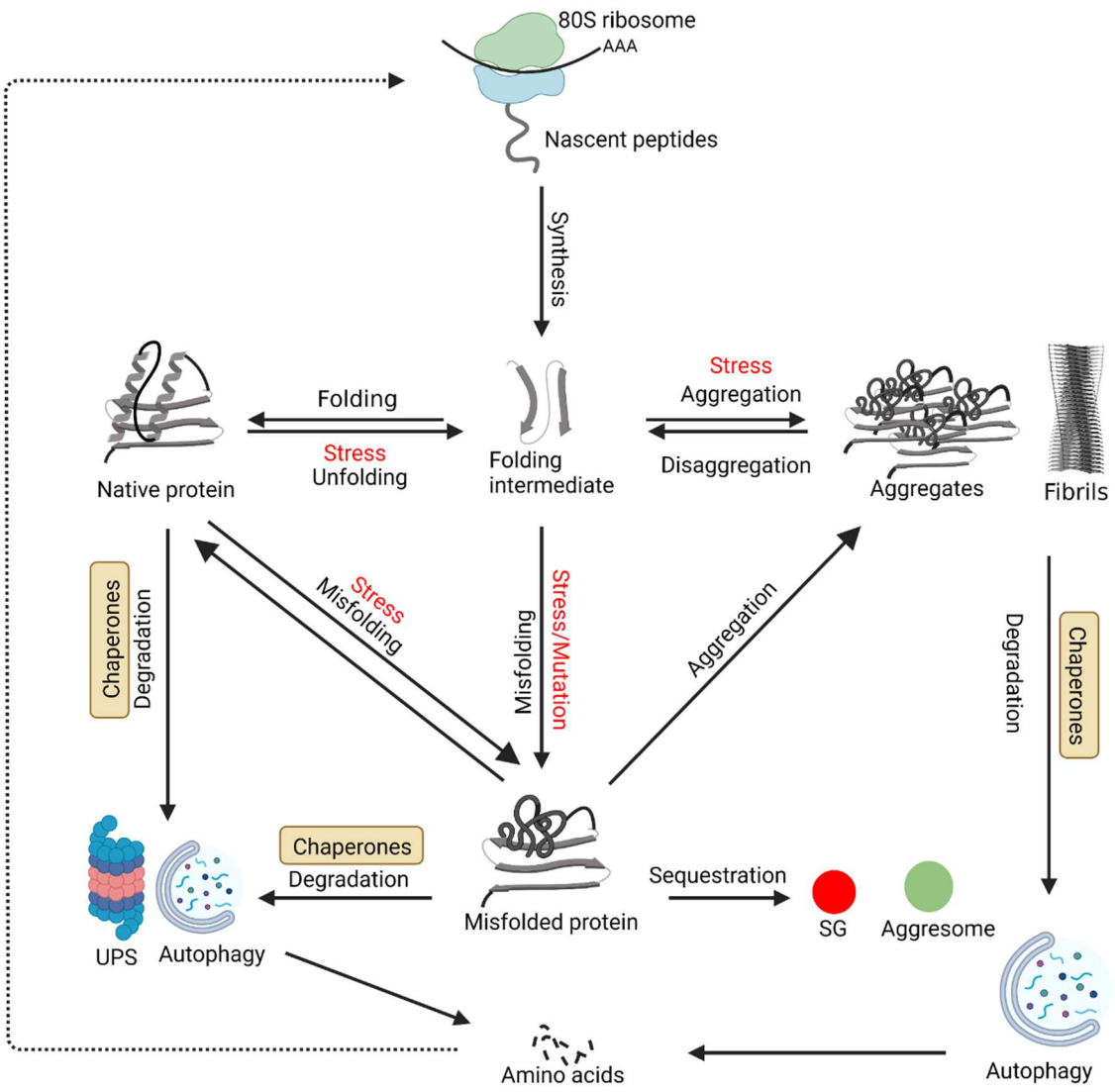

Figure 1. The proteostasis network. The proteostasis networks consists of several modulators that coordinate protein synthesis, folding, sequestration and degradation. Modified based on reference 1. Figure created with BioRender.

2. Stress response pathways

2.1 Integrated stress response

The integrated stress response (ISR) is a conserved signaling network that couples cellular stress detection to reprograming of protein translation thereby adapting cells to the physiological changes and maintaining proteostasis[3,4]. The ISR is activated in response to diverse extracellular stress, (hyperthermia, hypoxia, amino acid or glucose deprivation, viral infection, etc), and intracellular stress conditions (endoplasmic reticulum (ER) stress, oxidative stress, etc) and induces a general translation initiation reduction while at the same time increasing translation of specific mRNAs encoding proteins involved in the stress response[5] (**Figure 2**). The diverse stress conditions are sensed by four kinases: HRI (heme-regulated inhibitor), PKR (protein kinase R), PERK (PKR-like ER kinase), and GCN2 (general amino acid control non-depressible 2)[6,7,8,9]. All four kinases share a similar signal transduction mechanism in which the divergent regulatory domains detect stress signals and trigger dimerization of the kinase domain and trans-autophosphorylation[10].

Upon stress, the four kinases phosphorylate the serine 51 of the eukaryotic initiation factor 2 (eIF2) α subunit[11]. eIF2 is a heterotrimeric GTPase composed of α, β, and γ subunits, which forms a ternary complex with GTP and methionine initiator tRNA[12]. This ternary complex is essential for translation initiation at the AUG start codon of open reading frames of transcripts[13]. Once the start codon is decoded, eIF2-bound GTP is hydrolyzed to GDP, and eIF2-GDP is released from the ribosome subunit, allowing the entire ribosome to assemble, followed by the elongation phase of protein synthesis. After its release, eIF2-GDP is recycled back to its activated GTP-bound state. The GDP/GTP nucleotide exchange is catalyzed by the guanine nucleotide exchange factor eIF2B, which is considered to be the rate limiting, regulatory phase of the ternary complex formation and AUG-initiated mRNA translation[14].

eIF2B is a symmetric decamer composed of two copies of five subunits (α, β, γ, δ and ε). The nucleotide exchange activity of eIF2B relies on the bipartite interaction between eIF2B-ε and eIF2-γ, which stabilizes the nucleotide binding pocket for GDP release and GTP loading[15,16]. Phosphorylation of eIF2α leads to a

conformational rearrangement, forming a hydrophobic surface patch that has higher affinity to a binding site of eIF2B. Interaction of phosphorylated eIF2α with eIF2B interferes with proper positioning of eIF2 to the catalytic domain of eIF2B. In this way, phosphorylated eIF2α acts as a competitive inhibitor of eIF2B nucleotide exchanging activity, thereby limiting recycling of eIF2B and the formation of the eIF2 ternary complex, which causes a reduction in general translation initiation[17,18,19].

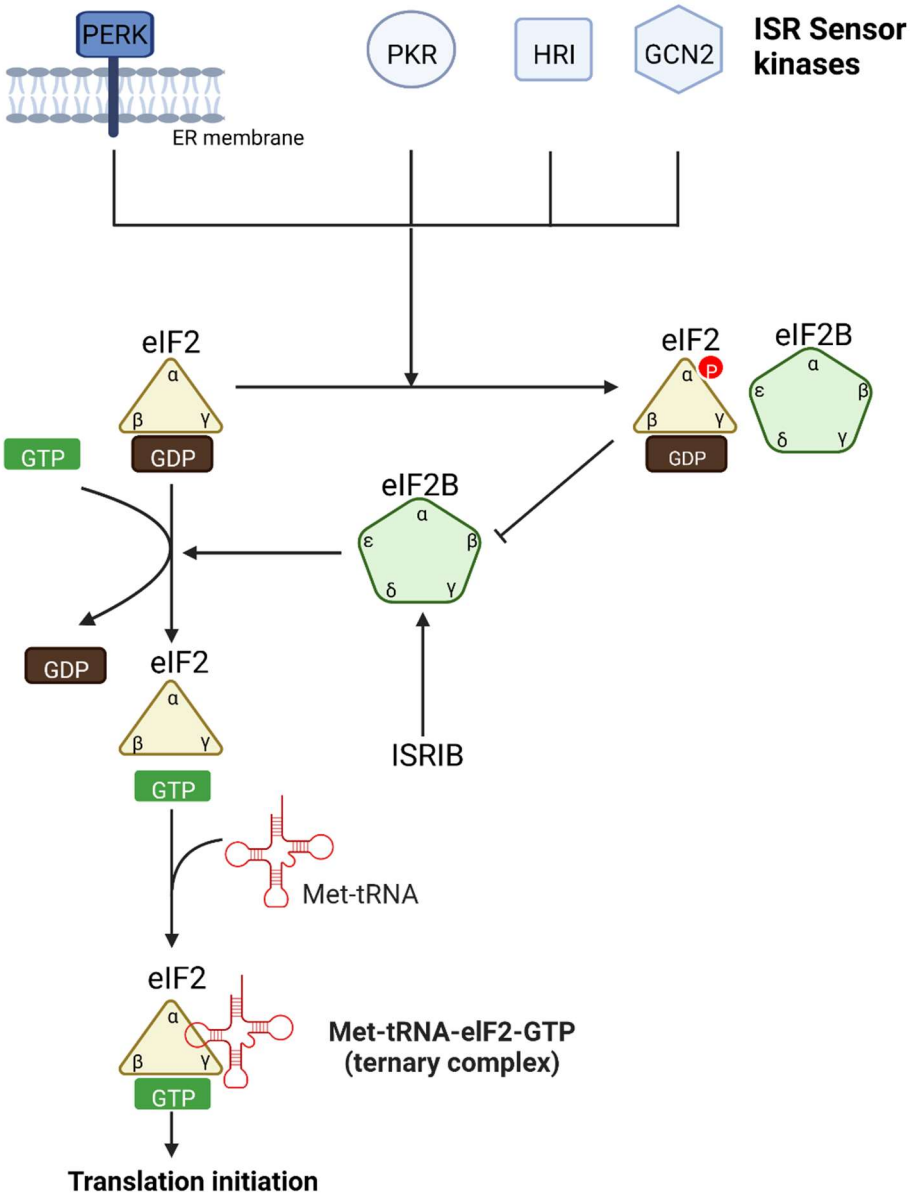

Figure 2. Mechanism of integrated stress response. Four sensor kinases phosphorylate eIF2α and inhibit the eIF2B nucleotide exchange factor activity, limiting the eIF2·GDP exchange to eIF2·GDP and inhibiting the formation of the eIF2·GDP·tRNA translation initiation complex. Figure created with BioRender.

Depletion of the ternary complex as a consequence of ISR activation results in a general decrease in translation with the exception of a subset of stress response related mRNA transcripts containing upstream open reading frames[20]. The inhibition of protein translation is restored by the reduction of the interaction between phosphorylated eIF2α and eIF2B[21]. Overall, ISR activation is a response to a disbalance in proteostasis and adjusts protein expression at the translational level[22].

Recently, a small molecule inhibitor for the ISR, ISRIB (Integrated stress response inhibitor) has been developed[23]. When the ISR is activated, assembled eIF2B decamers are only present in the inactive phosphorylated eIF2α -eIF2B complex as described above. ISRIB binds to eIF2B and promotes the free eIF2B subunits to assemble, increasing the level of functional eIF2B[24,25]. Opposite to phosphorylated eIF2α, ISRIB functions as an activator of eIF2B. The intriguing result of this mode of action is that ISRIB only works when free eIF2B subunits are available[26]. If the phosphorylated eIF2α concentration exceeds a certain threshold, induced by acute and strong ISR activation, ISRIB fails to activate eIF2B and fails to prevent protein translation inhibition[27]. This threshold-controlled mechanism ensures that ISRIB does not disturb ISR's cytoprotective function under conditions of severe stress.

Aberrant modulation of protein production by ISR is related to several diseases, such as cognitive and neurodegenerative disorders[28,29], metabolic disorders[30,31] and cancer[32]. Given the pharmacological properties of ISRIB, such as efficient penetration of blood-brain barrier and a low half maximal inhibitory concentration (IC_{50})[23], ISRIB is expected to be clinically potent. In line with this perspective, it was further reported that ISRIB inhibits neurodegeneration, facilitates long-term memory formation, and reverses cognition deficits after brain injury in mouse models[33,34,35].

2.2 Heat shock response

The heat shock response (HSR) is another protective signaling pathway in response to environmental or pathological stress[36] (**Figure 3**). The HSR is manifested by a general feature of rapid and massive induction of molecular chaperones and other protective proteins, which is conserved from bacteria to mammals[37].

In eukaryotes, the master regulator of the HSR are the heat shock transcription factors (HSFs)[38]. While there is only one HSF in lower eukaryotes, such as yeast, four HSFs have been identified in mammals, namely HSF1, 2, 3 and 4[39]. Of the four

HSFs in mammalian cells, HSF1 exerts a predominant and essential function in the HSR. HSF2 is considered to coordinate the response with HSF1 during HSR activation, whereas HSF3 and HSF4 have tissue-specific expression and their functions in HSR remain uncovered[39,40].

Under non-stress conditions, the inactive HSF1 monomer forms a complex with the chaperones HSP70 and HSP90[41]. Upon proteotoxic stress, the unfolded and misfolded proteins compete with HSF1 for binding to HSP70, resulting in release of HSF1 from HSP70. Following dissociation of the complex, HSF1 monomers form homo-trimers mediated through the leucine zipper in the oligomerization domain of HSF1[42,43]. The activated HSF1 trimers translocate into the nucleus, where they bind to the cis-regulatory nGAAn pentamers called heat shock elements (HSE), which are localized in the promoter region of genes encoding heat shock proteins (HSPs), strongly inducing transcription of these genes[44]. The transcriptionally induced HSPs competitively bind to HSF1 and inhibit the activity of the HSF1 trimers, thereby attenuating the HSF1 mediated transcription activity. This negative feedback loop provides a self-regulatory mechanism of HSR activation based on the cellular HSP concentration[45].

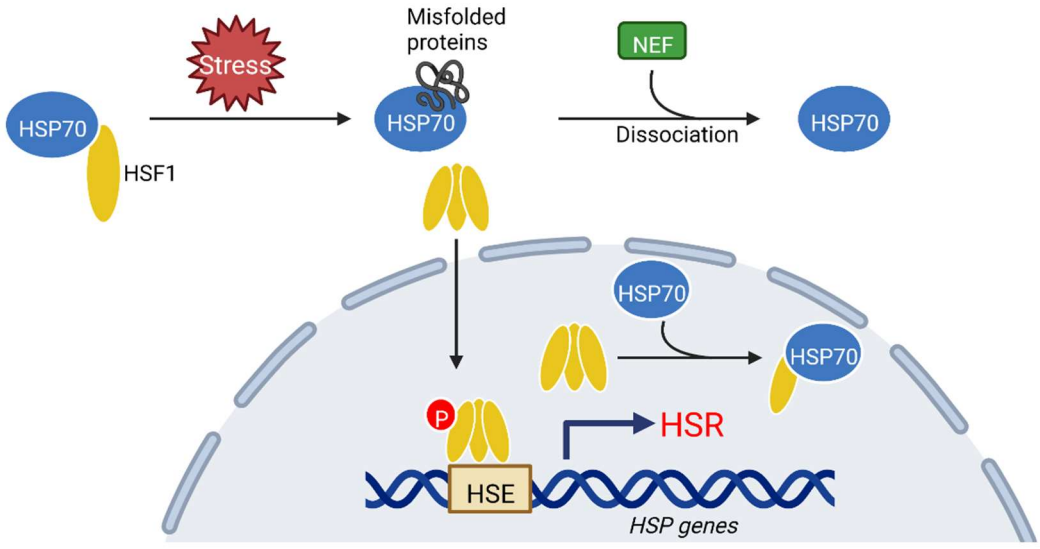

Figure 3. Regulation of heat shock response (HSR). Cytosolic and nuclear HSF1 constituently binds to HSP70. Misfolded proteins titrate away HSP70 from HSF1 and release HSF1. HSF1 monomers assemble to trimer and translocate to nucleus, where they bind to HSE and enhance the transcription of downstream genes (including but not limiting to *HSP70*). The interaction of HSP70 and misfolded proteins is disassociated by NEF (nucleotide exchange factor). The released and newly synthesized HSP70 binds to HSF1 and attenuates the HSR. Figure created with BioRender.

Both the numbers of HSEs in promoter regions and HSF1 post translation modifications affect the extent and duration of the HSF1 mediated transcription. In addition to the ON/OFF chaperone switch, the HSF1 phosphorylation at serine 230, serine 326 and threonine 142 is another positive finetuning mechanism of HSR activation[46,47,48]. Besides, HSF1 forms puncta triggered by stress, which is previously considered to be positively related to the HSR activation[49,50]. However, a recent report shows that the disassembly of nuclear HSF1 granules after stress insults is another feature that positively regulates HSR activation[51]. Furthermore, the nucleotide exchange factors responsible for substrate release from HSP70 are involved in HSF1 transcriptional activity regulation[52,53]. These multiple pathways allow cells to flexibly couple HSF1 to signaling pathways for proteostasis maintenance.

2.3 Unfolded protein response

The ER is the organelle for chaperone-dependent processing and folding of secreted, membrane-bound, and organelle-targeted proteins[54]. The folding status is actively monitored within the ER. If the balance of protein folding capacity and protein folding load is interrupted by various stresses, such as glucose deprivation or viral infection, the unfolded protein stress response (UPR) is activated[55,56,57].

The UPR constitutes of three pathways involving the ER transmembrane receptors, the double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), the inositol requiring kinase 1 (IRE1), and the activating transcription factor 6 (ATF6)[58]. PERK phosphorylates eIF2α, reducing general mRNA translation initiation as described above. Inhibition of translation initiation reduces the misfolded protein load by limiting the influx of newly synthesized protein to the ER. IRE1 splices the mRNA encoding X box binding protein 1 (XBP1) resulting in translation of the activate form of transcription factor XBP1, ultimately upregulating genes related to ER protein translocation, folding and degradation of misfolded ER proteins[59,60,61]. ATF6 is a transmembrane protein that is cleaved to produce soluble ATF6p50 when it transits from the ER to the Golgi apparatus in response to ER stress. The ATF6p50 translocates to nucleus and activates transcription of ER folding homeostasis genes[62,63].

These three branches function to globally attenuate protein translation and specifically promote expression of chaperones that enhance protein folding. If these adaption steps fail, the UPR will activate proapoptotic members of B-cell lymphoma 2 (BCL2) family, and eventually induce apoptosis[64,65,66].

3. Protein synthesis

Protein synthesis is the process in which cytosolic ribosomes decode mRNAs to produce polypeptides. The ribosomes recruit specific amino acid-carrying complementary tRNA anticodons to mRNA codons, resulting in incorporation of amino acids into the growing polypeptide chain that is produced as the mRNA passes through the ribosome. The polypeptides will further fold into their native state[67,68].

Protein synthesis is regulated downstream of several signaling pathways activated by stress sensors, such as GCN2 and PERK kinases, introducing an additional regulatory mechanism in response to a disbalance in proteostasis. Protein translation is regulated by kinases that phosphorylate the eIF2α as described above. The straightforward way to reduce accumulation of misfolded protein is to avoid the production of unstable proteins. However, protein synthesis is error-prone due to an average misincorporation rate of 1 in 10,000 amino acids, producing errors in approximately every twentieth protein[69].

Besides the intrinsic spontaneous error of protein synthesis, the quality of mRNA and codons also affect the translation rate and the fate of the synthesized protein[70]. Hydrophobic stretches in newly synthesized polypeptides have a tendency to be aggregation prone and require chaperone binding before they are released from ribosome complexes, in order to avoid aggregate formation[71]. Furthermore, the tRNA availability could also be another limiting factor for the translation rate, which can result in protein aggregation by increasing the risk of misreading or frameshift errors[72].

Aberrant, or stalled translation products are strictly controlled by the ribosome quality control (RQC) system[73]. This system is coordinated by chaperones and E3 ubiquitin ligase components of the ubiquitin-proteasome system (UPS)[74]. There are still arguments about the exact role of ubiquitination in RQC, which will require further exploration.

4. Protein degradation

4.1 Ubiquitin-proteasome system (UPS)

Almost all soluble misfolded proteins are degraded through the UPS, a major proteolytic pathway in eukaryotic cells for degradation of short-lived proteins. The UPS consists of two sequential steps: ubiquitination and proteasomal degradation[75] (**Figure 4**). Ubiquitination is executed in sequential enzyme-catalyzed steps. First, the ubiquitin-activating E1 enzyme activates ubiquitin using ATP, producing ubiquitin-E1 thiol ester formation via a ubiquitin-AMP intermediate[76]. The ubiquitin-conjugating E2 enzymes recognize the ubiquitin-E1 thiol ester and transfer activated ubiquitin to substrate proteins guided by specifically binding to an E3 ubiquitin ligase, or transfer activated ubiquitin to an E3 after which the E3 transfers ubiquitin to the substrate proteins[77]. E2 conjugases and E3 ligases catalyze covalent attachment of ubiquitin to the substrate, which is the final step of the conjugation process[78]. Ubiquitination may take the form of one single ubiquitin attached to one lysine (monoubiquitination) or multiple lysine residues (multiple monoubiquitination) or polyubiquitin chains (polyubiquitination)[79]. In ubiquitin chains, ubiquitin molecules can be linked to N terminal methionine residue (M1) or one of 7 lysine residues (K6, K11, K27, K29, K33, K48, K63)[80]. K48-linked chains is the canonical degradation signal recognized by proteasome[81]. Emerging evidence show the other chains except for K63 can also target substrate for degradation[82]. K63-linked chains were found that to trigger proteasomal degradation by seeding K48/K63 branched ubiquitin chains[83]. Besides degradation signal, ubiquitin chains are also involved in signal transduction and regulation as non-proteolytic signal[84]. Deubiquitinating enzymes (DUBs) function to reverse ubiquitination by removing ubiquitin molecules, which makes DUBs important regulators of ubiquitin-mediated degradation and other ubiquitin-dependent functions[85].

The second phase of the UPS comprises the actual proteolysis of the ubiquitinated substrate by the 26S proteasome. The 26S proteasome is a complex of approximately 2.5 MDa that consists of 31 subunits assembled in two subcomplexes: the 20S catalytic core particle and 19S regulatory particle. The 20S complex contains proteolytic active sites while the 19S regulatory complex contains multiple ATP hydrolysis sites, ubiquitin binding sites and DUBs[86].

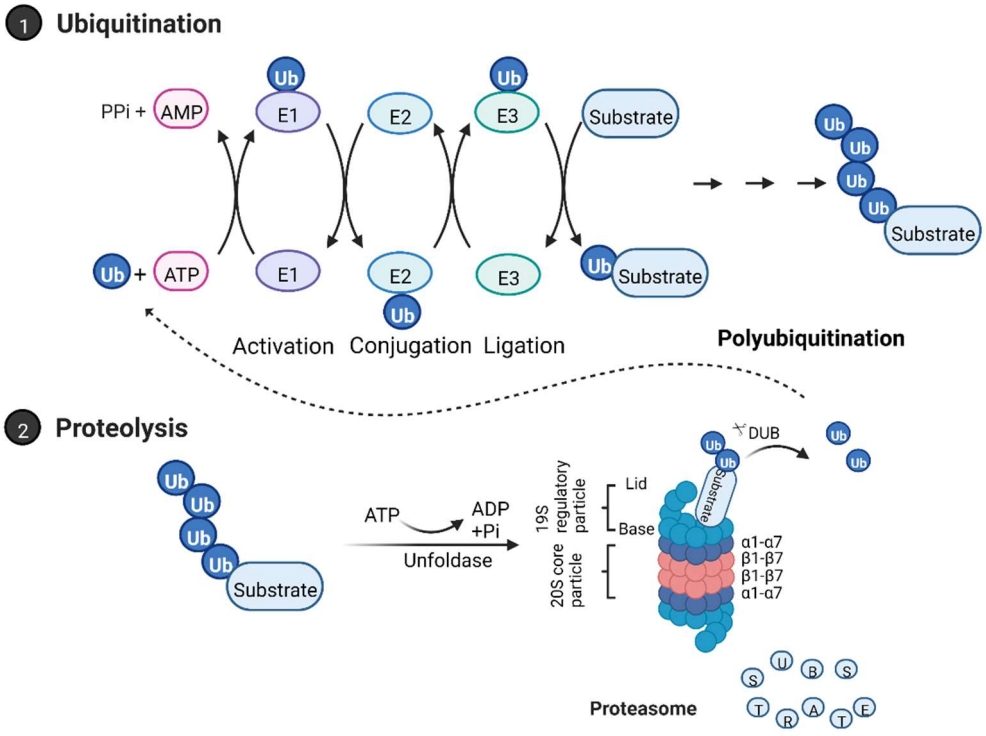

Figure 5. The ubiquitin proteasome system. The substate is tagged with a polyubiquitin chain by cascade reactions catalyzed by E1, E2 and E3 sequentially. The polyubiquitinated substrates are unfolded dependent on ATP before degradation can take place. The ubiquitin chain is removed by DUBs before substrate entering the proteolysis chamber, releasing free ubiquitin monomers, which can be used for a next round of ubiquitination. The substrate is hydrolysed in the 20S core particle. Figure created with BioRender.

The 20S subcomplex is a cylindrical stack composed of four heptameric protein rings assembled like four doughnuts[87]. Seven related α subunits assemble the two peripheral rings while seven related β subunits assemble the two inner rings. The central rings house the β1, β2 and β5 catalytic subunits, which have caspase-like, trypsin-like and chymotrypsin-like hydrolyzing activities, respectively[88]. The flexible amino termini of the α subunits gate the channel to control substrate import. Thus, only unfolded substrates can access the inner cavity of the proteasome via the narrow pores[89]. The 19S regulatory particle flanks each terminus of the 20S core particle to form the 26S proteasome[90]. Each 19S particle comprises of approximately 20 subunits, which can be subclassified as Regulatory particle of triple-ATPase (Rpt) subunits and Regulatory particle of non-ATPase (Rpn) subunits. The outer lid subcomplex of the 19S component is involved in the recognition and deubiquitination of the captured substrates[91]. The base complex has three functions: capturing and

unfolding of substrates as well as opening the α-ring channel upon substrate engagement[92]. The base subcomplex is composed of four non-ATPase subunits and six homologous ATPases subunits which provide the necessary energy for substrate unfolding and opening of the α-ring channel[93]. Proteins are degraded in a processive way by the proteasome, with the next substrate only entering the proteolytic core once the former one is properly hydrolyzed [94]. The proteasome cleaves the substrate in peptides with an average length of 6 to 10 amino acids peptides, which are further hydrolyzed into individual amino acids by amino peptidases[95].

4.2 Autophagy

Autophagy is a multi-step catabolic degradation process through which primarily long-lived proteins, protein aggregates and damaged/redundant organelles are recycled. Based on the mode of substrate delivery to the lysosome, autophagy can be divided into macroautophagy, microautophagy and chaperone-mediated autophagy[96]. Macroautophagy (hereafter referred to as autophagy) is characterized by the formation of double membrane vesicles sequestering cellular cargo, so-called autophagosome, that fuse with the proteolytic cell organelle, the lysosome[97] (**Figure 5**).

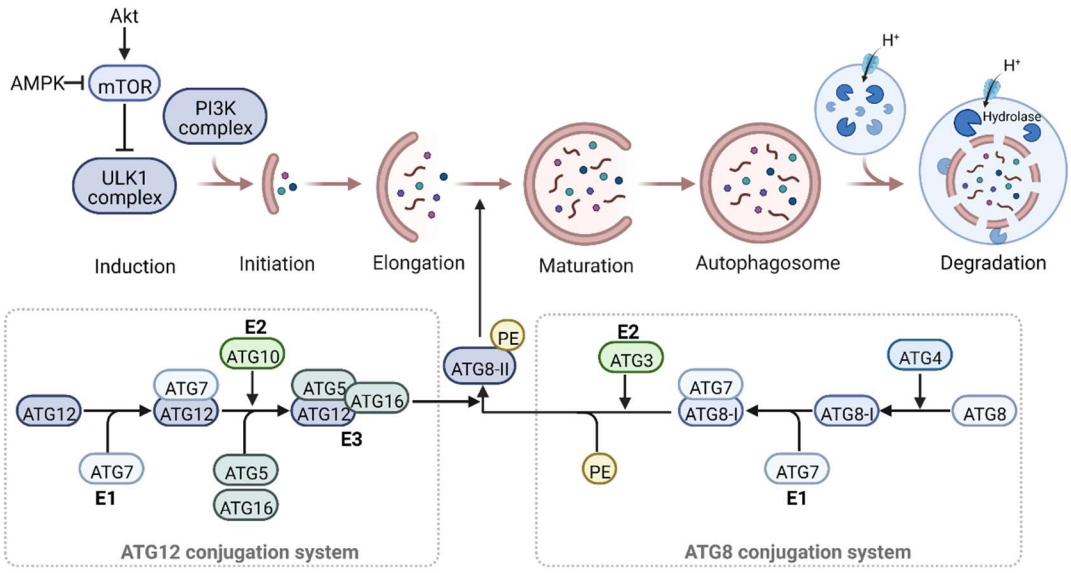

Figure 6. Macroautophagy. Schematic representation of key steps of autophagy. Autophagy is initiated by inhibition of the mTOR complex. Conjugation system of the ubiquitin-like proteins ATG8 and ATG12 function to catalyze conversion of ATG8 to ATG8-II, resulting in autophagosome membrane biogenesis. Figure created with BioRender.

Autophagy comprises several precisely controlled steps involving a series of autophagy related proteins. Autophagy induction is controlled by the serine/threonine kinase mTORC1 (mTOR complex 1) and the AMPK kinase that are regulated by diverse input signals, such as nutrient depletion, ATP abundance and accumulation of misfolded proteins[98]. Under steady-state conditions, activated mTORC1 inhibits autophagy induction by phosphorylating the uncoordinated-5 like kinase 1 or 2 (ULK1 or ULK2) and Atg13 in the autophagy initiation ULK complex[99]. Consequently, repression of mTORC1 by nutrient or growth factor depletion leads to its release from the ULK complex, resulting in dephosphorylation and activation of the ULK complex[100]. Activated ULK complex initiates autophagy and resides in the phagophore during starvation.

Upon autophagy induction, the class III phosphatidylinositol 3-kinase (PI3K) complex is recruited to the putative site of autophagosome biogenesis, where it is responsible for PtdIns3P generation and phagophore nucleation[101]. Following nucleation, the autophagosome membrane begins to expand involving two ubiquitin-like conjugation systems[102]. Firstly, Atg12 is covalently linked to Atg5, which is dependent on the E1-like enzyme Atg7 and E2-like enzyme Atg10[103]. The Atg12-Atg5 conjugate interacts with Atg16L in a noncovalent way and forms a heterotrimer, which acts as an E3-like enzyme[104]. The second ubiquitin-like conjugation system facilitates Atg8 lipidation[103]. The Atg8 protein family consists of seven LC3 and GABARAB protein variants of which LC3B is the most commonly studied family member[105].

LC3 is cleaved by the cysteine protease Atg4, exposing the C-terminal glycine residue and resulting in the LC3-I form[106]. Next, LC3-I is activated by the E1-like Atg7, transferred to the E2-like Atg3, and finally covalently linked to the amino group of the major membrane phospholipid phosphatidylethanolamines (PE) by the E3-like Atg12-Atg5: Atg16L complex, producing LC3-PE (commonly referred to as LC3-II)[103]. Unlike LC3-I, LC3-II localizes at the autophagosome membrane, which makes it useful as an autophagy specific marker. When the autophagosome closes, a double membrane vesicle is generated which normally lacks the Atg12-Atg5: Atg16L complex[107]. The pool of LC3-II that is present in the autophagosome outer membrane is cleaved from PE by Atg4 and recycled for another round of autophagosome biogenesis[108].

After completion of the autophagosomes, they fuse with lysosomes, releasing the inner membrane and cargo into the lumen of the lysosome. The fusion event is characterized as a multi-step process, involving multiple cellular complexes and AAA-ATPase[109,110]. The proteolytic lysosomal enzymes are also recruited into the autolysosomes, working as endopeptidase in acidic environment that is established by the vacuolar ATPase on the lysosomal membrane[111]. After the substrates are digested, the amino acids and peptide fragments are released into the cytosol by permeases[112].

5. Protein sequestration

5.1 Stress granules

Disturbances on protein translation by various stress causes ribosomes disassembly, releasing untranslated mRNAs and thereby triggering cytoplasmic structure stress granules formation. Stress granules are non-membrane structures ranging in size from 100 to 2000 nm, which are generated as a consequence of stress-induced inhibition of protein translation[113]. The formation of stress granules shows characteristics of liquid-liquid phase separation (LLPS)[114]. Structurally, stress granules are composed of a stable core surrounded by a dynamic shell[115].

Stress granules are variable and dynamic structures as different type of stress insults results in stress granules with different constituents (**Figure 6**). Stress granules are rapidly resolved when the cells are relieved from the stress insult. Proteomic analyses revealed that predominant components of stress granules are translation arrested mRNAs, 40S ribosome subunits, translation initiation factors and RNA binding proteins (RBP)[115,116]. Analysis of RNA sequences shows that the mRNA content in stress granules is heterogeneous and depends on the nature of the stress[117,118,119]. Remarkably, the *HSP70* transcripts are selectively excluded from stress granules and preferentially targeted for translation during the stress condition[115], suggesting a delicate control of mRNA content in stress granules.

The RBPs that nucleate in the stress granule core share the presence of intrinsically disorder regions (IDRs) that can drive spontaneous liquid-liquid phase separation. IDRs comprises of two subtypes: prion related and low complexity (LC) regions[120]. The RBPs are recruited by RNA-protein or protein-protein interactions, thereby producing a local high concentration of IDR-containing proteins, which is a prerequisite for liquid-liquid phase separation[121]. Mutations in or malfunctioning of IDR domain-containing proteins have been reported to be related to neurodegenerative diseases, such as TAR DNA binding protein of 43 kDa (TDP43), fused in sarcoma (FUS) and T cell internal antigen-1 (TIA1)[122,123]. The stress granules nucleation factors, including the 48S translation initiation complex, RNA and RBPs, form liquid cytosolic droplets under multivalent weak interactions[124]. The composition of the shell part is rather complicated and dynamic as more than 100 cellular and viral proteins are found in stress granules[125]. Also, the proteome of

stress granules is dependent on the stress condition. For example, it is reported that subunits of 19S and 20S proteasome particles colocalize with stress granules only in cells exposed to oxidative stress but not heat shock[126]. Stress granules can merge with each other and become larger, which is referred to as maturation of stress granules[127].

There are still controversies about the mechanism responsible for the assembly of stress granules[127]. It is widely accepted that RNA-RNA, RNA-protein, and protein-protein interactions contribute to the assembly process. Recent evidence suggests that trans intermolecular RNA-RNA interactions contribute to stress granules formation. Exogenous injecting RNA induces stress granules formation, implying substantial non-translating RNA pool is necessary for stress granule[128]. It has also been demonstrated that the translation initiation factor eIF4A functions as an ATP-dependent RNA chaperone that reverse RNA condensation by limiting intermolecular RNA-RNA interactions, which reduce stress granules formation consequently[129]. These studies suggest that RNA condensation is an event that lies upstream of stress granules formation.

RBPs are also involved in regulation of stress granules formation. Among the stress granules constituent proteins, the central regulators are G3BP1 (Ras GTPase-activating protein-binding protein 1) and G3BP2 (Ras GTPase-activating protein-binding protein 2) because stress granules formation is interrupted only in G3BP-deficient cells[130]. There are three IDRs in G3BP protein, which makes G3BP function as a tunable switch of phase separation[131]. The IDR3 and RRM (RNA recognition motif) are RNA binding domains, which determine RNA-dependent phase separation[132]. Although the IDR1 and IDR2 are dispensable for phase separation, deletion of IDR1/2 still promote liquid-liquid phase separation at lower threshold concentration. Besides, the IDR1/2 regulates stress granules dynamic and composition[132].

Stress granules serve as protective structures in multiple ways. Firstly, they are considered as a temporary storage place for untranslated mRNA. The fate of mRNAs is decided by RBPs. This means that part of the mRNAs may be degraded while other transcripts may be re-allocated for translation after stress granule disassembly when the stress is released. By selectively preserving a subpopulation of mRNAs, stress granules potentiate effective re-assignment of cellular resource to

adaptive proteome reprogramming[133,134]. Secondly, stress granules serve as hubs that intercept signaling molecules, thereby communicating an emergency state to other signaling pathways, which regulate cell metabolism, growth and survival. Signaling proteins and enzymes that are recruited to stress granules share the presence of IDRs, indicating that multivalent interactions are also needed for recruitment. Some proteins affect stress granule assembly and hence influence signaling pathways which components are involved in the stress granules formation. For example, it is shown that DDX3, which is a DEAD box RNA helicase, promotes stress granule formation in influenza virus infected cells and functions as antiviral protein[135].

Other proteins that are sequestered in stress granules cause inhibition of signaling pathways without affecting stress granules formation. It has been published that stress granules assembly regulates mTORC1 signaling in yeast and mammalian cells by sequestering mTORC1 and downstream kinases during stress[136]. When cells are stressed by amino acid deprivation, mTORC1 is inactivated and released from vacuolar or lysosomal membranes in yeast and mammalian cells, respectively, losing its ability to promote protein synthesis and inhibit autophagy. Inactivated mTORC1 accumulates in stress granules in response to stress activated phosphorylation of eIF2α[136]. Reactivation of mTORC1 is linked to its release when stress granules are disassembled during recovery. Additionally, cytosolic DYRK3 (dual specificity tyrosine-phosphorylation regulated kinase 3) promotes mTORC1 activity by phosphorylating and repressing the mTORC1 inhibitory subunit PRAS40[137], and allows stress granule dissolution to release sequestered mTORC1[138]. DYRK3 is recruited to stress granules by its LC domain, preventing stress granules to dissolve and activation of mTORC1[139]. These data suggest that stress granule--dependent regulation of mTORC1 activity occurs in stressed cells. RACK1 is a scaffold protein for multimerization of the mitogen-activated protein kinase MTK1, which acts upstream of JNK and p38, resulting in induction of apoptosis[140]. This process is abolished when RACK1 is recruited to stress granules by binding to the translation initiation factor 3 complex[140]. It is noteworthy that studies defining stress granules associated signal molecules typically include only one or two cell lines and one type of stress, so it is still hard to generalize cross talk between stress granules and signaling pathways[120].

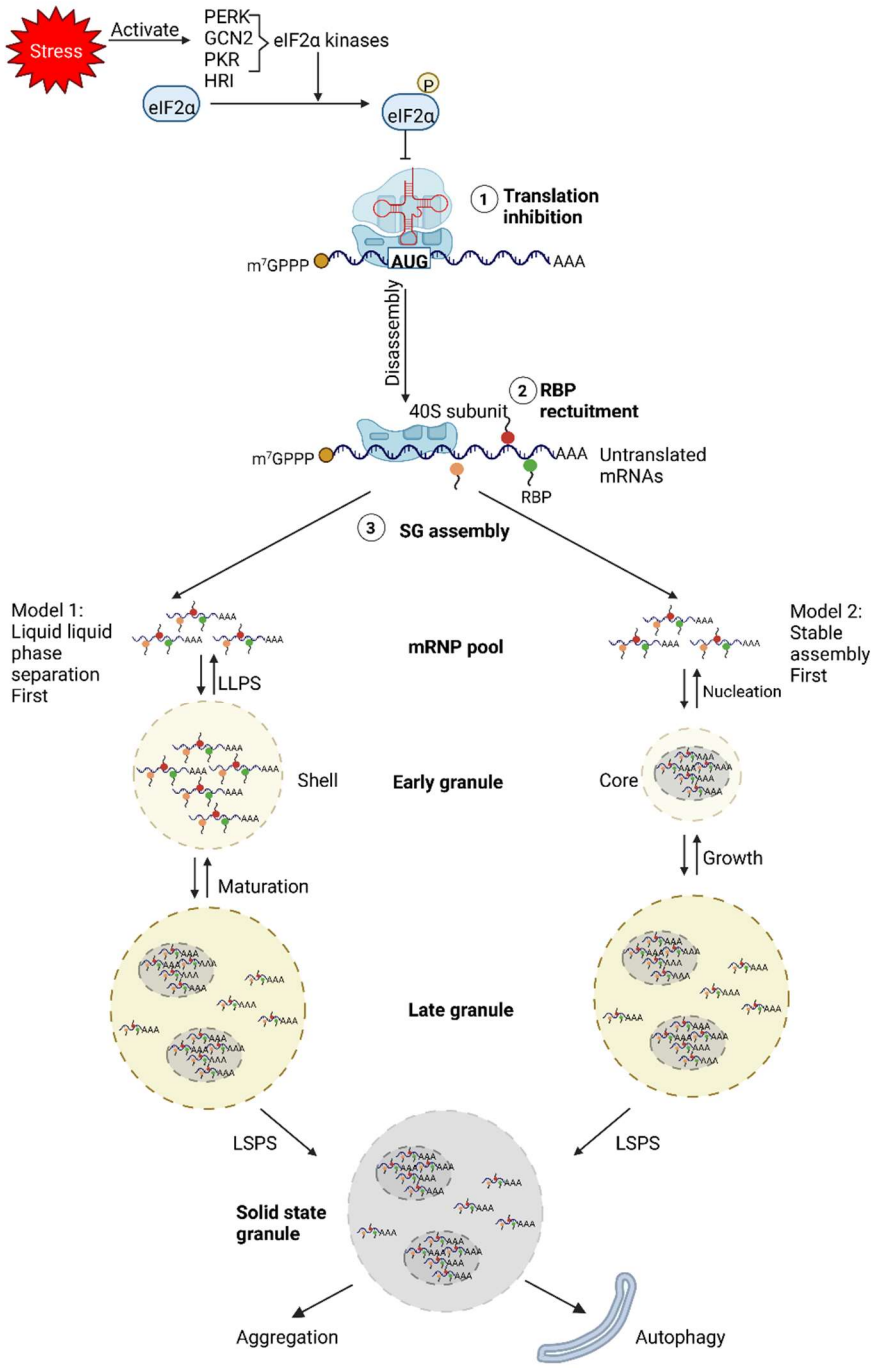

Figure 6. Two models for stress granules formation. The first model argues that the granule core part forms because of compression of granule components with time after initial formation. The second model argues that the dense core forms first and expands with time. The liquid state stress granules are reversible. The liquid stress granules can undergo liquid-solid phase separation (LSPS) and covert to a solid-state granule, that are either removed by autophagy or behave as precursors for aggregates. Figure created with BioRender.

Finally, stress granules are used for transient storage of unfolded and misfolded proteins to avoid irreversible aggregation[141,142]. Also, the proteasome and chaperones are recruited to stress granules and may assist in the clearance of

components[126,143]. Dysfunctional stress granules are destined for autophagy for further clearance[144,145].

5.2 Nucleolus

The nucleolus is a large nuclear non-membrane compartment where ribosome biogenesis takes place[146]. It regulates cell growth and survival as well as stress responses[147]. The layered tripartite organization of nucleolus consists of the fibrillar center, the dense fibrillar component and the granular component phase[146]. The granular component is enriched in negatively charged proteins with IDRs combined with RNA. Its assembly is dependent on liquid-liquid phase separation and exhibits liquid-like properties[146]. Besides its function in genome integrity maintenance and repair[148], recent reports have revealed that misfolded proteins produced during heat shock are translocated to the granular component phase of the nucleolus, where they interacted with the granular component protein nucleophosmin[149]. As companion, the chaperone HSP70 shuttles into the nucleolus and is responsible for the release of misfolded proteins from the nucleolus for degradation[150]. Thus, the nucleolus functions as a protective cellular structure that sequesters misfolded and damaged proteins thereby reducing the impact of proteotoxic stress for cells[149].

5.3 Promyelocytic leukemia (PML) body

The promyelocytic leukemia (PML) body is a nuclear structure composed of the PML protein associated with other proteins that regulate transcription, DNA repair, DNA replication, and RNA transport[151]. Conjugation of SUMO (small ubiquitin modifier) to PML is required for proper formation of nuclear PML bodies[152]. SUMOylation is a post-translation modification with the ubiquitin-like protein SUMO, which is catalyzed by a cascade reaction with specific E1, E2 and E3 enzymes, a process similar to ubiquitination. Both the PML body and nucleolus are in a coordinated way involved in nuclear protein quality control as has been shown recently[153]. Inhibition of proteasomal protein degradation induces PML-associated proteins to accumulate in the nucleolus[154]. Another study showed that inhibition of ubiquitination of nuclear substrates causes SUMOylation of proteins, resulting in their accumulation in PML bodies[155]. PML bodies colocalize with the SUMO-targeted ubiquitin ligase (StUbL) RNF4 (RING finger protein 4) and RBPs after thermal stress[156]. Briefly, PML bodies

function as a platform for SUMO-targeted ubiquitination by RNF4 of nuclear substrates, which targets these proteins for degradation by the proteasome.

5.4 Aggresome

The aggresome is a cytoplasmic juxtanuclear structure, composed of misfolded proteins and aggregates. Formation of the aggresome involves recognition of misfolded and aggregated proteins, dynein complex coupling, and transport along to the microtubule to the microtubule organizing center (MTOC)[157]. While assembling, the aggresome is surrounded by cage like structure made of cytoskeleton intermediate filament, which is supposed to function in promoting aggresome stability and preventing nonspecific interaction[158]. The cage-like structure is cell type specific. In non-neuron cells, the aggresome is encircled by vimentin intermediate filament, while in neurons, the aggresome is encircled by neurofilaments[159].

In addition to aggregated proteins, chaperones, UPS- related proteins, centrosomal proteins, and proteins of the autophagy machinery are reported to reside in aggresomes[160]. Although ubiquitinated proteins and UPS components are observed in aggresomes, aggregated proteins are destinated for autophagy instead of proteasomal degradation[161]. The UPS is likely to deliver the polyubiquitinated proteins to aggregates for recognition by HDAC6[162].

The aggresome is considered to function as a protective structure by sequestering toxic aggregates[163]. Emerging evidence implies that the intermediates during aggregate formation are primary responsible for cytotoxicity caused by aggregation-prone proteins[163]. Because the small intermediates contain more exposed surface area, this may increase the risk of abnormal interaction with cellular membranes, proteins, or other macromolecules[164]. Therefore, transport of intermediates to the aggresome limits the exposed surface area, thereby reducing proteotoxicity. Besides, the aggresome functions as a transient storage structure by clustering the aggregates and autophagic machinery, which may also provide efficient degradation of aggregates[160]. These aggregates can subsequently be degraded by the lysosomes. Evidence for this is provided by studies where induction promotes clearance while impairment blocks aggregate degradation[165166]. Further indications for a link between aggresome and autophagy is provided by the

finding that the master inhibitor of autophagy mTOR is localized in aggresomes, suggesting a potential function for aggresomes in autophagy induction[167].

RESEARCH AIMS

The studies presented in this thesis focus on the proteostasis network with the following three major aims:

Identifying the crosstalk between two branches of the proteostasis network: protein degradation and sequestration (Paper I and II).

Reducing the burden on the proteostasis network by introducing a natural solubility domain in an aggregation-prone protein (Paper III).

Study the effects of compound CBK79 on the proteostasis network (Paper IV).

METHODOLOGICAL CONSIDERATIONS

1. Reporters for UPS activity

To monitor activity of the UPS in living cells, the green fluorescent protein (GFP) and yellow fluorescent protein (YFP) have been converted from stable proteins (half-life >24 hours) to short-lived proteasome substrates by introducing several unique degradation signals.

According to the N-end rule pathway, the N terminal amino acid residue determines the stability of proteins[168]. We constructed an artificial N-end rule substrate, Ub-Arg-YFP, that generated the instable Arg-YFP with a half-life is approximate 10 minutes[169], by cleavage of the ubiquitin moiety by DUBs[170]. Another ubiquitin fusion reporter, Ub-Gly76Val-YFP (Ub-YFP) has the glycine residue at position 76 in the ubiquitin moiety substituted by a valine residue, which prevents removal of the ubiquitin moiety by DUBs. The lysine residues at position 29 and 48 in the N-terminal ubiquitin moiety are recognized by the ubiquitin fusion degradation pathway and act as acceptors for polyubiquitin chains that target the fusion for proteasomal degradation[171]. A third UPS reporter substrate is YFP-CL1, where CL1 is a hydrophobic peptide motif that was originally characterized as a degradation signal recognized by the E2s Ubc6 and Ubc7 in budding yeast[172] and reduces the half-life of stable proteins to approximately 20-30 minutes[173]. Unlike the soluble Ub-Arg-YFP and Ub-YFP substrate, the YFP-CL1 is aggregation prone and requires more time to be degraded after thermal stress[174]. In Paper I, I also used variations on the GFP-CL1 substrate that contained a nuclear localization signal (NLS) or a nucleus export signal (NES) to detect the compartmental (nucleus or cytoplasm) UPS activities[175].Ornithine decarboxylase (ODC) is an extremely short-lived enzyme, which is recognized by the proteasome independently of ubiquitination[176]. GFP-ODC and ZsGreen-ODC fusion proteins are usually employed to measure the ubiquitin-independent UPS activity. To monitor the degradation of aberrant translation products by ribosome quality control system, I generated an open reading frame that encodes the GFP protein but lacks a stop codon ($GFP^{nonstop}$)[177].

A major advantage of the engineered fluorescent substrates of the UPS is that they are not biologically active in vivo, avoiding potential artificial side effects. Most endogenous substrates, p53 e.g., are multifactional executors, and their degradation

is related to the regulation of diverse cellular processes. Additionally, the fluorescent proteins make it convenient and efficient for detection by various approach: microscopy, flow cytometry and immunoblotting. The phenotype-based function detection also allows high throughput screening strategies to identify extracellular added compounds or intracellular proteins that inhibit or promote UPS degradation.

To detect two cellular processes simultaneously, mCherry-G3BP1 was stably inserted in the MelJuso Ub-YFP cell line. The mCherry-G3BP1 is used to visualize the formation of stress granules, while the Ub-YFP is used to monitor protein degradation by the UPS.

2. Protein labelling

To compare protein translation and turnover rates, proteins need to be metabolically labelled.

Puromycin labelling

In paper I, I conducted puromycin labelling to monitor synthesis of proteins during proteotoxic stress conditions. Structurally, puromycin is a tyrosine amino acid covalently linked to an adenosine base, mimicking the 3′ ends of aminoacylated tyrosyl tRNA (aa tRNA). Puromycin enters the A site of the ribosome, where its amino group accepts the nascent polypeptide from the peptidyl tRNA in the P site, catalysed by the ribosomal peptidyl transfer centre. However, the peptide bond of puromycin cannot be cleaved by the incoming aminoacylated tRNA, preventing translation elongation and resulting in irreversible premature translation termination. This process is independent on energy and causes the 80S ribosome to disassemble. The truncated polypeptides are recognized as Defective Ribosome Products (DRiPs) and targeted for degradation by the UPS through the ribosome quality control system[178]. The incorporation of puromycin at the C terminus can be recognized by a puromycin-specific antibody and detected by immunoblotting or immunostaining.

Classically, radioactive amino acids were considered as the golden standard to measure changes in translation, as the incorporation of radioactive amino acids resembles normal physiological protein synthesis. The fact that more than one radioactive amino acid can be incorporate in a single polypeptide chain increases

also the final signal intensity. Recently, puromycin labelling has become an alternative way to measure translation rate because incorporation of puromycin in polypeptides is proportional to overall translation. In the simplest application, puromycin is added to cultured cells, and the puromycin labelled peptides are detected by immunoblotting using anti-puromycin antibodies, generating a smear pattern which reflects the broad range in molecular weight of newly synthesized proteins. By measuring the intensity of the puromycin signal, the translation rates can be compared between conditions. Notably, since the puromycin containing peptides are aggregation-prone DRiPs, they can at the same time provide insight in the functionality of protein quality control.

Metabolic labelling by click chemistry

In paper II, I employed metabolic labelling by using click chemistry in order to follow the synthesis of the Ub-YFP reporter. In this context, click chemistry refers to the copper catalysed azide-alkyne cycloaddition (CUAAC)[179]. L-homopropagylglycine (HPG) is an analog of methionine bearing an alkyne group. HPG is randomly incorporated in peptides instead of methionine during translation, which makes it possible to label nascent peptides using click chemistry. An azido conjugated dye is covalent linked to the nascent peptides by the CUAAC coupling reaction between azide and alkyne group of HPG, which forms a stable triazole ring as a linker. Afterwards, the fluorescent dye is detectable under an excitation laser. The YFP pulldown was performed to separate the Ub-YFP reporters from other labelled polypeptides. The amount of the HPG incorporated Ub-YFP reporters was evaluated by measuring the fluorescent intensity after polyacrylamide gel electrophoresis.

In paper II, the turnover of the Ub-YFP reporters is followed by a pulse chase approach combined with the click chemistry reaction. As the methionine is competitive with HPG for incorporation during translation, a 2-hours pulse of HPG labelling was followed by a 1 hour chases with an excess of L-methionine. Fluorescent labelling of HPG-containing proteins by the click chemistry reaction followed by YFP pulldown and analysis of YFP levels by polyacrylamide gel electrophoresis provided insight in the half-life of the Ub-YFP reporter. The intensities of the YFP-CL1 bands in the pulse chase samples were measured by their fluorescent signal after polyacrylamide gel electrophoresis.

3. Stress granules induction and detection

In paper I and II, I employed thermal stress to induce stress granule formation. Multiwells plates with cell cultures were sealed with parafilm and placed in a 43°C water bath for 30 minutes. Stress granules were visualized either by immunostaining of the stress granule marker TIA1 (paper I) or the mCherry-tagged stress granule marker G3BP1 (paper II).

4. Ethical considerations

All the studies were performed with established cell lines.

RESULTS AND DISCUSSION

Paper I: Cytosolic stress granules relieve the ubiquitin-proteasome system in the nuclear compartment

Summary of results

G3BP1 and G3BP2 are critical components of stress granules[132]. First, I compared the effect of G3BP1/2 depletion on UPS activity in MelJuso cells stably expressing different types of reporter substrates. The results show that the G3BP1/2 deficiency impairs degradation of ubiquitin-dependent UPS reporters in response to thermal stress. In particular, the accumulation of the nuclear localized UPS reporter was aggravated in G3BP1/2-depleted cells, while the levels of the cytoplasm localized UPS reporter accumulation were comparable to control and G3BP1/2-depleted cells. It is surprising that the inability to form cytosolic stress granules ultimately impairs the nuclear UPS.

The stress granule is considered to function as a transient storage structure for misfolded or unfolded proteins caused by proteotoxic stress[126,145]. If the stress is revoked, the stress granule disassembles, and the misfolded proteins are released and targeted for degradation or refolding. If the stress is prolonged, the liquid state of stress granules can convert to a solid state, resulting in protein aggregation. The solid-state content of stress granules has been reported to be removed by autophagy and chaperone-dependent proteasomal degradation[180].

I also analyzed the localization of misfolded or unfolded proteins by puromycin labeling. Puromycin is incorporated in the C termini of polypeptides that are being synthesized by the translation machinery, which results in halting of translation and release of polypeptides. These polypeptides are usually randomly truncated and dysfunctional, and being referred to as defective ribosome products (DRiPs)[181]. During stress, the DRiPs are aggregation prone and targeted as substrates for proteasomal degradation. Consistent with previous findings[145], the DRiPs colocalized with stress granules in stress granule-proficient cells. In contrast, I found that in stress granule-deficient cells the DRiPs are shuttled to nucleoli.

DRiPs are usually kept soluble by binding to chaperones after being produced[182]. By immunofluorescent staining, I observed that HSP70 colocalizes with DRiPs directly

after heat shock. After 1 hour recovery, the HSP70 still colocalizes with DRiPs in stress granule-deficient cells, whereas the colocalization is not detectable in stress granule-proficient cells. By detecting HSF1 phosphorylation, formation of HSF1 nuclear dots and *HSP70* mRNA, which are readouts of the HSR[183], I found that the HSR is prematurely activated in stress granule-deficient cells.

SUMO2/3 modification has been reported to be involved in enhancing nuclear protein degradation by the UPS in response to proteotoxic stress[184]. Substrates are tagged by poly SUMO2/3 chains and in turn subjected to ubiquitin-dependent proteasomal degradation. Interestingly, the SUMO2/3-dependent degradation has been reported to function downstream of HSF1 activation[185]. I analysed the turnover of SUMO2/3 modified proteins during the recovery phase after heat shock. The results show that thermal stress results in a build-up of SUMOylated proteins independent on the ability of cells to form stress granules. However, the SUMOylated proteins are degraded faster during the recovery phase in stress granule-deficient cells. Additionally, the immunostaining showed that the SUMO2/3 conjugates localize in nucleoli in stress granule-proficient cells, while, in stress granule-deficient cells, the SUMO2/3 conjugates colocalize with PML bodies, which is a structure for ubiquitination and degradation for nuclear substrates[156]. These results imply that SUMOylated proteins are degraded preferentially in stress granule-deficient cells.

To further explore SUMO2/3-dependent degradation in stress granule-deficient cells, I analysed the turnover of the detergent insoluble and soluble fractions of the aggregation-prone protein TDP43. The detergent insoluble fraction of TDP43 has been found to be degraded in a SUMO2/3-dependent way[184]. Thermal stress increased the pool of detergent insoluble TDP43, which is consistent with its role in stress granule formation[186]. The detergent insoluble TDP43 was removed during the recovery phase, and degradation of TDP43 was found to be accelerated in stress granule-deficient cells. The turnover of detergent insoluble TDP43 further supports that the SUMO2/3-dependent degradation is prioritized in stress granule deficiency cells.

Based on these observations, we proposed that the accelerated SUMO2/3-dependent proteasomal degradation causes an overload of the nuclear UPS in stress granule-deficient cells, resulting in impaired degradation of other ubiquitin-

dependent proteasome substrates in the nuclear compartment. To test this hypothesis, I knocked down the SUMO-targeted ubiquitin ligase (STUbL) RNF4, which specifically catalyses the ubiquitination of SUMOylated proteins[187]. RNF4 depletion reduced the degradation of SUMO2/3 conjugates in stress granule-deficient cells, and partially restored the degradation of the Ub-YFP reporter in the recovery phase. Furthermore, RNF4 knockdown also decreased the nucleolar accumulation of YFP-CL1 in stress granule-deficient cells.

Our data suggest that stress granule deficiency causes impairment of the nuclear UPS. To illustrate the importance of stress granule formation in a more pathological relevant model, I checked the effect of stress granule deficiency on the accumulation of mutant Ataxin-1, a protein causative for the neurological disease called spinocerebellar ataxia type 1 (SCA-1)[188]. More and larger Ataxin-1 aggregates were observed in nuclei of stress granule-deficient cells after thermal stress, suggesting a failure of nuclear protein quality control.

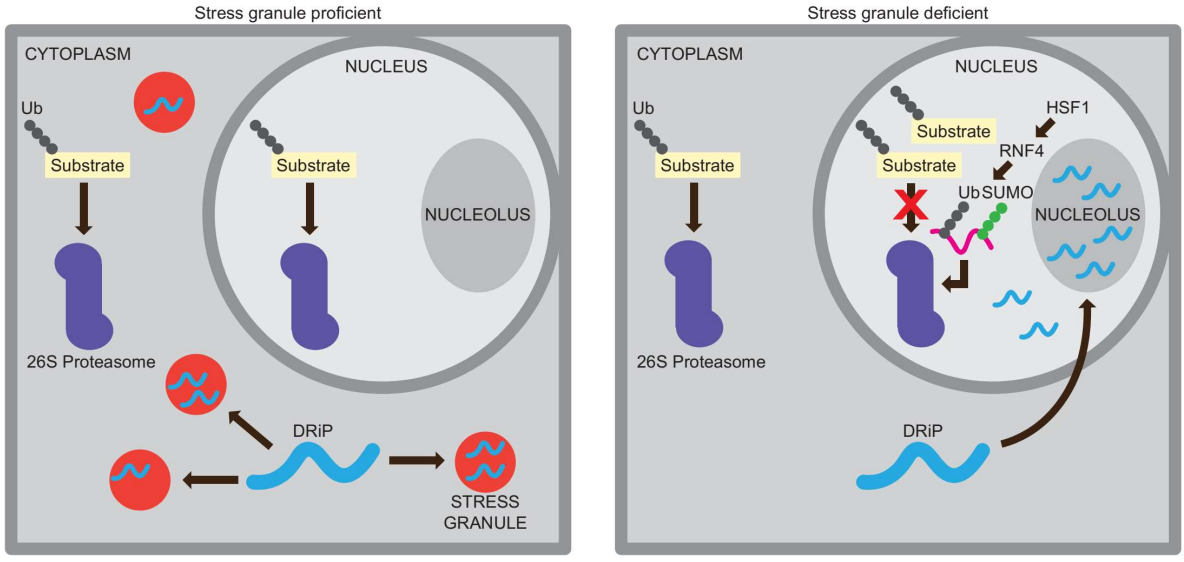

Figure 7. Schematic illustration of the model. Upon stress, the DRiPs distribute differently in stress granule-proficient and -deficient cells. DRiPs that fail to be sequestered in stress granule translocate instead to nucleoli and induce a premature HSR. This boosts SUMO2/3-dependent degradation of nuclear substrates, thereby overloading the nuclear UPS and impairing degradation of other proteasome substrates.

Discussion

Many studies focus on modulators of the proteostasis network individually, ignoring that the proteostasis network is complex and exhibits multiple crosstalk mechanisms

between individual components. Here I investigated the effect of the stress granules on protein degradation by the UPS. Our data suggest that the inability to sequester misfolded proteins in stress granules is initially compensated by sequestration of these protein species in the nucleolar compartment. Consequently, this causes premature activation of HSF1 and enhances the RNF4-mediated degradation of SUMO2/3 substrates. I propose that this premature activation of HSR is responsible for the aggravated impairment of the nuclear UPS in stress granule-deficient cells.

Several components of stress granules have been linked to neurodegenerative diseases, suggesting that stress granules can potentially be deleterious for cells[189]. It has been shown that liquid stress granules can phase transition to a solid state, forming detergent insoluble inclusion bodies[190,191]. Currently, there are some studies focusing on reversing the liquid to solid phase transition of stress granules in order to prevent aggregate formation[192]. Since a potential side effect of inhibition stress granule formation is demonstrated in our study, therapies aiming on inhibition of stress granule formation need to take into consideration adverse effects on protein quality control.

The thermal stress approach that we used is acute insult and rapidly induces stress granules within a couple of minutes. However, some phenomena are detected still hours after the thermal stress has been released, such as the distribution of SUMO2/3 conjugates and UPS functionality. This could be because the degradation is compromised at several steps: targeting of substrates with polyubiquitin chains, binding of ubiquitinated substrates to the proteasome, and unfolding and proteolysis in the proteasome. Therefore, the UPS reporter accumulation is gradually in time after thermal stress.

DRiPs, like other newly synthesized aggregation-prone proteins that are produced during stress, have the potential to threaten critical cellular functions. Therefore, they need to be cleared from the cellular environment, either by degradation or, in case the DRiPs production is too massive, by transient sequestration in specific cellular structures. Our data suggest that they passively diffuse into the nucleus when the cytoplasmic transient storage is limited. As a result, in stress granule-deficient cells, the HSP70 mediates the DRiPs translocate to the nucleolus, which has been recently characterized as a transient storage place for misfolded proteins[149,193](**Figure 7**). During recovery, HSP70 appears to be trapped in nucleoli

because the DRiPs are not released from nucleoli and not delivered for degradation until 1 hour after the thermal stress. Both HSF1 and DRiPs bind in a competitive manner to the substrate binding domain of HSP70[45,194]. In this way, DRiPs make HSF1 dissociate from HSP70, resulting in activation of HSF1, promoting transcription of heat shock protein genes. The transcriptional activity could be detected until 2 hours after the thermal stress. After 2 hours of recovery, the HSF1 transcriptional activity quickly declined. This could be explained by elevated production of chaperones caused by prematurely HSR activation.

The SUMO2/3 modification has recently been reported to be involved in the degradation of nuclear substrates in response to thermal stress[155,185]. Interestingly, the degradation of SUMOylated proteins is facilitated by activation of the HSR[185]. Although there is no direct explanation for the correlation between SUMO2/3-dependent degradation and HSF1 activation yet, it is reasonable to speculate that the premature HSR caused by HSF1 transcriptional activation promotes degradation of SUMOylated substrates. In this scenario, the chaperone production has a dual function: promote degradation of nuclear misfolded proteins and titrate HSF1 away from the DNA to turn off the HSR, generating a regulatory feedback loop. It was shown that inhibition of HSF1 counteracts degradation of SUMOylated proteins [185]. In this study, we showed that the enhanced HSR promotes SUMO2/3-targeted degradation of detergent insoluble TDP43. This makes the HSR a potential regulator of protein degradation, beyond its previous transcription functionality.

Paper II: Chemical inhibition of the integrated stress response impairs the ubiquitin-proteasome system

Summary of results

In paper II, I studied the effect of the integrated stress response inhibitor (ISRIB) on protein degradation. Under proteotoxic stress, the ISR is activated to maintain protein homeostasis. The protein eIF2α is phosphorylated by kinases, and as a result, protein translation is interrupted[3]. ISRIB functions as an inhibitor of ISR, preventing translation inhibition and inhibiting stress granule formation[195,196].

In this study, I employed a MelJuso cell line stably expressing the reporters Ub-YFP and G3BP1-mCherry. The Ub-YFP reporter was used for monitoring the UPS activity, and the G3BP1-mCherry is a stress granules marker that was used to validate that ISRIB prevented stress granule formation. Following the same setup as paper I, I employed thermal stress to disturb the proteostasis network.

I compared the Ub-YFP level and G3BP1-mCherry localization in control and ISRIB-treated MelJuso reporter cells without heat shock, directly after heat shock and during the recovery phase. ISRIB treatment indeed inhibited the stress granule formation during heat shock. Interestingly, administration of ISRIB caused an enhanced accumulation of Ub-YFP reporter during recovery phase. I also compared degradation of compartmentalized UPS reporters in presence and absence of ISRIB treatment. Opposite to G3BP1/2 depletion (paper I), ISRIB predominantly impaired the degradation of the cytosolic UPS reporter. Additionally, ISRIB treatment caused accumulation of the $GFP^{nonstop}$ reporter for ribosome quality control in nucleoli during recovery phase. Pulse-chase experiments confirmed that the increase in reporter levels were caused by a delay in their degradation.

An earlier study reported that thermal stress impairs UPS degradation by limiting the pool of free ubiquitin[174]. Interestingly, we found that ISRIB treatment increased the insoluble fraction of ubiquitin conjugates under thermal stress, which could potentially contribute to the depletion of free ubiquitin. In parallel, protein synthesis rates were determined by puromycin labelling. The immunoblotting results show that ISRIB treatment increased the insoluble pool of newly synthesized proteins. To confirm that the newly synthesized peptides are also a source of insoluble ubiquitin conjugates, we performed a pull down with tandem ubiquitin binding entities (TUBEs)

to detect ubiquitinated proteins. Consistent with our hypothesis, enhanced ubiquitination of puromycin-labelled peptides was detected in ISRIB-treated cells. In summary, ISRIB reverses the translation arrest caused by ISR activation under thermal stress, resulting in an increase in newly synthesized insoluble proteins that are ubiquitinated. This pool of insoluble proteasome substrates may titrate away the free ubiquitin pool, thereby aggravating the impairment of ubiquitin-dependent proteasomal degradation during recovery phase.

Discussion

The UPS is a tightly organized machinery in cells. In this study, we speculate that depletion of free ubiquitin negatively affects other cellular functions. During proteotoxic stress, the main function of the activated ISR is to reduce protein biogenesis and promote protein degradation in order to minimize the burden on the proteostasis network. Therefore, it is not unexpected that chemical inhibition of ISR compromises proteostasis and results in UPS impairment.

ISRIB is considered as a preclinical compound because it counteracts the ISR activation at relatively low concentrations[26]. However, its side effects remain unclear. Here we characterized a negative effect of ISRIB on proteostasis. The increased production of DRiPs caused by ISRIB treatment are normally monitored and polyubiquitinated by ribosome quality control and subjected for proteasomal degradation. Moreover, DRiPs are typically aggregation-prone polypeptides. ISRIB treatment increases the amount of the detergent insoluble DRiPs, which thereby forms a large fraction of the ubiquitin linkages in the detergent insoluble protein pool.

ISRIB also functions as an inhibitor of stress granule formation. However, we observed differences in the DRiPs distribution between ISRIB-treated cells and the G3BP1/2 knockout cells. One explanation could be that the reverse effect on translation inhibition of ISRIB prevent the release of DRiPs from ribosome subunits, thereby sequestering the DRiPs in the cytoplasm instead of them being translocated into the nucleus. This assumption is also supported by the observation that the UPS is primarily impaired in the cytosolic and nuclear compartment in ISRIB-treated cells and G3BP1/2 knockout cells, respectively. This hypothesis still needs to be explored further.

Paper III: A spider silk-derived solubility domain inhibits nuclear and cytosolic protein aggregation in human cells.

Summary of results

In paper III, we developed a strategy to prevent protein aggregation in mammalian cells. The NT* domain is a monomeric tag derived from the N-terminal domain of spider silk protein spidroin, which can enhance solubility of recombinant proteins[197]. We fused the NT* domain with the aggregation-prone domain AgDD[198] that was fused to the super fold green fluorescent protein (sfGFP)[199]. The aggregation-prone property of AgDD is regulatable. In presence of the ligand Shield, AgDD aggregation is prevented[200]. Biochemical and microscopic analysis showed that the NT* domain counteracted the AgDD aggregate formation in human cells, comparable to Shield treatment. The nuclear AgDD inclusion bodies generated by adding NLS signal to the recombinant protein were also prevented by the NT* domain as well, suggesting the NT* domain exerts its anti-aggregation property in both the nucleus and cytoplasm. Moreover, the relative position of NT* domain to AgDD domain had no effect on the anti-aggregation function of NT* domain.

Discussion

Many neurodegenerative diseases show a gradual accumulation of aberrant proteins in inclusion bodies and aggregates[201]. The formation of aggregates is irreversible, and these structures can in principle only be degraded by autophagy, putting a heavy burden on this proteolytic process[202].

An alternative therapeutic approach would be to prevent protein aggregation by introducing small molecule modulators. An interesting observation derives from a spider silk protein, that switches from a soluble to an insoluble state dependent on the pH value[203]. A challenging, translational idea is to employ this type of solubility tag to prevent aggregation of diseases-related proteins. We chose the ligand regulatable aggregation-prone AgDD domain as a model protein instead of a disease-related protein for our proof-of-principle study. Overexpression of toxic disease-related proteins might cause cellular dysfunction and potential adaptive response. The rational is that the ligand regulatable property of AgDD domain mimics the aggregation of disease-associated proteins in neurons while at the same time minimizing activation of adaptive responses.

When aggregation was prevented through the administration of Shield the nuclear NLS-AgDD-sfGFP distributed towards the nucleoli, which is consistent with observations in paper I and II that show that aggregation-prone proteins accumulate in nucleoli. In contrast, introduction of the NT* domain fusion in NLS-AgDD-sfGFP not only prevented aggregation but also inhibited nucleolar redistribution of the reporter, suggesting the NT* domain renders NLS-AgDD-sfGFP less aggregation prone.

Paper IV: Identification of a novel compound that simultaneously impairs the ubiquitin-proteasome system and autophagy.

CBK79 is a derivative of a small molecule compound that was identified as an inhibitor of Ub-YFP reporter degradation in an automated high-content microscopy screen. CBK79 caused a strong accumulation of the ubiquitin-dependent reporter Ub-YFP reporter and ubiquitin-independent reporter ZsGreen-ODC, which correlated with reduced viability of cancer cells. Furthermore, CBK79 treatment also increased the levels of endogenous UPS substrates, such as p53 and HIF1α, without having an effect on proteasome functionality based on the proteolytic activity of the β5 subunit of the 20S proteasome. Additionally, CBK79 treatment resulted in an impairment of autophagy as an increase in the GFP-LC3 autophagosomes was detected by microscopy. A defect in autophagy was further confirmed by detection of impairment in the autophagic flux based on the RFP-GFP ratio in cells stably expressing the tandemly tagged RFP-GFP-LC3B reporter. Western blot analysis revealed that CBK79 increased the LC3-II levels, implying that autophagy is impaired. These results suggest that CBK79 impairs both proteolytic pathways.

CBK79 induces various proteotoxic stress responses as well. The compound induces aggresome formation in the MTOC area. Besides, CBK79 induces stress granule formation in puromycin sensitized cells. CBK79 induces the HSR, which is characterized by induction of HSF1 nuclear stress bodies. Interestingly, preconditioning of cells by a mild thermal stress could rescue the Ub-YFP reporter accumulation caused CBK79 but not the accumulation of LC3-II.

Discussion

The characterization of the small molecule CBK79 as a dual inhibitor of the two main proteolytic pathways, UPS and autophagy, offers an opportunity for the development of a new type of proteostasis drugs in cancer therapy. Both pathways are involved in the survival of cancer cells and therefore the potential to target these pathways simultaneously may give an advantage[204,205]. Current proteostasis drugs are either targeting the UPS or autophagy, and face challenges such as drug resistance through activation of compensatory mechanisms.

Based on the observations that CBK79 causes HSF1 foci formation, upregulation of HSP proteins, and formation of aggresomes, we speculate that CBK79 causes

an acute proteotoxic stress. This is supported by the finding that preconditioning reduces the UPS impairment caused by CBK79. Thermotolerant cells have many components of the proteostasis network upregulated and thereby a larger capacity to deal with additional proteotoxic stress conditions. Recently, it has been reported that thermal stress elevates also proteasome activity[206], and that this effect lasts until 16 hours after heat shock. In line with this theory, the elevated proteasomal activity in thermally conditioned cells may contribute to the decrease of UPS impairment caused by CBK79 in preconditioned cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

Protein homeostasis is tightly linked to several types of diseases. The work presented in this dissertation not only deciphers some mechanisms behind these proteostasis-related phenomena, but also provides potentially new approaches for clinical therapy strategies.

In paper I, we found that the formation of cytoplasmic structure stress granules is critical to preserve the functionality of the nuclear UPS. We uncovered that the subcellular localization of DRiPs, activation of the HSR and the degradation of SUMO2/3 conjugated substrates are correlated with each other. Although stress granules can be a precursor of aggregates, their formation is still indispensable for cells to maintain nuclear protein homeostasis.

Functional studies on stress granules mainly focus on signal transduction, as several kinases involved in AKT/mTOR signal pathways are sequestered in stress granules[207]. Our work describes the importance of sequestering aberrant ribosomal polypeptides, or DRiPs, in cytosolic stress granule to preserve nuclear UPS activity. This may also provide a hint for interpreting the function of other misfolded protein transient storage structures, such as nucleoli and PML bodies.

In paper II, we observed that a chemical inhibitor of the ISR impairs UPS activity. The ISRIB inhibitor reverses translation inhibition, producing more misfolded, newly synthesized products. These newly synthesized products form a pool of insoluble ubiquitinated proteins that may trap ubiquitin and limit the free ubiquitin pool, thereby inhibiting ubiquitin-dependent proteasomal degradation.

This work uncovered a potential side effect of ISRIB, which has shown beneficial therapeutic effects in previous reports[208,209]. Our data suggest that if protein biogenesis is not reduced under proteotoxic stress, the persistent translation may result in compromising protein degradation.

Protein synthesis, sequestration and degradation are not independent executors. The crosstalk not only provides deeper insight in mechanisms that control proteostasis, but also suggests new therapeutic targets in the proteostasis network.

From paper III, the main conclusion is that protein aggregation can be prevented in the cytosolic and nuclear compartment by introducing the spider silk-derived NT* tag in mammalian cells. The NT* tag can be applied as a tool in in vitro studies on aggregation of disease-related proteins but may be harder to apply in clinical treatment of neurodegenerative diseases.

In paper IV, we observed that the compound CBK79 impairs ubiquitin-dependent and -independent proteasomal degradation and simultaneously inhibits the autophagy flux. As a defensive response, the HSR is activated and protein synthesis is attenuated by CBK79. Although the underlying mechanism remains to be elucidated, CBK79 may have clinical potential as anti-cancer drug because of the multiple effects it has on proteostasis modulators.

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