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Prevention of aberrant protein aggregation by anchoring the molecular chaperone αB-crystallin to the endoplasmic reticulum

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ABSTRACT

The chaperone αB-crystallin (αBC) is a member of the small heat shock protein family and its point or truncated mutants cause the muscular disorder α-crystallinopathy. The illness is histologically characterized by accumulation of protein aggregates in muscle cells. Expression of the myopathy-causing R120G mutant of αBC, harboring an arginine-to-glycine mutation at position 120, results in aggregate formation. We demonstrated that tethering αBC to the endoplasmic reticulum (ER) membrane represses the protein aggregation mediated by the R120G mutant. ER-anchored αBC decreased the amount of the R120G mutant through autophagic proteolysis. In contrast, knockdown of ATG5, an E3 ligase essential for autophagy, in ER-anchored αBC-transfected cells restored the quantity of the R120G mutant. In this context, aggregate formation was still suppressed, indicating that ER-anchored αBC profoundly constrains aggregation competency of the R120G mutant separately from downregulating the abundance of the mutant. We have proposed that protein aggregation is prevented by manipulation of the ER microenvironment with αBC, and have shed light on a novel aspect of the ER as a therapeutic target.

1. Introduction

The molecular chaperone αBC is a member of the small heat shock protein and operates in cellular response to various stresses such as heat shock, ischemia and oxidation [1]. It prevents protein aggregation by binding to improperly folded proteins. Several mutants of αBC have been reported [2–4], among which the R120G mutant is the first to be identified to cause α-crystallinopathy. The illness is an autosomal dominant muscular disease characterized by compromised functions of cardiac and skeletal muscles. Histologically, a typical feature of α-crystallinopathy is the accumulation of protein aggregates containing αBC and desmin, an intermediate filament protein. Transfection of the R120G mutant into non-muscular cell lines resulted in aggregate formation, as observed in the specimens from α-crystallinopathy patients [4,5]. Although not yet clearly understood, defective chaperone activity of the R120G mutant is expected to trigger the accumulation of protein aggregates and underlie the development of α-crystallinopathy [6,7]. α-Crystallinopathy thus reflects the failure of protein quality control and is classified as a protein deposition disease [8,9], including Alzheimer's disease and Parkinson's disease.

The ER is intimately involved in protein quality control and has been recognized as a key regulator of stress responses [10]. It has been reported that misfolded proteins are sequestered to ER-associated subcellular compartments such as Q-body [11], JUNQ [12] and ERAC [13], further supporting the view that the ER deals with potentially harmful proteins. We recently have found that ultraviolet C-induced cell death is attenuated by tethering αBC to the ER, revealing a link between this organelle and the DNA damage response [14]. The finding led us to reason by analogy that other cellular responses could also be controlled by manipulating the ER using αBC as a biological tool.

Here we have demonstrated that ER-anchored αBC remarkably suppresses aggregate formation mediated by the disease-causing αBC mutant. Based on the results, we propose that modulation of the micromilieu surrounding the ER membrane is effective in preventing the accumulation of protein aggregates, a possible cause of the protein deposition disease.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells were maintained in α-MEM (nakalai tesque) supplemented with 10% fetal bovine serum (FBS). The expression constructs and siRNA were transfected into the cells using

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polyethylenimine and HiPerfect transfection reagent (Qiagen), respectively.

2.2. Expression vectors and small interfering RNAs

The construction of the following expression vectors were described previously [14]: pcDNA4/myc-αBC (WTαBC), pcDNA4/myc-TMαBC (TMαBC), pcDNA4/myc-TMαBCn (TMαBCn). Knockdown experiments were performed with the siRNA against the ATG5 mRNA sequence (5′-tgagataactgaaagggaa-3′) and the control siRNA employed previously [15].

2.3. Antibodies

Antibodies against each protein were purchased as follows: myc (Santa Cruz Biotech), GRP94 (Enzo Life Science), calumin (Atlas), GFP (MBL), actin (Sigma), and ATG5 (Cell Signaling).

2.4. Immunocytochemistry and fluorescence image

HeLa cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, treated with PBS including 0.1% triton X 100 for 10 min, and blocked in PBS containing 2% FBS for 1 h. The samples were then incubated with anti-myc antibody in PBS containing 2% FBS overnight at 4 °C, and then incubated with Alexa Fluor 555-conjugated anti-mouse IgG (Invitrogen) for 1 h at room temperature. The immunofluorescence and/or GFP images were acquired with a fluorescence microscope or a confocal laser-scanning microscope.

2.5. Immunoblot analysis

Cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail. After centrifugation at 15,000 rpm for 5 min, supernatants were subjected to SDS–PAGE and electrotransferred onto polyvinylidene difluoride membranes. Protein expression levels were determined by using specific antibody.

2.6. Immunoprecipitation assays

HeLa cells were solubilized in lysis buffer (50 mM Tris (pH 8.0), and 150 mM NaCl with 1% NP-40 plus protease inhibitor cocktail). Anti-myc antibody was added to the lysates and incubated with rotation for 1.5 h, followed by incubation with protein G-Sepharose (GE Healthcare Life Sciences) for 1.5 h. Immunoprecipitates captured with protein G-Sepharose were washed five times with lysis buffer. Samples were resolved by SDS–PAGE and immunoblotted with specific antibody.

2.7. Statistical analysis

All data are expressed as means ± S.E.M. The data were accumulated under each condition from at least three independent experiments. Statistical significance was evaluated using the *t*-test for comparisons between two mean values.

3. Results

3.1. ER-anchored αBC represses aggregate formation mediated by the R120G mutant

Expression of the R120G mutant C-terminally tagged with GFP in HeLa cells, a human cervical cancer cell line, led to formation of protein aggregates (Fig. 1A, vector). We then determine whether tethering αBC to the ER affects the R120G mutant-mediated protein aggregation. Upon co-transfection with either an empty vector or the myc-tagged wild-type αBC construct (WTαBC [14]), aggregates were found in ~30% of the GFP-positive cells (Fig. 1A and B, vector and WTαBC). Meanwhile, the rate dropped to ~5% upon simultaneous expression of the ER-anchored αBC, referred to as TMαBC [14] (Fig. 1A and B, TMαBC). Unlike TMαBC, the N-terminal half of αBC tethered to the ER (TMαBCn [14]) did not attenuate aggregate formation (Fig. 1A and B, TMαBCn). Consistent with this difference, a majority of the R120G mutant co-localized with TMαBC, but not with TMαBCn, as shown by microscopy (Fig. 1C). It is therefore suggested that TMαBC specifically prevents the R120G mutant from aggregating.

3.2. ER-anchored αBC decreases the abundance of the R120G mutant

Protein aggregates are formed when misfolded proteins excessively accumulate to overwhelm the capacity of the protein disposal system [16–19]. In this regard, we hypothesized that TMαBC reduces the abundance of the R120G mutant, the burden on the proteolysis system, thereby blocking aggregate formation. To test this possibility, the R120G mutant levels were quantified based on the immunoblotting data. The amount of the R120G

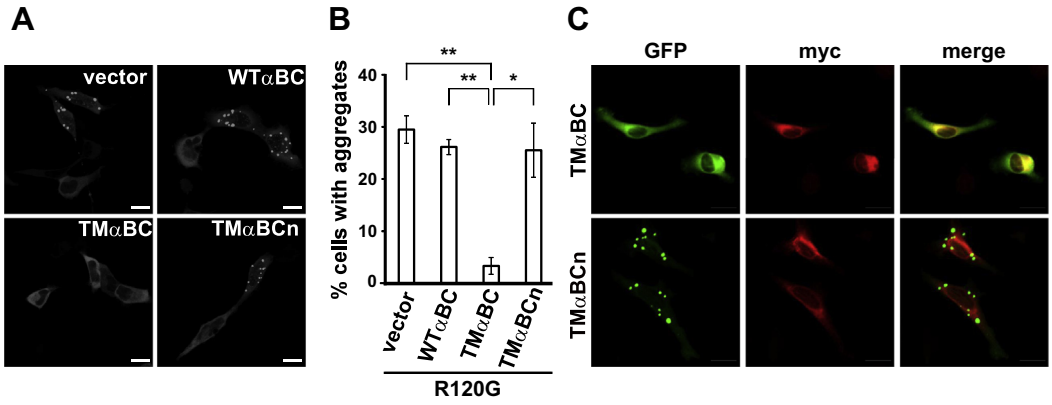

Fig. 1. ER-anchored αBC inhibits aggregation of the R120G mutant. (A) HeLa cells were transfected with pEGFP-N1-αBC R120G mutant (R120G) in combination with pcDNA4/myc-αBC (WTαBC), -TMαBC (TMαBC), -TMαBCn (TMαBCn) or the insertless vector (vector). Fluorescent imaging of the R120G mutant-GFP was performed at 16 h post-transfection. Scale bar: 20 μm. (B) Shown is the mean percentage of cells with aggregates in GFP-positive cells. The data represent mean ± S.E.M. from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (C) HeLa cells were transfected with the R120G mutant in combination with TMαBC or TMαBCn, subjected to staining with anti-myc antibody. The dual fluorescence images (GFP, green; myc, red) were acquired with a confocal laser-scanning microscope. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutant in TMαBC-transfected cells was reduced to ~50% of that in mock- or WTαBC-transfected cells (Fig. 2A). The reduction appeared selective because the amounts of actin, the ER luminal protein GRP94 or the ER transmembrane protein calumin were comparable between groups. Co-immunoprecipitation assays showed that the R120G mutant associates with TMαBC and WTαBC, but not with TMαBCn (Fig. 2B). Therefore, binding to the R120G mutant was necessary but insufficient for αBC to decrease the amount of the mutant. Considering its localization to the ER, TMαBC was most likely to concentrate the R120G mutant in the immediate vicinity of the ER membrane, whereby targeting the mutant for selective proteolysis.

3.3. Defects in autophagy abrogate the quantitative control over the R120G mutant

Two major proteolysis sites have been well known, the proteasome and the lysosome, coupled with protein ubiquitination and autophagy, respectively [20,21]. We next investigated the relative contribution of each site to the TMαBC-mediated reduction of the R120G mutant. The level of the mutant in TMαBC-transfected cells was ~80% elevated by the lysosome inhibitor chloroquine plus the protein synthesis inhibitor cycloheximide (Fig. 3A). In contrast, the level was not significantly affected by the proteasome inhibitor MG132 plus cycloheximide, suggesting that the R120G mutant is subjected to lysosomal degradation. Given that the R120G mutant was degraded predominantly in the lysosome, we directly examined involvement of autophagy by knocking down autophagy-related protein 5 (ATG5), an E3 ligase vital for autophagy [22,23]. Upon ATG5 knockdown in TMαBC-transfected, the quantity of the R120G mutant was increased relative to that upon control knockdown (Fig. 3B). Moreover, the levels of the mutant were comparable between TMαBC-transfected cells depleted of ATG5 and WTαBC-transfected cells enriched in ATG5, demonstrating that defects in autophagy abolishes the negative effects of TMαBC on the abundance of the R120G mutant.

3.4. ER-anchored αBC renders the R120G mutant aggregation-incompetent

Because ATG5 knockdown in TMαBC-transfected cells restored the amount of the R120G mutant to the control level, we reasoned that aggregates are formed in this setting to a similar extent in WTαBC-transfected control cells. Upon ATG5 knockdown, however, less than 5% of the TMαBC-transfected cells harbored aggregates (Fig. 4), indicating that the quantity of the R120G mutant does not significantly correlate with the extent of aggregate formation. Collectively, it was suggested that TMαBC renders the R120G

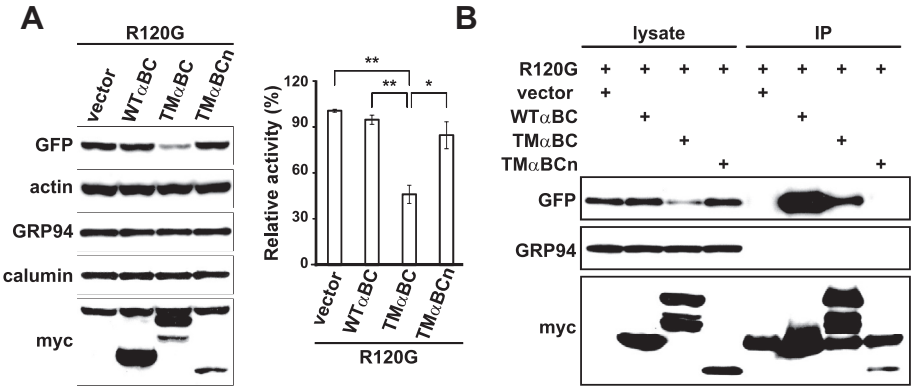

Fig. 2. ER-anchored αBC reduces the amount of the R120G mutant. (A) Transfections were performed as described in Fig. 1A. Immunoblots were probed with antibodies against GFP, actin, GRP94, calumin or myc. Shown in the right panel is the GFP immunoreactivity normalized with the values acquired from the cells cotransfected with the R120G mutant and the insertless vector. The data represent mean ± S.E.M. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) The cells transfected with the indicated combination of expression constructs. Myc immunoprecipitates from the cell lysates were probed with antibodies against GFP, myc or GRP94.

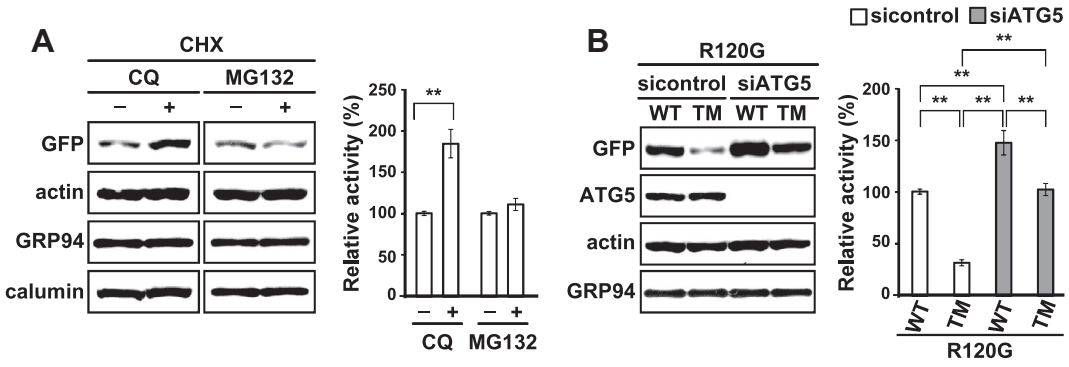

Fig. 3. The R120G mutant is degraded predominantly through autophagy. (A) HeLa cells were cotransfected with the R120G mutant and TMαBC. At 16 h post-transfection, the cells were treated with 10 μg/ml cycloheximide (CHX) in combination with either 50 μM chloroquine (CQ) or 5 μM MG132, incubated for another 6 h, and subsequently lyzed. The lysates were subjected to immunoblotting performed as in the legend to Fig. 2A. Shown in the right panel is the GFP immunoreactivity normalized with the values acquired from the mock-treated cells. The data represent mean ± S.E.M. from four independent experiments. $^{**}p < 0.01$. (B) HeLa cells were transfected with either control (sicontrol) or ATG5 (siATG5) siRNA. After 40 h, the cells were transfected with the R120G mutant (R120G) in combination with WTαBC (WT) or TMαBC (TM). At 16 h post-transfection, the cells were lyzed for subsequent analysis performed as in (A). Shown in the right panel is the GFP immunoreactivity normalized with the values acquired from the cells sequentially transfected with the control siRNA, the R120G mutant and WT. The data represent mean ± S.E.M. from five separate experiments. $^{**}p < 0.01$.

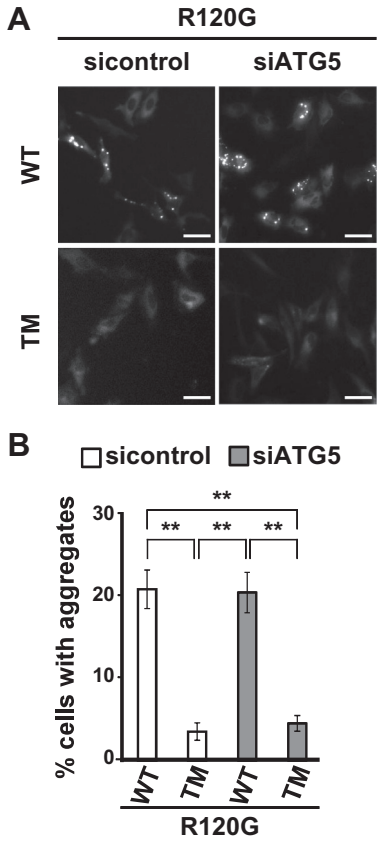

Fig. 4. Aggregate formation is repressed by ER-anchored αBC in autophagy-defective cells. (A) HeLa cells transfected as described in Fig. 3B were subjected to fluorescent imaging of the R120G mutant-GFP. Scale bar: 50 μm. (B) Shown is the mean percentage of cells with aggregates in GFP-positive cells. The data represent mean ± S.E.M. from five separate experiments. $^{**}p < 0.01$.

mutant aggregation-incompetent independently of targeting the mutant for autophagic proteolysis.

4. Discussion

We have revealed that aggregation of the disease-causing R120G mutant is remarkably suppressed by TMαBC. It was highly expected that the reduction of the abundance of the R120G mutant diminishes burden on the protein disposal system. Indeed, it has been known that aggregate formation is closely associated with disturbance of protein homeostasis [18]. We therefore postulated that TMαBC corrects the imbalance between the quantity of aberrant proteins and the capacity of protein disposal system, thereby exerting its negative effect on protein aggregation. The results of ATG5 knockdown analysis, however, argued against this hypothesis. While the abundance of the R120G mutant was restored, the aggregation was still repressed by TMαBC. In other words, quantitative control of the protein burden is not the driving force of TMαBC's anti-aggregation activity.

The finding that TMαBC blocks aggregate formation separately from its proteolysis-enhancing function has raised the question about why αBC needs to be situated on the ER membrane to impact on the R120G mutant. Owing to its subcellular localization, WTαBC would have difficulty in getting access to the ER membrane proteins. Meanwhile, such geographical obstacles are most likely to be circumvented by tethering αBC to the ER. In this regard, one simple explanation would be that TMαBC might operate primarily by increasing the chance that ER membrane proteins encounter the R120G mutant. A vast array of ER proteins could be in contact with the R120G mutant bound to TMαBC, whereby counteracting the mutant's propensity to form aggregates. Considering that TMαBC could be juxtaposed to the ribosome on the ER membrane, it might also be possible that TMαBC imposes conformational constraints on the nascent polypeptide chain of the R120G mutant, averting its misfolding. The previous study showed that the ribosome-bound chaperone NAC (nascent polypeptide-associated complex) is involved in clearance of aggregates [24]. It is tempting to speculate that TMαBC disassembles protein aggregates by employing those ribosome-associated components.

α-Crystallinopathy is histologically characterized by both accumulation of aggregates and degenerative myofibrils in muscle fibers [7,25]. If introduced into muscle cells in a relatively early phase of the disease, TMαBC would repress further protein aggregation, thereby delaying or even blocking the disease progression. From a therapeutic standpoint, it is also critical whether TMαBC disassembles preexisting aggregates. Induced-expression of TMαBC in the R120G mutant transgenic mice is required to address this issue. If this is the case, TMαBC could ameliorate muscular dysfunction elicited by the R120G mutant. Moreover, TMαBC might be applied to other protein deposition diseases such as amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease, because aggregate formation is common characteristics of the protein deposition disease. Considering that aggregate clearance is mediated by autophagy [16,26,27], TMαBC, functioning through autophagy, could possibly be a useful therapeutic tool.

Here we have demonstrated that manipulation of the microenvironment on the ER membrane would be a promising strategy for α-crystallinopathy. The findings have shed light on a novel aspect of the ER as a potential target in developing pharmacological interventions for the protein deposition disease.

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References

[1] Y. Sun, T.H. MacRae, Small heat shock proteins: molecular structure and chaperone function, Cell. Mol. Life Sci. 62 (2005) 2460–2476.
[2] D. Selcen, A.G. Engel, Myofibrillar myopathy caused by novel dominant negative alpha B-crystallin mutations, Ann. Neurol. 54 (2003) 804–810.
[3] P. Reilich, B. Schoser, N. Schramm, S. Krause, J. Schessl, W. Kress, et al., The p.G154S mutation of the alpha-B crystallin gene (CRYAB) causes late-onset distal myopathy, Neuromuscul. Disord. 20 (2010) 255–259.
[4] P. Vicart, A. Caron, P. Guicheney, Z. Li, M.C. Prévost, A. Faure, et al., A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy, Nat. Genet. 20 (1998) 92–95.
[5] A.T. Chavez Zobel, Distinct chaperone mechanisms can delay the formation of aggresomes by the myopathy-causing R120G αB-crystallin mutant, Hum. Mol. Genet. 12 (2003) 1609–1620.
[6] M.D. Perng, P.J. Muchowski, P. van Den IJssel, G.J. Wu, A.M. Hutcheson, J.I. Clark, The cardiomyopathy and lens cataract mutation in alphaB-crystallin alters its protein structure, chaperone activity, and interaction with intermediate filaments in vitro, J. Biol. Chem. 274 (1999) 33235–33243.
[7] A. Sanbe, Molecular mechanisms of α-crystallinopathy and its therapeutic strategy, Biol. Pharm. Bull. 34 (2011) 1653–1658.
[8] R.R. Kopito, Aggresomes, inclusion bodies and protein aggregation, Trends Cell Biol. 10 (2000) 524–530.
[9] F. Chiti, C.M. Dobson, Protein misfolding, functional amyloid, and human disease, Annu. Rev. Biochem. 75 (2006) 333–366.
[10] C. Xu, B. Bailly-Maitre, J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions, J. Clin. Invest. 115 (2005) 2656–2664.
[11] S. Escusa-Toret, W.I.M. Vonk, J. Frydman, Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress, Nat. Cell Biol. 15 (2013) 1231–1243.
[12] S. Specht, S.B.M. Miller, A. Mogk, B. Bukau, Hsp42 is required for sequestration of protein aggregates into deposition sites in *Saccharomyces cerevisiae*, J. Cell Biol. 195 (2011) 617–629.

[13] X.-D. Liu, S. Ko, Y. Xu, E.A. Fattah, Q. Xiang, C. Jagannath, et al., Transient aggregation of ubiquitinated proteins is a cytosolic unfolded protein response to inflammation and endoplasmic reticulum stress, J. Biol. Chem. 287 (2012) 19687–19698.
[14] A. Yamashita, T. Taniwaki, Y. Kaikoi, T. Yamazaki, Protective role of the endoplasmic reticulum protein mitsugumin23 against ultraviolet C-induced cell death, FEBS Lett. 587 (2013) 1299–1303.
[15] S. Yamamoto, T. Yamazaki, S. Komazaki, T. Yamashita, M. Osaki, M. Matsubayashi, et al., Contribution of calumin to embryogenesis through participation in the endoplasmic reticulum-associated degradation activity, Dev. Biol. 393 (2014) 33–43.
[16] J. Tyedmers, A. Mogk, B. Bukau, Cellular strategies for controlling protein aggregation, Nat. Rev. Mol. Cell Biol. 11 (2010) 777–788.
[17] M.S. Hipp, S.-H. Park, F.U. Hartl, Proteostasis impairment in protein-misfolding and -aggregation diseases, Trends Cell Biol. 24 (2014) 506–514.
[18] E.M. Sontag, W.I.M. Vonk, J. Frydman, Sorting out the trash: the spatial nature of eukaryotic protein quality control, Curr. Opin. Cell Biol. 26 (2014) 139–146.
[19] N.P. Dantuma, L.C. Bott, The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution, Front. Mol. Neurosci. 7 (2014) 1–18.
[20] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[21] Y. Ohsumi, Molecular dissection of autophagy: two ubiquitin-like systems, Nat. Rev. Mol. Cell Biol. 2 (2001) 211–216.
[22] A. Kuma, M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, et al., The role of autophagy during the early neonatal starvation period, Nature 432 (2004) 1032–1036.
[23] T. Hara, K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, et al., Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice, Nature 441 (2006) 885–889.
[24] J. Kirstein-Miles, A. Scior, E. Deuerling, R.I. Morimoto, The nascent polypeptide-associated complex is a key regulator of proteostasis, EMBO J. 32 (2013) 1451–1468.
[25] L.G. Goldfarb, M. Olivé, P. Vicart, H.H. Goebel, Intermediate filament diseases: desminopathy, Adv. Exp. Med. Biol. 642 (2008) 131–164.
[26] R.I. Morimoto, A.M. Cuervo, Proteostasis and the aging proteome in health and disease, J. Gerontol. A Biol. Sci. Med. Sci. 69 (Suppl. 1) (2014) S33–S38.
[27] Y.E. Kim, M.S. Hipp, A. Bracher, M. Hayer-Hartl, F.U. Hartl, Molecular chaperone functions in protein folding and proteostasis, Annu. Rev. Biochem. 82 (2013) 323–355.