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The Role of the protein quality control system in SBMA

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2 **ABSTRACT**
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4 Spinal and Bulbar Muscular Atrophy (SBMA) or Kennedy's disease is an X-linked disease
5 associated with the expansion of the CAG triplet repeat present in exon-1 on the androgen receptor
6 (AR) gene. This results in the production of a mutant AR containing an elongated polyglutamine
7 tract (polyQ) in its N-terminus. Interestingly, the ARpolyQ becomes toxic only after its activation
8 by the natural androgenic ligands, possibly because of aberrant androgen-induced conformational
9 changes of the ARpolyQ, which generate misfolded species. These misfolded ARpolyQ species
10 must be cleared from motoneurons and muscle cells, and this process is mediated by the protein
11 quality control (PQC) system. Experimental evidence suggested that failure of the PQC pathways
12 occurs in disease, leading to ARpolyQ accumulation and toxicity in the target cells. In these review
13 we summarized the overall impact of mutant and misfolded ARpolyQ on the PQC system and
14 described how molecular chaperons and the degradative pathways (ubiquitin-proteasome system
15 (UPS), the autophagy-lysosome pathway (ALP), and the unfolded protein response (UPR), which
16 activates the endoplasmic reticulum-associated degradation (ERAD)) are differentially affected in
17 SBMA. We also extensively and critically reviewed several molecular and pharmacological
18 approaches proposed to restore a global intracellular activity of the PQC system. Collectively, these
19 data suggest that the fine and delicate equilibrium existing among the different players of the PQC
20 system could be restored in a therapeutic perspective by the synergic/additive activities of
21 compounds designed to tackle sequential or alternative steps of the intracellular defense
22 mechanisms triggered against proteotoxic misfolded species.
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INTRODUCTION

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4 Almost all inherited and sporadic neurodegenerative diseases are characterized by the presence of
5 proteins that, either because of their natural structure, and/or as a consequence of genetic
6 mutation(s), have a tendency to aberrantly fold (misfold), generating uncommon conformations.
7 When this happens, these proteins become prone to oligomerize and/or aggregate. These misfolded
8 proteins, either in their monomeric, oligomeric or aggregated form, are recognized by the protein
9 quality control (PQC) system, which surveys proteostasis, by facilitating the removal of potentially
10 toxic species and/or attenuating their translation (Carra, et al., 2013, Seguin, et al., 2014). However,
11 when not properly disposed, these aberrant misfolded species can exert toxic effects, thereby
12 contributing to cell vulnerability and death. In presence of aberrantly folded species, the PQC
13 system activates the so called proteotoxic stress response, which involves: a) the chaperone proteins,
14 including members of the heat shock protein (HSP) family, and the two major intracellular
15 degradative systems, comprising the b) ubiquitin-proteasome system (UPS) and c) the autophagy-
16 lysosome pathway (ALP). Molecular chaperones recognize the misfolded species and assist their
17 refolding; when this fails, as in case of misfolded proteins, chaperones assist their clearance by
18 cooperating with the degradative systems. ALP can be subdivided into at least three different
19 pathways, the macroautophagy (herein referred to autophagy), microautophagy and chaperone-
20 mediated autophagy (CMA). In addition, even if more indirectly, the unfolded protein response
21 (UPR), which involves the endoplasmic reticulum-associated degradation (ERAD), is part of this
22 protective mechanism which aims to reduce proteotoxic stress to neurons. The UPR system works
23 commonly in conjunction with molecular chaperones to determine the fate of misfolded proteins,
24 leading to either re-folding of the protein or UPS/ALP-mediated degradation. Imbalance in the PQC
25 system has been described in a number of neurodegenerative diseases (Carra, et al., 2012, Carra, et
26 al., 2013, Ciechanover and Kwon, 2015, Kakkar, et al., 2014, Kampinga and Craig,
27 2010, Nikolettou, 2015 #4737, Klionsky, et al., 2012, Senft and Ronai, 2015, Xilouri and
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1 Stefanis, 2015). Here, we will focus on the role of the PQC in the pathogenesis of Spinal and Bulbar
2 Muscular Atrophy (SBMA). In addition, we will discuss why and how boosting specific molecular
3 chaperones and/or enhancing the activity of the degradative systems may have beneficial and
4 therapeutic implications for SBMA.
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10 11 **SBMA is a protein misfolding disease**

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15 SBMA, also known as Kennedy's disease, is an inherited X-linked motor neuron disease (MND)
16 mainly characterized by motor neuron loss in the brain stem and in the anterior horns of the spinal
17 cord. However, other cell types are also directly affected, such as dorsal root ganglia (DRG)
18 sensory neurons and some cells involved in male reproductive functions (Atsuta, et al.,
19 2006, Fischbeck, 1997, Kennedy, et al., 1968, La Spada, et al., 1991, Malena, et al., 2013, Soraru, et al.,
20 2008). In recent years, a significant contribution of muscle has emerged in SBMA as in other
21 MNDs (Boyer, et al., 2013a, Boyer, et al., 2013b). Indeed, recent data suggests that muscle
22 pathology maybe a primary player in the pathogenesis of SBMA, underscoring the importance of
23 exploring therapeutic approaches targeting muscle as well as motor neurons (Cortes, et al.,
24 2014a, Lieberman, et al., 2014, Rinaldi, et al., 2014). Most of the cells that are affected in SBMA are
25 postmitotic (Campisi and d'Adda di Fagagna, 2007, Grunseich, et al., 2014b), and their loss give rise
26 to a variety of symptoms. The most debilitating of these symptoms are caused by atrophy of bulbar,
27 facial and limb muscles (Fratta, et al., 2014b, Kennedy, et al., 1968, Sobue, et al., 1989), alterations in
28 sensory function (Adachi, et al., 2005, Atsuta, et al., 2006, Polo, et al., 1996, Suzuki, et al., 2008), as
29 well as specific endocrine alterations (Fischbeck, 1997, Fischbeck, 2012). The molecular basis of
30 the disease is associated with an expansion of a CAG triplet repeat sequence, that codes for the
31 amino acid glutamine (Q), located in exon 1 of the androgen receptor (AR) gene (La Spada, et al.,
32 1991). In the normal population, this CAG repeat is comprised between 9 and 37 repeats
33 (average=22), but in SBMA patients, expansions of more than 38 (up to 68) repeats (Fratta, et al.,
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2014a, Grunseit, et al., 2014a) results in disease. The CAG repeat codes for a polyglutamine (polyQ) tract, which is located in the N-terminal transactivation domain of the AR (ARpolyQ) protein. The elongated polyQ confers toxicity to the ARpolyQ, but in a ligand dependent manner (figure 1) (Katsuno, et al., 2003, Katsuno, et al., 2002). This toxicity has been linked to the acquisition of polyQ-induced aberrant protein conformations (misfolding), which are prone to aggregation. The excess of misfolded ARpolyQ may perturb the PQC system, specifically in cells expressing high levels of ARpolyQ, which include both neuronal and non-neuronal cells including lower spinal cord motor neurons, DRG neurons, muscle cells, Sertoli and Leydig cells.

ARpolyQ nuclear inclusions were first identified back in 1998 by Li and colleagues in several autopsy tissues from SBMA patients (Li, et al., 1998a). These inclusions are heavily ubiquitinated, a clear index of alteration of UPS-mediated degradation. They are also particularly reactive with antibodies recognizing the N-terminus of the AR, suggesting that they originate from proteolytic cleavage of the AR. The nuclear inclusions were found in spinal and brainstem motor neurons, but not in unaffected neural tissues, although they were also present in several different non-neural tissues (Li, et al., 1998b). Subsequent studies further characterized their intracellular distribution in affected neurons, and showed that ARpolyQ inclusions are present in nuclei of spinal cord motor neurons and the cytoplasm of DRG sensory neurons (Adachi, et al., 2005, Suzuki, et al., 2008). In muscle, inclusions were mainly found in nuclei (Li, et al., 1998b). Subsequently, we and others were able to show that in reconstituted motor neuronal systems these inclusions are formed exclusively after ARpolyQ activation either by its endogenous ligand testosterone (or the testosterone derivative, dihydrotestosterone, DHT) (Simeoni, et al., 2000, Stenoien, et al., 1999), or by some (but not all, i.e. bicalutamide or casodex) specific AR selective modulators (SARMs), such as cyproterone acetate (Rusmini, et al., 2007, Whitaker, et al., 2004). However, it is still not clear why ARpolyQ toxicity strictly depends on androgens. Several data suggest that testosterone triggers ARpolyQ toxicity by inducing a switch in its structure from “non-toxic” to “toxic” conformations, which generates ARpolyQ aggregates (Simeoni, et al., 2000). Thus, both the process of misfolding

1 and the consequent aggregation possibly occur during the androgen-induced AR maturation to its
2 active form (Poletti, 2004,Poletti, et al., 2005). The AR maturation/activation process requires the
3
4 ligand-induced dissociation from HSPs proteins (Figure 1). This step allows the unmasking of the
5
6 polyQ tract of the AR, which may then act either intramolecularly (intrinsic propensity to misfold),
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8 preventing proper folding because of its elongated size, or intermolecularly (extrinsic propensity to
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10 oligomerize and possibly aggregate), by its interaction with other polyQs in different ARpolyQ
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12 molecules. Moreover, ARpolyQ could interact with non-polyQ proteins able to exacerbate
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14 ARpolyQ misfolding (see (Poletti, 2004) for review) and possibly ARpolyQ cleavage, with the
15
16 consequent release of a potentially "super-toxic" N-terminal fragment containing the polyQ repeat.
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18 This process has long been considered one of the major steps required for the acquisition of
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20 ARpolyQ toxicity. However, recent data have raised the possibility that caspase mediated release of
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22 ARpolyQ fragments may not be the cause of cell death. In fact, while the "toxic fragment" may
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24 directly induce neuronal dysfunction and death in a soluble form, its toxicity could be buffered by
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26 its sequestration into amyloid-like inclusions. The fragment containing the polyQ may also
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28 contribute to (or initiate) the seeding process required to generate aggregates in many misfolded
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30 protein diseases. However, the ARpolyQ proteolysis is a late event in SBMA mice, and most of the
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32 ARpolyQ aggregates that originate in the early stage of the disease contain the full-length protein.
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34 Finally, the ARpolyQ undergoes cleavage to a smaller fragment via the UPS, and only after its
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36 incorporation into intranuclear inclusions (Heine, et al., 2015), further supporting the possibility
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38 that ARpolyQ fragments may not be the "culprit".
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48 An alternative, but not exclusive mechanism proposed to explain the ligand-induced toxicity to
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50 ARpolyQ is based on the fact that testosterone induces nuclear translocation of ARpolyQ. Several
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52 lines of evidence have demonstrated that the nucleus is the site where ARpolyQ exerts most of its
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54 toxic effects (Montie, et al., 2009, Montie, et al., 2011, Nedelsky, et al., 2010). A specific
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56 interdomain interaction, which is required for the nuclear activities of AR, is also crucial for
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58 ARpolyQ aggregation and toxicity. This is the amino-terminal FXXLF motif capable of interacting
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1 in a ligand-dependent manner with the carboxyl-terminal AF-2 domain (N/C interaction). Genetic
2 mutation of the FXXLF motif can prevent ARpolyQ aggregation and toxicity (Orr, et al., 2010).
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4 Apart from the molecular mechanisms of aggregation, during this phenomenon, the aggregating
5 ARpolyQ also sequesters several other proteins which are fundamental for cell functions. These
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7 include proteins that can associate to the AR (e.g.: CREB and the steroid receptor coactivator 1;
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9 SRC-1), or proteins recruited by misfolded species during their processing (e.g. proteins involved in
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11 the PQC, like NEDD8, HSP70, HSP90 and HDJ-2/HSDJ or also components of the PA700
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13 proteasome caps, but not 20S core particles) (Stenoien, et al., 1999).
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19 While the original interpretation suggested that aggregates/inclusions of ARpolyQ were responsible
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21 for motor neuronal death, other data revealed that this process may be more complex than expected.
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23 A recent study performed with atomic force microscopy (AFM) has focused attention to inclusions
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25 normally generated by the wild-type (wt) AR (a protein particularly prone to aggregate even in its
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27 normal aminoacid sequence) in aged tissue, and compared these inclusions with those produced by
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29 the ARpolyQ in SBMA (Jochum, et al., 2012). The results evidenced the existence of annular
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31 oligomers (120-180nm in diameter) for the wtAR and of small oligomeric fibrils (up to 300-600nm
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33 in length) for the ARpolyQ, similar to those described by us and others in SBMA (Li, et al.,
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35 2007, Palazzolo, et al., 2008) and also similar to inclusions described in other related CAG/PolyQ
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37 neurodegenerative diseases. Thus, the elongated polyQ seems to exacerbate the natural propensity
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39 of wtAR to aggregate, but with a distinct aggregate maturation.
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46 Whether these steps are associated with the intrinsic properties of the ARpolyQ, to a deleterious
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48 activity of the ARpolyQ on the degradative system, or rather due to its insufficient handling and
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50 clearance by an overwhelmed PQC system is still matter of discussion. It is conceivable that all of
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52 these mechanisms are involved, and the accumulation of ARpolyQ depends on a reduced capability
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54 of aged cells to counteract the intrinsic tendency of ARpolyQ to generate immature inclusions. In
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56 general, it is accepted that the aggregation process occurs through several successive steps that
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58 require misfolded protein oligomerization into soluble deposits, with generation of different
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1 peculiar structures, followed by maturation into insoluble fibrils. In SBMA mice, ARpolyQ
2 oligomers appear several weeks prior to symptom onset and are rather soluble, since they disappear
3 after testosterone removal by castration. Thus these aggregates are structurally distinct from
4 intranuclear inclusions that appear at later stages (Li, et al., 2007). With these subsequent steps, the
5 formation of ARpolyQ aggregates may initially serve to protect cells from the aberrant toxic
6 conformation of ARpolyQ. In fact, during aggregation, these toxic species are sequestered into a
7 physically defined subcellular compartment (the aggregate), waiting for their possible intracellular
8 degradation (see below) (Arrasate, et al., 2004, Klement, et al., 1998, Rusmini, et al., 2007, Saudou,
9 et al., 1998, Simeoni, et al., 2000). However, if not removed, at later stages and possibly because of
10 reduced PQC activity in aged cells, ARpolyQ aggregates may then have impact on several other
11 intracellular mechanisms, thus becoming toxic to cells (Piccioni, et al., 2002, Piccioni, et al.,
12 2001, Poletti, 2004), by affecting several different intracellular functions. Therefore, the toxic role of
13 aggregates is rather controversial, although the search for the "toxic specie(s)" of ARpolyQ (and of
14 other polyQ containing proteins) is still in progress.

36 **The protein quality control system in SBMA**

37 Besides the search for the "toxic specie(s)", the presence of insoluble ARpolyQ in affected SBMA
38 cells is the index of defective activity of the PQC system.

43 a) The Chaperones

44 The first line of defense of the PQC system is represented by several families of HSPs. HSPs act as
45 molecular chaperones capable of both recognizing and stabilizing misfolded proteins, including
46 ARpolyQ (figure 1). The effect of a variety of chaperones has been tested on ARpolyQ clearance,
47 accumulation and aggregation in different models of SBMA. The heat shock factor-1 (HSF-1),
48 which is a transcriptional regulator of HSPs, strongly reduces ARpolyQ aggregation by enhancing
49 the levels of different HSPs; thus, with a rather aspecific mechanism associated with the pan-
50 activation of the heat shock response (Kondo, et al., 2013). The mediators of the anti-aggregation
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1 chaperone activity have been identified mainly in the combined activities of HSP70 and HSP40
2 (Figure 1), and of HDJ2/HSDJ (Adachi, et al., 2003, Bailey, et al., 2002, Howarth, et al.,
3 2007, Kobayashi, et al., 2000, Stenoien, et al., 1999). Other chaperones active against ARpolyQ
4 aggregation include HSP90, HSP105 (Adachi, et al., 2009, Ishihara, et al., 2003, Katsuno, et al.,
5 2005) and the DnaJ-like-1 (HSJ1) proteins (which contain DnaJ and ubiquitin-interacting motifs)
6 (Howarth, et al., 2007). As mentioned above, these chaperones are often sequestered into ARpolyQ
7 inclusions, and/or their levels in affected cells may be significantly reduced. In general, their
8 overexpression correlates with a reduction in the total amount of insoluble ARpolyQ accumulated
9 in neuronal cells (Adachi, et al., 2009). The complex interplay between the chaperones and the
10 ARpolyQ has been extensively reviewed in (Pratt, et al., 2014), in which the Authors proposed that
11 the misfolded ARpolyQ accumulates as a consequence of being a "client" of HSP90, which is then
12 recognizable by the other HSPs. The same concept is applied to other misfolded polyQ containing
13 proteins (e.g. huntingtin, (HD)) or mutant proteins associated with other neurodegenerative diseases
14 (e.g.: tau (AD), α -synuclein (PD)). In these models, HSP90 exerts its action by acting on a specific
15 protein-folding cleft, which can be used to drive the clearance of the client protein (Pratt, et al.,
16 2014). In addition, the overall power of molecular chaperones against ARpolyQ aggregation may
17 depend both on the type of chaperones, and on their specific combinations (e.g.: synergic activity of
18 HSP70 with its co-chaperone HSP40) (Kobayashi, et al., 2000, Stenoien, et al., 1999). For example,
19 HSP70, HSP40, HSJ1a and HSJ1b have all been shown to reduce ARpolyQ inclusions, HSP70 and
20 HSP40 increase chaperone-mediated refolding, while HSJ1 proteins mainly avoid ARpolyQ
21 aggregation and assist its ubiquitination and UPS-mediated degradation, without promoting its
22 refolding (Howarth, et al., 2007). Another HSP70 interactor, the C-terminus of HSC70 (heat shock
23 cognate protein 70)-interacting protein (CHIP), which has a U-box type E3 ubiquitin ligase activity,
24 has been shown to be a potent modulator of ARpolyQ accumulation. Following interaction with
25 HSP70, CHIP can ubiquitinate the ARpolyQ entrapped by the chaperone machinery, allowing its
26 subsequent degradation by the PQC degradative pathway (Figure 1) (Adachi, et al., 2007). Indeed,
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in studies performed on neuronal models of SBMA, CHIP greatly reduced ARpolyQ accumulation, while CHIP overexpression in a SBMA transgenic mouse model resulted in a clear amelioration of the motor behavior of affected mice, accompanied with a reduced neuronal nuclear accumulation of ARpolyQ (Adachi, et al., 2007).

It is not only the classical ATP-dependent HSPs that can exert anti-aggregation activity on ARpolyQ, but also small ATP-independent HSPs, such as HSPB8 (Carra, et al., 2005, Rusmini, et al., 2013) which are potent inhibitors of ARpolyQ aggregation. In vitro, HSPB8 displays chaperone activity (Carra, 2009, Carra, et al., 2012, Carra, et al., 2013, Carra, et al., 2008a, Carra, et al., 2008b, Rusmini, et al., 2013). In cells, HSPB8 forms a complex with BAG3, a co-chaperone of HSP70, and promotes the degradation of misfolded proteins resistant to HSPs-dependent refolding (Figure 1). Degradation of the bound substrate (ARpolyQ and several other disease-associated misfolded proteins) occurs via autophagy rather than via the UPS and in different cell types, including motor neurons, HeLa cells, COS cells and HEK293T cells (Carra, et al., 2012, Crippa, et al., 2010b, Gamerding, et al., 2011, Rusmini, et al., 2013).

b) The Proteasome

When the first PQC defense line is activated, but refolding fails, specific chaperones assure cell protection against proteotoxicity by routing misfolded proteins to the degradative systems (UPS and autophagy) (Figure 1). With regards to proteasome, misfolded proteins are recognized by several different chaperones (mainly the HSP70/HSP40 complex), which allows the selective ubiquitination of the target misfolded substrates (mediated by the complex system of the ubiquitin ligases) to be directed to the 20S core of the proteasome for degradation (Figure 1). Both the wtAR and the ARpolyQ are efficiently processed by the proteasome, and inhibition of the UPS results in accumulation of large amounts of AR (either wt or mutant) in neuronal and non-neuronal cells (Dossena, et al., 2014, Rusmini, et al., 2010, Rusmini, et al., 2013, Rusmini, et al., 2007, Rusmini, et al., 2011). It is unclear whether neuronal cells have a different UPS vs autophagic power compared

1 to other cell types (Crippa, et al., 2013b, Galbiati, et al., 2014, Onesto, et al., 2011), but possible
2 modifications to the equilibrium between these two pathways may be responsible for selective
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4 modification of ARpolyQ clearance in different cells.
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7 It has been shown that the propensity of ARpolyQ to form insoluble species is accentuated in
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9 neuronal cells, possibly because of a less efficient PQC system compared to other cell types. In fact,
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11 using SBMA patient derived iPSCs cells, Nihei and colleagues showed that ligand-induced
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13 ARpolyQ aggregation can be detected in filter retardation assays (FRA) only in cells differentiated
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15 to a motor neuronal phenotype, and not in undifferentiated iPSCs or fibroblasts from SBMA
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17 patients (Nihei, et al., 2013). However, in non-neuronal cells, such as adipose mesenchymal cells
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19 derived from SBMA patients, inhibition of the PQC (in this particular case, the inhibition of
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21 proteasome, but not of autophagy) induces ARpolyQ accumulation into ubiquitinated and HSP70
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23 positive nuclear aggregates (Dossena, et al., 2014). Because of its structure, the wtAR is already
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25 prone to aggregate (Jochum, et al., 2012), and the AR utilizes the same route of degradation, which
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27 is normally based on the proteasome. However, the presence of the elongated polyQ makes this
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29 protein more resistant to UPS handling and degradation, and the long polyQ may impair and/or
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31 overwhelm the proteasome capabilities; as a consequence this phenomenon will require the
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33 involvement of the autophagic pathway, which is an alternative pathway of degradation
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35 characterized by lower selectivity, but higher capacity than the UPS (see (Rusmini, et al., 2010) for
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37 review). The aggregates therefore serve to temporarily sequester those fractions of ARpolyQ
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39 misfolded species that may have direct impact on the UPS. Indeed, by analyzing UPS functions
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41 during ligand-induced ARpolyQ cytoplasmic aggregation, we clearly noted that the proteasome
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43 reporter, YFPu, accumulates in absence of ARpolyQ aggregates (untreated ARpolyQ is cleared by
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45 the UPS, thus impairing the pathway), but testosterone-induced ARpolyQ aggregation correlates
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47 with a normal YFPu clearance (Rusmini, et al., 2007). Thus, aggregation contributes to proteasome
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49 desaturation (but this effect is not related to AR nuclear translocation), protecting the proteasome
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1 from an excess of misfolded protein to be processed, and the forming aggregates then have to be
2 processed by the autophagic pathway.
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4 c) Autophagy

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6 Autophagy may take place in different cytoplasmic regions and the insoluble forms of ubiquitinated
7 ARpolyQ are recognized by specific proteins, including p62/SQSTM1. p62/SQSTM1 interacts with
8 the autophagosome associated lipidated form of LC3 (LC3-II) and is engulfed into nascent
9 autophagosomes; later, these autophagosomes fuse to lysosomes, where misfolded proteins and
10 aggregates are degraded (Figure 1). Misfolded species can also be directed to the microtubule
11 organization center (MTOC) in a microtubule and dynein dependent manner (Figure 1) (Johnston,
12 et al., 2002, Webb, et al., 2004). Here, misfolded species can form the so called "aggresomes" to
13 facilitate engulfment into the nascent autophagosomes (Fujinaga, et al., 2009, Iwata, et al.,
14 2005, Johnston, et al., 2002, Johnston, et al., 1998, Kopito, 2000, Kopito and Ron, 2000, Taylor, et al.,
15 2003). The MTOC region is particularly enriched of autophagosomal components (Johnston, et al.,
16 2002). Depletion of the essential autophagic component p62/SQSTM1 results in an earlier disease
17 onset and a worsening of motor deficits in a transgenic SBMA mice model (Doi, et al., 2013),
18 which correlate with increased levels of insoluble ARpolyQ in affected cells. In addition, in an
19 autophagy related manner, p62/SQSTM1 silencing increased, while p62/SQSTM1 overexpression
20 reduced, the total amounts of monomeric soluble and oligo/heteromeric insoluble complexes of
21 ARpolyQ in cultured cells and in transgenic SBMA mice (Doi, et al., 2013). If the proteasome is
22 responsible for wt and ARpolyQ degradation in basal conditions, and autophagy takes over only
23 when the UPS is impaired or overwhelmed, the accumulation of ARpolyQ (in particular in nuclear
24 inclusion) may be linked to a defect in the secondary activation of the protective cytoplasmic
25 autophagic pathway, rather than to an initial failure in proteasome function. A delicate equilibrium
26 exists between the UPS and autophagy, which are both essential for the correct functioning of the
27 PQC system (Carra, et al., 2012, Carra, et al., 2013, Rusmini, et al., 2010, Rusmini, et al., 2013). The
28 ARpolyQ re-routing from the proteasome to autophagy may be of relevance for its clearance and it
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1 requires specific chaperones that are able to sense proteasome inhibition, such as HSPB8 (Crippa, et
2 al., 2010a,Crippa, et al., 2010b) and the co-chaperones BAG3 (for autophagic clearance) and BAG1
3 (for UPS clearance) (Figure 1) (Gamerding, et al., 2011). The complex HSPB8 and BAG3 (in a
4 2:1 ratio) interacts both with misfolded proteins and with HSC70/CHIP for substrate ubiquitination
5 (Figure 1) (Arndt, et al., 2010,Crippa, et al., 2010a,Crippa, et al., 2010b). It is of note that
6 conditions that lead to proteasome overwhelming and/or impairment result in a selective "*de novo*"
7 transcriptional activation of the HSPB8 promoter (Crippa, et al., 2010b). The raised levels of
8 HSPB8 permit an increase in the total amount of the 2x(HSPB8)/BAG3 complex, with an
9 enhancement of its overall activity against misfolded species (Crippa, et al., 2010a). Interestingly,
10 we found that HSPB8, by interacting with BAG3, decreases ARpolyQ aggregation, by increasing
11 its solubility and clearance (Rusmini, et al., 2013). In motor neuronal cells, HSPB8 does not modify
12 the expression of p62 and LC3 (the two key autophagic molecules). However, HSPB8 prevents p62
13 body formation, restoring a normal autophagic flux, which is known to be impaired by testosterone-
14 activated ARpolyQ (Rusmini, et al., 2013). As a consequence of the restored autophagic flux,
15 HSPB8 facilitates the ARpolyQ autophagic removal, thereby limiting its aggregation and toxicity in
16 motor neuronal cells (Carra, et al., 2013,Crippa, et al., 2010a,Crippa, et al., 2010b,Rusmini, et al.,
17 2013). Upregulation of the HSPB8-BAG3 complex may thus play a key role in the defense against
18 ARpolyQ and in the maintenance of motor neuron viability. Notably, HSPB8 is mutated (at K141
19 with E or N) in some forms of motor neuron diseases (Charcot-Marie-Tooth type 2L disease and in
20 hereditary distal motor neuropathy type II (dHMNII) (Fontaine, et al., 2006,Irobi, et al., 2010)), and
21 the chaperone activity of HSPB8 is lost in the mutant dHMNII-HSPB8 (Kwok, et al., 2011). This
22 supports the possibility that HSPB8 is crucial for motor neuron function and viability. Moreover, in
23 ALS mice, both anterior horn spinal cord motor neurons and skeletal muscle cells respond to
24 proteotoxicity by activating a robust HSPB8-mediated PQC system response (Crippa, et al.,
25 2013a,Crippa, et al., 2013b,Crippa, et al., 2010b). Furthermore, the motor neurons that survive in
26 the spinal cord at disease end stage have particularly enhanced levels of HSPB8 protein in
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1 association with soluble mutant SOD1 (Crippa, et al., 2010b). Similar data have been reported in
2 autopsy specimens of ALS patients spinal cord (Anagnostou, et al., 2010). It must be noted that in
3 mouse spinal cord, HSPB8 expression level decline with age (Crippa, et al., 2010b), suggesting that
4 motor neuronal cells may become more vulnerable to misfolded protein toxicity during aging, as a
5 result of low levels of this pro-autophagic protein. HSPB8 also exerts a physiological role in the
6 PQC response in muscle (Arndt, et al., 2010), another target of ARpolyQ toxicity. However,
7 although the expression levels of HSPB8 dramatically increase with disease progression in muscle
8 of ALS mice (Carra, et al., 2013, Crippa, et al., 2013a, Crippa, et al., 2013b), nothing is known at
9 present about the possible involvement of the HSPB8 and BAG3 machinery in SBMA skeletal
10 muscle. Our preliminary results suggest that in symptomatic SBMA male mice, the expression level
11 of HSPB8 in muscle is increased, but not at levels comparable to those found in the corresponding
12 muscles of symptomatic ALS mice.
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28 As stated above, many of the genes involved in autophagy have been found altered in response to
29 ARpolyQ expression or activation by ligands. However, one of the most relevant defects is
30 associated with the fact that ARpolyQ is able to reduce the long-term proteins turnover and to block
31 the cytoplasmic autophagic flux (Carra, et al., 2012, Cortes, et al., 2014b, Giorgetti, et al.,
32 2014, Rusmini, et al., 2010, Rusmini, et al., 2013, Rusmini, et al., 2007, Rusmini, et al., 2011). As will
33 be discussed in the next sections, the pharmacological restoration of a normal autophagic flux, for
34 example by treatment with trehalose, a TFEB activator (Dehay, et al., 2010), greatly increases the
35 clearance of the mutant misfolded ARpolyQ (Giorgetti, et al., 2014, Rusmini, et al., 2013). Defects
36 in autophagy flux have been clearly identified in SBMA mice (Cortes, et al., 2014b), and the
37 importance of autophagy in SBMA is confirmed by several recent studies (Cortes, et al.,
38 2014b, Settembre and Ballabio, 2011). The ARpolyQ processing may differ considerably in
39 neuronal and muscle cells, both targets of ARpolyQ toxicity, and the identification of specific
40 autophagy related molecular markers of skeletal muscle degeneration in SBMA might represent a
41 valuable diagnostic tool for monitoring disease progression. In SBMA muscle, the toxicity of
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1 activated ARpolyQ is rather complex and controversial, and autophagy may exert either a
2 protective or a detrimental effect. Autophagy is dysregulated in muscle of AR113Q knock-in
3 SBMA mice, and autophagic genes appear to be regulated by a physiological antagonism between
4 TFEB and ZKSCAN3 (Chua, et al., 2014), which surprisingly, are both greatly increased. In muscle
5 of SBMA mice, TFEB levels are increased, and consequently most of the TFEB-target genes are
6 upregulated, including LC3, Vps11, Vps18 and Lamp1 (Chua, et al., 2014). Inhibition of Beclin-1
7 induced autophagy diminished skeletal muscle atrophy in AR113Q knock-in SBMA mice,
8 improved motor behavior and extended lifespan (Yu, et al., 2011). On the other hand, over-
9 activation of autophagy worsened the motor phenotype (Yu, et al., 2011). Therefore, restoration of
10 both a normal flux and a dysregulated autophagy may help in the ARpolyQ clearance in muscle of
11 SBMA mice.
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29 *d) Endoplasmic Reticulum-Associated Degradation in stress conditions and the Unfolded Protein*
30 *Response.*
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32 Alteration of the proteasomal and/or autophagic pathways may involve a proteotoxic response to
33 cell stress that induces the unfolded protein response (UPR) at the level of the endoplasmic
34 reticulum (ER). The UPR is normally activated in the ER lumen to restore normal cell function via
35 transient translational blockage, activation of degradative pathways and induction of molecular
36 chaperones. When protein misfolding occurs, hydrophobic aminoacids are usually exposed to the
37 surface of the protein, which can be recognized by proteins such as BiP/Grp78, a member of the
38 HSP70 family. These proteins can be driven to the ER-associated degradation (ERAD) to initiate
39 the signaling of the UPR, involving factors including PKR-like endoplasmic reticulum kinase
40 (PERK) and inositol requiring enzyme 1 (IRE1) (Figure 1). Translation attenuation is mediated by
41 the PERK receptor soon after UPR initiation, and induced by PERK oligomerization and
42 autophosphorylation, and involves phosphorylation of the eIF2 mRNA translation machinery. At
43 the same time, the mRNA coding for the transcription factor XBP1 is activated by alternative
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1 splicing and the resulting protein product upregulates the transcription of UPR stress genes. In
2 addition, the activating transcription factor 6 (ATF6) is activated by protein cleavage, migrates from
3 the ER to the nucleus, where it controls other UPR genes. These simultaneous activities lead to the
4 restoration of normal ER functions. It has been shown, using a disease-associated fragment of the
5 ARpolyQ, that this is capable of activating an ER stress-inducible promoter. When the contribution
6 of the three proximal sensors of ER stress, ATF6, IRE1, and PERK, to the UPR was analysed in
7 detail, it was found that ARpolyQ toxicity increased when ATF6 was blocked, but decreased by
8 overexpression of a constitutively active mutant of ATF6. Conversely, modification of the IRE1
9 arm of UPR did not modify ARpolyQ toxicity, whilst PERK activation resulted in increased
10 ARpolyQ toxicity (Thomas, et al., 2005). Thus, the UPR is activated by ARpolyQ and UPR
11 activation may reduce its toxicity. ARpolyQ induced toxicity in skeletal muscle also induces the
12 UPR. In knock-in SBMA mice, characterized by muscle atrophy induced by Beclin-1 mediated
13 autophagy, deletion of the transcription factor C/EBP homologous protein (CHOP), which is
14 normally induced following ER stress, significantly increased muscle atrophy, proving the existence
15 of a cross-talk between the UPR and macroautophagy in SBMA (Yu, et al., 2011).

16 In mouse embryonic stem cells (ESCs), ARpolyQ is able to induce a testosterone-dependent cell
17 stress phenotype during cell differentiation, characterized by the formation of ARpolyQ inclusions
18 positive for E3 ubiquitin ligase, CHIP and BiP/GRP78, as well as by Caspase-3 activation.
19 ARpolyQ aggregation was accompanied by ER stress and increased susceptibility to apoptosis.
20 These modifications were paralleled by a robust increase in levels of ER chaperones (GRP78/BiP
21 and GRP94) and ER stress markers (ATF6, phosphorylated PERK, GADD153/CHOP and spliced
22 XBP-1). ARpolyQ activation was also responsible for the dissociation of BiP/GRP78 from ATF6.
23 Interestingly, exogenous overexpression of GRP78/BiP reduced ARpolyQ ubiquitination and
24 aggregation, while GRP78/BiP silencing of GRP78/BiP correlated with increased ARpolyQ
25 aggregation, caspase-3 activity and cell apoptosis (Yang, et al., 2013).

1 The ER may be regarded as a quality control system for newly synthesised proteins in cells. The ER
2 is associated not only with protein folding, but also the control of Ca²⁺ homeostasis and is the main
3 intracellular Ca²⁺ reservoir (Hetz and Mollereau, 2014). Therefore, accumulation of misfolded
4 proteins within the ER and/or depletion of ER Ca²⁺ may compromise normal cellular function and
5 subsequently trigger ER stress. The mechanisms that attempt to restore ER homeostasis include
6 store-operated Ca²⁺ influx, which replenishes Ca²⁺ levels, as well as activation of the UPR
7 (Szegezdi, et al., 2006). Sustained activation of ER stress diminishes the ability of the UPR to
8 restore ER homeostasis and results in ER stress-induced apoptosis, mediated by caspase 12
9 (Yoneda, et al., 2001). The role of ER stress and Ca²⁺ homeostasis was recently examined in the
10 AR100 mouse model of SBMA. AR100 mice develop a late onset, slowly progressive
11 neuromuscular phenotype, accompanied by motor neuron degeneration and muscle atrophy (Malik,
12 et al., 2011, Montague, et al., 2014, Sopher, et al., 2004). Dysregulation of Ca²⁺ homeostasis in
13 embryonic motor neurons of AR100 mice results in ER stress, which enhances the vulnerability of
14 AR100 motor neurons to ER-stress-induced apoptosis (Montague, et al., 2014). These findings
15 suggest that in cultured motor neurons of AR100 SBMA mice, ligand-dependant DHT activation of
16 ARpolyQ triggers a depletion of ER Ca²⁺ levels and a reduction of store-operated Ca²⁺ influx. As a
17 consequence, ER stress is more likely to occur in AR100 embryonic motor neurons, as the normal
18 function of the ER is dependent on Ca²⁺ levels (Hetz and Mollereau, 2014, Tadic, et al., 2014). The
19 reduction in ER Ca²⁺ may in part be explained by the finding that the expression of the SERCA2b
20 pump was significantly lower in AR100 motor neurons than in wildtype motor neurons. SERCA2b
21 belongs to the sarcoendoplasmic reticulum Ca²⁺ ATPases (SERCAs) which enable ER Ca²⁺ re-
22 uptake (Foradori and Handa, 2008). Furthermore, the levels of the ER stress markers BiP, ATF4
23 and CHOP were all also significantly elevated in DHT-treated AR100 embryonic motor neurons *in*
24 *vitro*. As a result, ER stress-mediated apoptosis was activated in AR100 motor neurons *in vitro*,
25 leading to an increase in the expression of activated caspase 12. Similarly, there was elevation of
26 ER stress markers *in vivo*, in spinal cord motor neurons of AR100 mice (Montague, et al., 2014).
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Importantly, this ER stress was detected in presymptomatic mice, long before the onset of any pathology and development of disease, suggesting that ER stress may be an early event in the pathogenesis of SBMA which possibly plays a causal role in the development of the disease. Furthermore, preliminary findings suggests that there is a difference in activation of the ER stress in muscle compared to spinal cord in AR100 mice, indicating that there may be an alternative mechanism responsible for muscle dysfunction in SBMA (Malik and Greensmith, unpublished observation).

Approaches aimed to enhance the activity of the protein quality control system to counteract ARpolyQ toxicity in SBMA

The findings discussed so far indicate that the PQC system exerts protective effects against ARpolyQ toxicity. On the other hand, ARpolyQ may impair the PQC system, generating a vicious circle in which the reduced activity of the PQC leads to less protection against cell stress induced by the ARpolyQ, thereby enhancing its toxicity. As a result, several investigators have developed different approaches aimed to improve the overall activity of the PQC system in neurons or muscle cells. Indeed, the simple overexpression of chaperones and/or the stimulation of autophagic pathway have been shown to positively modulate ARpolyQ deposition, and to reduce its toxicity.

a) Enhancement of the chaperone response

One of the first attempt to increase the chaperone response in models of SBMA involved the use of Geranylgeranylacetone (GGA), a nontoxic anti-ulcer drug, which has been shown to stimulate Hsp70, Hsp90 and Hsp105 expression (Katsuno, et al., 2005). In transgenic SBMA mice, oral GGA treatment induced a significant enhancement of the chaperone activity mediated by these HSPs, resulting in an amelioration of neuromuscular deficits and an increase in survival. This protective effect of GGA was mediated by a significant reduction in ARpolyQ accumulation in the affected areas of the central nervous system (Adachi, et al., 2009, Katsuno, et al., 2005). Unfortunately,

1 although oral GGA was well tolerated in mice, the effective dose was very high, so that translation
2 to humans patients would be likely to cause significant side effects. Further studies by other groups
3 have shown that specific lysine (Lys) residues of the wtAR (acetylated in a ligand-dependent
4 manner) may significantly affect AR dynamics, inducing a behavior (in term of trafficking,
5 misfolding, and aggregation) similar to that caused by ARpolyQ. When the Lys 630 or 632 and 633
6 are converted to alanine (K/A conversion), wtAR nuclear translocation is greatly delayed,
7 accompanied by the formation of inclusions that entrap HSP40, HSP70 and the ubiquitin-protein
8 isopeptide ligase (E3) CHIP. This aberrant activity can be blocked with radicicol, an inhibitor of
9 Hsp90, suggesting that the ligand-dependent K/A ARpolyQ intracellular distribution, folding and
10 aggregation are linked to Hsp90 functions (Thomas, et al., 2004). Whether or not the effect of
11 radicicol is extended to ARpolyQ, and whether this approach may have relevance for SBMA
12 remains to be tested.

13 Following these two initial proofs of principle, other molecules targeting the chaperone response
14 have been tested in SBMA models. For example, geldanamycin and other related HSP90 inhibitors
15 can activate heat shock factor 1 (HSF-1), which in turn results in an enhanced expression of HSP70
16 and HSP40. Surprisingly, treatment with geldanamycin completely prevented ARpolyQ aggregate
17 formation in HSF-1(-/-) mouse embryonic fibroblasts, in which HSP70 and HSP40 cannot be
18 induced by HSF-1 (Thomas, et al., 2006). Since geldanamycin also inhibits HSP90-mediated
19 trafficking in cells it may also act via HSP90-dependent trafficking that involves the immunophilins
20 (IMM). As a proof of concept, overexpression of p23, a co-chaperone of HSP90, was able to inhibit
21 both ARpolyQ trafficking and aggregation (Thomas, et al., 2006). Thus, different druggable sites
22 can be identified in the chaperone system that plays a role in the response to proteotoxicity.

23 A study using *Drosophila* models of SBMA has demonstrated that some small molecules may act
24 by mimicking the activity of HSP70 interacting protein (Hip) (Wang, et al., 2013). Hip is a co-
25 chaperone that can enhance the binding of HSP70 to the target misfolded substrates. Together with
26 HSP70, Hip promotes the ubiquitination of the client ARpolyQ, facilitating its degradation. In this
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1 study, a series of small molecules were designed based on the rhodocyanine MKT-077, a known
2 interactor of HSP70, and capable of binding the nucleotide-binding domain of ADP, but not ATP-
3 bound HSP70. A derivative of MKT-077, named YM-1, was able to promote HSP70-dependent
4 steps in nNOS maturation, thus possibly favoring the tight-binding form of HSP70 (Wang, et al.,
5 2013). YM-1 potently stimulated the binding of HSP70 to unfolded substrates, and converted
6 HSP70 to its tight-affinity conformation. Hip blocks binding of YM-1 to HSP70, indicating that this
7 synthetic co-chaperone uses a mechanism similar to Hip to interact with HSP70. Using this
8 mechanism, YM-1 was able to increase ubiquitination and degradation of client ARpolyQ (Wang,
9 et al., 2013). YM-1 reduced the accumulation of RIPA-insoluble ARpolyQ species and the number
10 of intranuclear inclusions induced by testosterone, but had no effect on soluble ARpolyQ,
11 suggesting that YM-1 acts primarily on unfolded AR species. Treatment with YM-1 was also able
12 to rescue toxicity in *Drosophila* expressing ARpolyQ. In fact the DHT-dependent pupal toxicity of
13 the ARpolyQ was significantly rescued by YM-1 (Wang, et al., 2013). Thus, the Hip mimetics act
14 allosterically, promoting the binding of HSP70 to unfolded substrates, like ARpolyQ, and enhance
15 its ubiquitination and degradation (Wang, et al., 2013).

16 An alternative approach to modulate chaperone function is based on the use of 17-allylamino-17-
17 demethoxygeldanamycin (17-AAG), which is a potent HSP90 inhibitor, and an anti-cancer agent
18 which is currently in Phase II clinical trials for a variety of cancers. HSP90 is a fundamental protein
19 involved in the assembly of the multichaperone complex responsible for the folding, activation and
20 assembly of the AR. HSP90 also has an important role in the response to misfolded proteins like
21 ARpolyQ (Poletti, 2004). The ARpolyQ has a higher affinity for the HSP90-p23 complex than for
22 wtAR (Waza, et al., 2005). Treatment of transgenic SBMA mice with 17-AAG markedly
23 ameliorates ARpolyQ-induced motor impairments (Waza, et al., 2005, Waza, et al., 2006b). This
24 effect was attributed to the ability of 17-AAG to reduce both the monomeric and aggregated forms
25 of ARpolyQ, an effect which is highly selective for ARpolyQ compared to wtAR (Waza, et al.,
26 2005). The effect of 17-AAG does not simply involve HSP90, but is associated with a mild

1 increase in HSP70 and HSP40 (Waza, et al., 2006a). Interestingly, 17-AAG acts on ARpolyQ by
2 enhancing the overall pro-degradative activity in motor neuronal cells, but without impacting on
3 proteasome function. 17-AAG clears the misfolded fraction of ARpolyQ and the resulting ARpolyQ
4 aggregates by activating the autophagic system (Rusmini, et al., 2011). The effect of 17-AAG on
5 the aggregation and clearance of ARpolyQ has been confirmed in iPSCs derived from SBMA
6 patients and induced to differentiate to motor neurons (Nihei, et al., 2013). Unfortunately, 17-AAG
7 is not well tolerated in humans and cannot be administered orally. A series of compounds have
8 therefore been developed to reduce these adverse effects. One of these is 17-
9 (dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), an oral HSP90 inhibitor,
10 that can ameliorate motor deficits in SBMA mice, by reducing the levels of monomeric and
11 nuclear-accumulated ARpolyQ, and which is far less toxic than 17-AAG (Tokui, et al., 2009).
12 Similarly to 17-AAG, 17-DMAG also increases HSP70 and HSP40 expression, thus contributing to
13 the enhancement of the chaperone response in motor neuronal cells.

14 Recently, Arimoclomol, a novel, well tolerated and orally administrable co-inducer of the heat
15 shock response (HSR) and chaperone system, was tested in SBMA mice (Malik, et al., 2013).
16 Arimoclomol is a hydroxylamine derivative of Bimoclomol, and has been shown to upregulate the
17 expression of HSPs via co-activation of HSF-1 (Hargitai, et al., 2003). Unlike other drugs which
18 target chaperone expression and the HSR, Arimoclomol acts as a ‘smart-drug’ and enhances the
19 HSR only in cells already under stress in which HSF-1 is already activated, thereby *co-inducing* the
20 HSR rather than actually activating this stress response (Kalmar, et al., 2008, Vigh, et al., 1997).
21 This precise targeting of only cells exhibiting a stress response reduces the possibility of non-
22 specific effects in otherwise unstressed cells, which occurs following the use of other compounds
23 that upregulate HSP expression by direct global activation of the HSR which may reflect a more
24 generalised stress response that actually results in cell toxicity and death (Kalmar, et al., 2008). In
25 the AR100 mouse model of SBMA, treatment with Arimoclomol increased HSP expression *only* in
26 affected tissue (spinal cord and muscle) and not in unaffected regions (e.g. cortex and liver).
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Moreover, oral administration of AR100 mice with Arimoclomol significantly delayed disease progression by preventing motor neuron degeneration, consequently improving the survival of functional motor units, reducing muscle atrophy and alleviating the significant deterioration in body weight. Importantly, Arimoclomol also improved hindlimb muscle force and contractile characteristics. Since Arimoclomol upregulates HSP expression in muscles and the spinal cord of AR100 mice, it is likely that its beneficial effects in AR100 mice are a result of its effects both in the periphery in muscles under stress, as well as within the CNS in motor neurons. In addition, Arimoclomol also upregulated the expression of vascular endothelial growth factor (VEGF), both in the spinal cord and hindlimb muscles of diseased AR100 mice. VEGF is known to have neurotrophic effects in the survival of motor neurons (Azzouz, et al., 2004, Cvetanovic, et al., 2011, Jin, et al., 2002, Lambrechts, et al., 2003, Sopher, et al., 2004, Storkebaum, et al., 2005), as well as protect against muscle degeneration in neuromuscular diseases (Lambrechts, et al., 2003, Storkebaum, et al., 2005). VEGF is regulated by the HIF1A transcription factor and as HIF1A has been shown to interact with HSPs (Huang, et al., 2009, Isaacs, et al., 2002), this may provide an explanation for the upregulation induced by Arimoclomol. Arimoclomol has been previously shown to be effective in delaying disease progression in the SOD1^{G93A} mouse model of ALS (Kalmar, et al., 2008, Kieran, et al., 2004), and to protect against retinal degeneration in rhodopsin retinitis pigmentosa (Parfitt, et al., 2014). It is therefore an attractive therapeutic candidate for SBMA, as it has already been shown to be safe and well tolerated in healthy volunteers and patients with ALS (Cudkowicz, et al., 2008), and is currently undergoing a phase II/III trial in the USA in SOD1-ALS (www.ClinicalTrials.gov), as well as in patients with the inflammatory myopathy inclusion body myositis (IBM) (Machado, et al., 2013).

b) Activation of autophagy

Several attempts have been made to enhance the overall cellular autophagy power to promote the clearance of toxic ARpolyQ species from SBMA affected cells. The advantage of this strategy is that autophagy is thought to be selectively regulated in cells which are specifically sensitive to its

1 activation (i.e. hepatocytes, muscle cells and neurons) and therefore it may be possible to identify
2 small molecules capable of selectively intervening in neuronal autophagy. The biological relevance
3 of autophagy in these cells is related to their energy requirements as well as to the need for
4 protection against different proteotoxic or organelle mediated stresses. Thus, the goal for SBMA is
5 to identify compounds that increase autophagic clearance of misfolded ARpolyQ selectively in
6 SBMA affected cells, motor neurons and/or muscle cells. Using primary cultures of motor neurons
7 derived from SBMA mice, Montie and Merry have shown that both the autophagy activator mTOR-
8 dependent phenoxazine (or AKTi, a potent Akt inhibitor or rapamicyn), and the mTOR-independent
9 trehalose, were able to rescue motor neurons from ARpolyQ mediated ligand-dependent death
10 (Montie, et al., 2009, Montie and Merry, 2009). In motor neurons, both AKTi (Montie, et al., 2009)
11 and trehalose (Giorgetti, et al., 2014, Montie, et al., 2009, Rusmini, et al., 2013) induced the classical
12 cytoplasmic punctate distribution of the lipidated form of LC3 (LC3-II) which associated with
13 nascent autophagosomes, a clear index of autophagy activation and this correlated with the full
14 removal of aggregated, insoluble ARpolyQ (Giorgetti, et al., 2014, Montie, et al., 2009, Montie and
15 Merry, 2009, Rusmini, et al., 2013). Other small molecules structurally related to trehalose may
16 have the potential to revert ARpolyQ accumulation and aggregation. Trehalose is considered a
17 natural osmolyte and/or "chemical chaperone" (Kumar, 2012). Its advantage as a small molecule,
18 which is shared by other osmolytes, is that it can cross the blood-brain barrier with high efficiency.
19 In addition, its activity can be enhanced by the synergic effects of other compounds that target
20 ARpolyQ. Indeed, since, testosterone activates ARpolyQ toxicity by inducing its nuclear
21 translocation, the prevention of ARpolyQ localization in the nucleus, combined with an increased
22 cytoplasmic clearance may be protective. Notably, cytoplasmic retention of ARpolyQ obtained
23 using the selective AR modulator (SARM) Casodex, not only prevented ARpolyQ aggregation and
24 toxicity, enhancing motor neuronal survival (Orr, et al., 2010, Rusmini, et al., 2007), but also
25 potentiated the pro-autophagic activity of trehalose (Giorgetti, et al., 2014). This synergistic effect
26 may be due to an increased time window for the recognition of misfolded ARpolyQ species in the
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1 cytoplasm, soon after AR dissociation from HSPs, and their autophagic engulfment prior to
2 migration into the nucleus. This, together with improved ARpolyQ autophagic clearance in the
3 cytoplasm, leads to an indirect reduction of ARpolyQ nuclear accumulation, preventing its nuclear
4 toxicity by maintaining normal motor neuron proteostasis and viability.
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11 Although the toxic effects of ARpolyQ are thought to be exerted at the nuclear level, cytoplasmic
12 autophagic degradation of ARpolyQ greatly reduces its toxic effects. Interestingly, the autophagic
13 activation that protects SBMA motor neurons has only mild effects on nuclear ARpolyQ
14 aggregation, but significant effects on cytoplasmic ARpolyQ aggregation. This pharmacological
15 data has been corroborated by evidence obtained in SBMA mice genetically manipulated to produce
16 an AR lacking the nuclear localization signal (NLS) (Montie, et al., 2009). In these ARpolyQ Δ NLS
17 mice, the AR is poorly translocated into nuclei upon androgenic activation, and this correlates with
18 a substantial improvement in motor function compared with classical SBMA mice (Montie, et al.,
19 2009). Furthermore, using a Drosophila model of SBMA, it has also been shown that the retention
20 of an ARpolyQ fragment in the cytoplasm ameliorates the disease (Takeyama, et al., 2002).
21 Moreover, in cultured motor neurons of ARpolyQ Δ NLS mice, the autophagic pathway was capable
22 of fully degrading the cytoplasmically-retained ARpolyQ, activated by androgens, suggesting that
23 autophagy may even protect against nuclear ARpolyQ toxicity. As mentioned above, trehalose is
24 not only a potent autophagic inducer, but also enhances the expression of HSPB8 (Rusmini, et al.,
25 2013), known to actively participate in the protection against cell stress by assisting the autophagic
26 removal of misfolded ARpolyQ. HSPB8 is able to restore a normal autophagic flux. We recently
27 screened for a large library of FDA approved drugs for their ability to stimulate the HSPB8
28 promoter in a reconstituted transcriptional assay. Several estrogenic compounds (and selective
29 estrogen receptor modulators, SERMs) were able to enhance the expression of HSPB8, suggesting
30 that these steroids may have a protective activity against proteotoxicity induced by misfolded
31 proteins. It will be of interest to evaluate whether SERMs, by modulating HSPB8, may exert
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1 pharmacological protection against misfolded ARpolyQ and related proteins (Crippa et al.
2 Unpublished results). In the same screening we also confirmed that proteasome inhibitors are potent
3 inducers of HSPB8 expression, leading us to hypothesize that when the proteasome is overwhelmed,
4 and consequently impaired, transcriptional activation of the HSPB8 gene may be triggered in order
5 to facilitate the removal of proteins via autophagy (Carra, et al., 2013, Crippa, et al., 2010b, Giorgetti,
6 et al., 2014, Rusmini, et al., 2013). Whilst the use of proteasome inhibitors is not appropriate for
7 disorders such as SBMA, their effects on HSPB8 can be taken as a proof of principle that proteins
8 that accumulate when the proteasome is blocked, may be molecularly re-routed by HSPB8 (in
9 complex with BAG3) to the autophagy pathway for clearance.
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23 c) Activation of the UPR response

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26 ER stress has been shown to be an early pathological feature of motor neuron disorders, in both the
27 SOD1^{G93A} mouse model of ALS and the AR100 model of SBMA (Montague, et al., 2014, Saxena, et
28 al., 2009). The effect of pharmacological targeting of ER stress was examined by treatment of
29 AR100 SBMA embryonic motor neuron cultures with the ER inhibitor, salubrinal, in order to
30 establish whether motor neuron survival was related to the ER stress observed in AR100 motor
31 neurons (Montague, et al., 2014). Salubrinal is a selective inhibitor of eIF2 α dephosphorylation, and
32 consequently diminishes ER stress-mediated apoptosis (Boyce, et al., 2005, Kessel, 2006).
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34 Treatment with salubrinal improved the survival of cultured motor neurons, in particular AR100
35 motor neurons. The expression of activated caspase 12 was significantly higher in AR100 motor
36 neurons, but treatment with salubrinal resulted in a marked decrease in expression in AR100 motor
37 neurons. Sustained ER stress is known to diminish the ability of the UPR to regulate ER
38 homeostasis, resulting in apoptosis. Caspase 12 plays a key role in this ER stress-induced apoptosis
39 in mice (Yoneda, et al., 2001). These results therefore show that treatment with an ER stress
40 inhibitor prevents activation of the ER-associated cell death pathway in AR100 motor neurons.
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42 Importantly, inhibition of ER stress in AR100 motor neurons *in vitro*, by treatment with salubrinal,
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1 significantly reduces activation of ER stress-associated apoptosis, by suppressing activation of
2 caspase 12. Treatment of mutant SOD1 transgenic mice with salubrinal has been shown to delay
3 disease progression and attenuate the disease phenotype (Saxena, et al., 2009). ER stress has also
4 been implicated in motor neuron degeneration in C.Elegans and Zebrafish models of TDP-43 ALS,
5 which is reduced by treatment with salubrinal, resulting in protection against TDP-43 neuronal
6 toxicity *in vivo* (Vaccaro, et al., 2013). In addition, treatment of α -synuclein transgenic mice with
7 salubrinal has also been shown to be neuroprotective in mouse model of Parkinson's disease (Colla,
8 et al., 2012a, Colla, et al., 2012b). Thus, pharmacological targeting of the ER stress pathway, one of
9 the earliest pathological mechanisms observed in SBMA motor neurons, may be a potential
10 therapeutic strategy for SBMA.
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26 d) Targeting other components of the proteostasis network

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31 The proteostasis network is an integrated system of pathways that regulate protein synthesis,
32 trafficking and degradation, to ensure proper protein folding and to eliminate misfolded proteins
33 when folding repair mechanisms are unsuccessful (Balch, et al., 2008). The overall purpose of this
34 network is to maintain the integrity of the proteome in response to environmental or genetic
35 stressors. The accumulation of aberrant protein species in SBMA, as in other diseases caused by
36 protein misfolding, challenges the proteostasis machinery and at the same time, may accelerate
37 aging by interfering with protein folding and clearance and other key cellular processes (Hipp, et al.,
38 2014). Along with the HSR, other critical components of this network are mediated by nuclear
39 factor (erythroid-derived 2)-like 1 (NFE2L1 or Nrf1) and nuclear factor (erythroid-derived 2)-like 2
40 (NFE2L2 or Nrf2). Nrf2 controls the antioxidant response by activating the expression of
41 antioxidant and detoxification enzymes. Oxidative stress plays a major role in the pathogenesis of
42 SBMA (Iida, et al., 2015, Ranganathan and Fischbeck, 2010) and neurodegeneration in general (Lin
43 and Beal, 2006). Nrf2 overexpression decreases neuronal loss in a mouse model of amyotrophic
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1 lateral sclerosis (Vargas, et al., 2008). The Nrf1 pathway is strongly linked to the antioxidant stress
2 response, which has been shown to enhance proteasome activity, thus representing a promising
3 target for therapeutic intervention in neurodegenerative diseases (Radhakrishnan, et al., 2010). To
4 date, attempts to improve UPS activity to counteract ARpolyQ toxicity in SBMA have been
5 unsuccessful. The major challenge is to find compounds that are safe, but capable of inducing
6 expression of several components of the proteasome, with the aim of enhancing its activity in cells.
7 Paeoniflorin, a major component of Paeonia plants has been shown to be effective in vivo in SBMA
8 mice, where it mitigates the behavioural and pathological impairments typical of these model mice
9 and induces ARpolyQ clearance via the UPS and autophagy (Tohnai, et al., 2014). The molecular
10 mechanism of Paeoniflorin involves upregulation of nuclear factor-YA (NF-YA), a potent inducer
11 of the PQC system. The effect of NF-YA is mediated by several molecular chaperones, CHIP and
12 TFEB, thus possibly activating a general response to proteotoxic stress, which is able to activate
13 both the UPS and autophagy, rather than to promote a focused and selective activity of the UPS.
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34 In the context of the regulation of UPS, HDAC6 modulators have also been shown to exert a
35 beneficial role. Their activity is mainly related to the tight functional relationship that exists
36 between the UPS and autophagy. Indeed, in a Drosophila model of SBMA, HDAC6 overexpression
37 was able to rescue the eye degeneration induced by ARpolyQ as a consequence of a malfunctioning
38 UPS (see (Beitel, et al., 2013) for review). The action of HDAC6 was initially related to its
39 potential to activate a compensatory activation of the autophagic pathway (see also below) in
40 response to UPS impairment (Pandey, et al., 2007), since HDAC6 also controls autophagosome
41 maturation. (J.Y. Lee, et al., 2010). In addition, an enhanced chaperone response may also be
42 involved (Hageman, et al., 2010). Importantly, several selective modulators of HDAC6 are now
43 available (d'Ydewalle, et al., 2012, Dallavalle, et al., 2012) and may have therapeutic potential in
44 SBMA. Efforts in targeting the proteostatic networks and the UPS with small molecules is starting
45 to show some promise: using a high-throughput screening, a selective small-molecule inhibitor of
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1 the deubiquitinating activity of human USP14 has been recently identified and shown to enhance
2 degradation of several proteasome substrates implicated in neurodegenerative diseases (Lee, et al.,
3 2012, B.H. Lee, et al., 2010). Therefore, small-molecules modulation of the proteostasis network
4 represents a promising strategy for the treatment of SBMA and other diseases caused by the
5 accumulation of misfolded proteins, as it offers the opportunity to both decrease levels of the toxic
6 protein and alleviate its pathogenic effects.
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18 **Conclusions**

19 Based on the data reported above, SBMA onset and progression may depend on several alterations
20 of the pathways composing the intracellular response to proteotoxic stress, known as the PQC
21 system. These alterations are associated with the formation of testosterone-inducible misfolded
22 species of the ARpolyQ responsible for SBMA, that, by escaping the PQC system, trigger a series
23 of downstream cytotoxic events and accumulate in motoneuronal and muscle cells. In the last
24 decades, the role of proteostasis in SBMA has been widely studied, not only for the understanding
25 of the disease, but also with the aim to find druggable targets to ameliorate disease course. All
26 major components of the PQC system are deregulated in SBMA and their functions could be
27 potentially rescued with appropriated molecular and pharmacological approaches, already tested
28 both on SBMA cell and/or animal models. In particular, the first intracellular response, represented
29 by molecular chaperones localized in the cytoplasm or associated with intracellular organelles
30 (including ER) can be boosted with commercially available drugs that may be promising for future
31 therapeutic approaches. It is possibly the failure or a down-regulation of the chaperones system that
32 in SBMA lead initially both to the ARpolyQ intracellular accumulation and to the activation of the
33 degradative pathways, the UPR, the UPS and the ALP. Since these three systems work synergically
34 and in concert for counteracting the ARpolyQ toxicity, it may be more difficult to act selectively on
35 one of these aiming for restoration of the global equilibrium on the PQC system. However,
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1 interesting approaches to enhance the functions of the degradative systems have been proposed (in
2 particular of the autophagic system) as it has been described in this review. In the perspective of
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4 future therapeutic approaches, it will be important to deepen the temporal and spatial cross-talk
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6 between UPS, UPR and ALP for identifying other key molecules involved in the clearance of
7
8 ARpolyQ.
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10 Although several different therapeutic approaches have already been tested, SBMA is still without a
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12 cure. Fortunately, among the various effective compounds, here reviewed, most of them able to
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14 modulate different players able to enhance the PQC system activity in SBMA, it might be worth to
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16 test the possibility of their combined additive/synergic action. This might allow to tackle different
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18 steps of the pathways damaged by the ARpolyQ in target cells. The combined approach might
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20 represent a novel therapeutic perspective and it could be combined even with several active
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22 compounds targeting other intracellular processes altered in SBMA.
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Legend to Figure

Fig. 1 Proteostasis regulation in Spinal and Bulbar Muscular Atrophy (SBMA)

Inactive ARpolyQ is located in the cytoplasm associated with HSPs proteins, in a “non-toxic” conformation. Testosterone (T) binding induces the HSPs dissociation unmasking the polyQ tract, which is prone to misfold. Misfolded ARpolyQ aggregate in cytoplasm and/or nuclei, An excess of misfolded ARpolyQ may alter the intracellular proteostasis. This leads to activation of the proteotoxic response mediated by the protein quality control (PQC) system. The PQC comprises: a) the chaperones proteins (including the heat shock proteins (HSPs)), the degradative pathways b) ubiquitin-proteasome system (UPS), c) autophagy-lysosome pathway (ALP), and d) the unfolded protein response (UPR), which activates the endoplasmic reticulum-associated degradation (ERAD). Misfolded ARpolyQ is recognized by the HSPs which may assist its refolding or alternatively, when folding cannot be reached, direct it to degradation. The UPR comprises three parallel signalling branches: PERK, IRE1 α and ATF6 and works with chaperones to determine the fate of misfolded proteins. ARpolyQ is mainly refolded by the ATP-dependent HSP70 and HSP40, and when this fails is ubiquitinated (by ubiquitin ligases E1, E2, E3) and degraded via UPS and/or ALP. HSP70 and HSP40 allow E1-E2-E3 ubiquitination of misfolded ARpolyQ degraded by UPS. ARpolyQ ubiquitination is also mediated by the E3-ligase C-terminus of HSC70-interacting protein (CHIP) complexed to HSC70 and misfolded ARpolyQ. The fate of this complex is determined by BAG family members acting as HSC70 co-chaperones. BAG1 links HSC70 to UPS through its ubiquitin-like (UBL) domain. BAG3 and its partner HSPB8 promote ALP degradation of HSC70 substrates. BAG3 interacts with the dynein complex moving misfolded ARpolyQ to the microtubule-organization center (MTOC) where aggresomes are formed. Alternatively, the histone deacetylase 6 (HDAC6) may also interact with dynein complex, but apparently after CHIP-mediated ubiquitination of substrates escaping UPS degradation. Polyubiquitinated proteins bound to the HSC70-BAGs are recognized by p62/SQSTM1, which interacts with the autophagy marker LC3 to allow substrates insertion into autophagosome for degradation.

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