Chaperone-like activity of the AAA+ proteins Rvb1 and Rvb2 in the assembly of various complexes

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Rvb1 and Rvb2 are highly conserved and essential eukaryotic AAA+ proteins linked to a wide range of cellular processes. AAA+ proteins are ATPases associated with diverse cellular activities and are characterized by the presence of one or more AAA+ domains. These domains have the canonical Walker A and Walker B nucleotide binding and hydrolysis motifs. Rvb1 and Rvb2 have been found to be part of critical cellular complexes: the histone acetyltransferase Tip60 complex, chromatin remodelling complexes Ino80 and SWR-C, and the telomerase complex. In addition, Rvb1 and Rvb2 are components of the R2TP complex that was identified by our group and was determined to be involved in the maturation of box C/D small nucleolar ribonucleoprotein (snoRNP) complexes. Furthermore, the Rvbs have been associated with mitotic spindle assembly, as well as phosphatidylinositol 3-kinase-related protein kinase (PIKK) signalling. This review sheds light on the potential role of the Rvbs as chaperones in the assembly and remodelling of these critical complexes.

1. What are Rvb1 and Rvb2?

Rvb1 and its parologue Rvb2, with 43 per cent sequence identity and 65 per cent sequence similarity to each other (for the human proteins), belong to the AAA+ (adenosine triphosphases associated with diverse cellular activities) superfamily of ATPases. This class of ATPases is present in all kingdoms of life and is divided into numerous groups, clades and families based on structural and sequence analyses [1–3]. AAA+ proteins usually form hexameric ring structures and are characterized by the presence of the AAA+ module, which contains the highly conserved Walker A and Walker B motifs responsible for nucleotide binding and hydrolysis, respectively [4].

Rvb1 and Rvb2 are known under diverse names such as Pontin/Reptin, TIP49/TIP48, RuvBL1/RuvBL2 and ECP54/ECP51, respectively, reflecting their appearance in many cellular protein complexes and their discovery by unrelated approaches in multiple organisms [5–9]. In this review, we refer to these two proteins as Rvb1 and Rvb2.

2. Discovery and roles of Rvb1 and Rvb2

Rvb1 was originally discovered in 1997 as part of a complex with the TATA-binding protein (TBP) in rat [10]. Rvb1 and Rvb2 were found in complex with the large RNA polymerase II holoenzyme oligomer in 1998 [11], and, subsequently, Rvb2 was identified as an interacting partner of Rvb1 in human cells in 1999 [12]. Rvb1 and Rvb2 share limited sequence similarity (approx. 30%) to the bacterial RuvB helicase [13,14]. RuvB drives the branch migration and resolution of the Holliday junction in complex with RuvA and RuvC during homologous recombination and DNA repair [15]. This sequence similarity suggested that the Rvbs might have helicase activity using ATP binding and hydrolysis, since the deletion of RVB1 and RVB2 genes in Saccharomyces cerevisiae was complemented by the overexpression of the bacterial RuvAB
complex [16] and since Rvb1 was found to be associated with the human replication protein (RP)A3 [11]. Indeed, the purified proteins exhibit weak helicase activity [12,17]. The RVB1 and RVB2 genes were found to be essential for viability in all model organisms examined so far, including S. cerevisiae [11], Drosophila melanogaster [8] and Caenorhabditis elegans [18], and are speculated to be also essential in mammalian cells. Since their discovery, the Rvbs have been found to be associated with many cellular pathways [19], including chromatin remodelling [9,20–23], transcription regulation [9,24], ribonuleoprotein complex biogenesis [25–29], mitotic assembly [30–32], telomerase complex assembly [33], RNA polymerase II assembly [26,34] and phosphatidylinositol 3-kinase-related protein kinase (PIKK) signalling [29] (figure 1).

3. The structure of Rvb1 and Rvb2

Based on the X-ray structure of human Rvb1 [18], the Rvb sequence can be divided into three domains (figure 2a, b): (i) an N-terminal αβα subdomain of the AAA+ domain, (ii) a 170 amino acid-insertion domain unique to the Rvbs among the AAA+ proteins which mediates DNA/RNA binding and shows similarity to the ssDNA binding domain of the replication factor replication protein A (RPA), and (iii) an α subdomain of the AAA+ domain. In the AAA+ domain (figure 2a, b), the Walker A and Walker B motifs are responsible for ATP binding and hydrolysis, respectively, while sensor I and sensor II motifs sense whether the protein is bound to di- or tri-phosphates. The arginine finger (Arg-finger) of one subunit extends into the ATPase site of the neighbouring subunit and allows coordination of ATP hydrolysis between the subunits in the hexamer [3].

The crystal structure of human Rvb1 has been solved as a hexamer [18] (figure 2b), however, the homohexamer was found to be inactive as a helicase and ATPase, suggesting that this might not be the physiologically relevant complex. There is no crystal structure of Rvb2 alone; however, more recently, the crystal structure of the human Rvb1–Rvb2 complex, with truncation of the insertion domain in both proteins, was solved [35] (figure 2c). The complex was found to be a dodecamer composed of two heterohexameric rings with alternating Rvb1 and Rvb2 monomers [35]. The study showed that the

**Figure 1.** Overview of Rvb1/2 function. Rvb1 and Rvb2 function in the assembly of multiple cellular complexes/processes. They are involved in the assembly of mitotic spindles, telomerase complex, box C/D snoRNPs, chromatin remodelling complexes, and PIKKs. They also exhibit other roles/functions in processes such as transcription, transformation and apoptosis by interacting with factors including β-catenin, c-Myc, Hint1 and TBP. TERT, telomerase reverse transcriptase.
A truncated version of the complex exhibits an enhanced ATPase and helicase activity compared with the wild-type (WT) complex, thus, suggesting that the insertion domain functions as a regulator of the activity of the complex.

Using multiple biophysical techniques including analytical ultracentrifugation, size exclusion chromatography, mass spectrometry and electron microscopy, it has been found that human Rvb1 and Rvb2 can form various oligomeric states that are modulated by the insertion domain [36,37]. The oligomeric state of yeast Rvb1 and Rvb2 was also found to be modulated by the presence of a tag at the N-terminus [17,38,39]. These observations seem to indicate that the Rvbs are capable of forming different oligomeric states depending on the complex or cellular process in which they are involved and that other proteins and cofactors might modulate the oligomeric state and, consequently, the activity of the Rvbs.

4. Chaperone-like activity of the Rvbs

Several studies have demonstrated a role of the Rvbs in the assembly of various complexes in different organisms suggesting that they might have a chaperone-like activity. The low abundance of Rvb1 and Rvb2 in eukaryotic cells relative to other components of several complexes which they are part of suggests that the Rvbs are not permanently associated with each complex, therefore providing further support for a general chaperone-like activity of the Rvbs rather than a defined catalytic activity within each complex [40]. In order
to understand the exact role of the Rvbs in each process/complex, many studies mutated different domains/motifs in the Rvbs and assessed their effects on the activity of the complexes or on the processes being studied. Table 1 summarizes the main reported mutations and their effects as relevant to this review. The chaperone-like activity of the Rvbs in different complexes is further discussed below.

5. Role of Rvb1 and Rvb2 in the assembly of chromatin remodelling complexes

Organisms use DNA as their genetic substance, therefore, DNA-related processes such as transcription, recombination, replication and repair are very critical. The eukaryotic DNA is packaged into chromatin in the nucleus. Nucleosomes form the fundamental repeating units of eukaryotic chromatin. The canonical nucleosome includes about 147 base pairs of DNA wrapped in approximately two superhelical turns around a histone octamer composed of two histone H2A–H2B heterodimers and a histone (H3–H4)_2 heterotetramer [49]. Non-canonical nucleosomes have one or more histone variants (e.g. H2A.Z) replacing the canonical histones [49]. The compaction of DNA into a smaller volume is critical for the regulation of the above-mentioned DNA-related processes; however, it also impedes DNA transcription, replication and repair. Several chromatin remodelling complexes modulate these processes by using one of the following mechanisms to facilitate the access of proteins/cofactors to the underlying DNA: (i) using the energy of ATP hydrolysis to slide nucleosomes along the DNA, (ii) adding or removing covalent modifications on the tails of the histones in the nucleosome core or (iii) exchanging canonical histones with histone variants [50,51]. Over the last few years, several studies revealed that Rvb1 and Rvb2 are associated with various chromatin remodelling complexes such as the Ino80 complex in *S. cerevisiae*, Homo sapiens and *D. melanogaster* [9,20,21, the SWR-C complex in *S. cerevisiae* [52], and its homologous SRCAP in *H. sapiens* [53–56], and the Tip60 complex in *H. sapiens* and *D. melanogaster* [53,57–59] (figure 1).

(a) The Ino80 complex

The multisubunit Ino80 complex is very well studied and was first purified from yeast by immunoprecipitation [20]. This complex is involved in transcription regulation, replication and repair of DNA double strand breaks by catalysing ATP-dependent mobilization of nucleosomes along the DNA [20,21]. The core subunits of the Ino80 complex are common between yeast and human: the SNF2 family helicase Ino80, which is the catalytic subunit of the complex, Rvb1, Rvb2, Act1, Ino80 subunit (Ies)2 and Ies6 [52], and the actin-related proteins Arp4, Arp5 and Arp8. In addition, the yeast and human Ino80 complexes have their own distinct set of additional subunits. Both yeast and human Ino80 complexes exhibit ATP-dependent nucleosome remodelling activity and DNA and nucleosome-activated ATPase activity [21].

In yeast, considerable overlap was found between genes regulated by Ino80 protein and those regulated by Rvb1 and Rvb2 [9]. The promoters of those genes were found to be associated with the Ino80 protein but not with Rvb1 or Rvb2 [9]. The Ino80 complex has ATPase activity ascribed largely to the Ino80 protein rather than the Rvbs since mutating the ATP-binding site of the Ino80 protein results in significant reduction in the ATPase activity of the complex without affecting the subunit composition of the complex [9]. However, loss of the Rvbs leads to the loss of Arp5 protein from the complex, and, consequently, the loss of the chromatin remodelling activity of the Ino80 complex [45]. The association between Arp5 and the Rvbs requires ATP but not the ATPase activity of the Rvbs [45]. Recently, Chen *et al.* [60] showed that in human Ino80 complex, Rvb1 and Rvb2 together with Arp5, Ies2 and Ies6 associate with an insertion region within the ATPase domain of the Ino80 protein (figure 3c).

The Ino80 complex in yeast causes the proximal eviction of nucleosomes surrounding double strand breaks [52]. The Rvb proteins were found to be required to the homothallic switching (HO) endonuclease-induced DNA double-strand break along with Arp8, Arp5 and Ino80 protein [52]. This recruitment of the Ino80 complex was dependent upon the phosphorylation of the histone variant H2AX. Deletion of Arp4 and Nhp10 (two subunits of the Ino80 complex) caused a reduction in the recruitment of the complex, including the Rvbs, to the double strand breaks, therefore suggesting that these two proteins are necessary for the recognition of the phosphorylated histones and for the interaction of the Ino80 complex with the double strand break [52]. The exact role of the Rvbs in this recruitment process is yet to be determined. It can be speculated that the Rvbs are required to recruit the rest of the Ino80 subunits to the double strand breaks to form a functional complex.

(b) SWR/SRCAP complex

The Swi/Snf2-related (SWR) complex in yeast, also known as the Snf2-related CREBBP activator protein (SRCAP) complex in mammalian cells, is yet another chromatin remodelling complex that contains both Rvb1 and Rvb2 as integral subunits. Both the SWR and SRCAP complexes were found to remodel chromatin by catalysing the ATP-dependent replacement of H2A–H2B histone dimers in nucleosomes by dimers containing the histone variant Htz1 in yeast or H2AZ in mammalian cells [54,55,61]. This mechanism is essential in a range of cellular processes, such as transcriptional regulation, chromosome segregation, cell cycle progression and DNA damage response. The catalytic subunit of the complex is the Swr1/SRCAP protein, which is an SNF2 helicase. Rvb1, Rvb2, Act1, Arp4, Arp6 and Yap9/GAS41 are shared subunits between the SWR complex in yeast and the SRCAP complex in mammalian cells [20,52,56]. The SWR/SRCAP complex shares several subunits with the Ino80 complex, namely Act1, Arp4, Rvb1 and Rvb2. In yeast, it was shown that the ATPase domain of the Swr1 protein binds Rvb1, Rvb2, Arp6, Swc2, Swc3 and Swc6 [62] (figure 3d), reflecting yet another similarity with the Ino80 complex. The exact function of the Rvbs in the SWR/SRCAP complex remains unexplored. However, given the significant similarity between the SWR complex and the Ino80 complex, it can be speculated that the Rvb proteins perform a role in the assembly of the SWR complex by binding and recruiting a subunit integral for the activity of the complex, just as they recruit Arp5 to the Ino80 complex [45].

(c) Tip60 complex

This complex, which is a histone acetyltransferase (HAT) found in both human and fly cells, remodels chromatin by acetylating histones converting chromatin to euchromatin, which is a
<table>
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<td>Walker B mutant D302N</td>
<td>inhibition of c-Myc-mediated cellular transformation but did not affect general growth of cells</td>
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<tr>
<td>Rvb1</td>
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<td>Rvb2</td>
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<td>expression of endogenous $\beta$-catenin/TCF target genes</td>
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<td>both</td>
<td>interaction with c-Myc</td>
<td>cell proliferation</td>
<td>deletion of Walker A ($\Delta70–77$ in Rvb1, $\Delta76–83$ in Rvb2) and Walker B ($\Delta302–306$ in Rvb1 and $\Delta299–303$ in Rvb2) motifs</td>
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(Continued.)
relaxed, transcriptionally active DNA [59,63]. It has been shown
that this complex also acetylates proteins such as the ataxia tel-
angiectasia mutated (ATM) protein kinase after DNA damage,
therefore activating ATM [64]. The Tip60 complex is involved in
transcription, DNA repair and apoptosis [65]. The catalytic
subunit in the complex is Tip60 (Tat interactive protein 60).
The complex has Rvb1 and Rvb2 as its integral subunits. The
Tip60 complex shares several subunits with the SWR complex
and several other subunits with the NuA4 (Nucleosomal
Acetyltransferase of H4) complex, which is an acetyltransferase
complex found in yeast but that does not contain Rvb1 or Rvb2,
suggesting that the Tip60 complex is a fusion of those two com-
plexes [53]. Esa1 in yeast, which is the orthologue of the Tip60
protein in mammals, is the catalytic subunit of the NuA4 com-
plex. Eaf1, a subunit found in the NuA4 complex, is the
orthologue of the mammalian p400/domino protein found in
the Tip60 complex; however, Eaf1 lacks the ATPase domain
found in p400/domino protein [53,57]. The absence of Rvb1
and Rvb2 in the NuA4 complex may be because of the absence
of the ATPase domain in Eaf1 since Rvb1 and Rvb2 were shown
to interact with the ATPase domain of p400/domino protein [53,57]
(figure 3a), similarly to the way they interact with the ATPase
domain of the Ino80 protein.

As mentioned above, Tip60 is involved in DNA damage
repair. DNA damage causes histone variant H2AX to be
phosphorylated by ATM and ATR protein kinases. The phos-
pho-H2AX acts as a marker that recruits other proteins to the
sites of DNA damage to amplify the damage signal and
repair the damage [52,63]. HAT activity of Tip60 is required
to acetylate H4 before the phospho-H2AX can be remodelled
and dephosphorylated in DNA damage response [63]. It has
been shown that depletion of either Rvb1 or Tip60 causes an
increase in the phosphorylated H2AX and that the Rvb1s
are required for the HAT activity of the Tip60 complex,
suggesting that Rvb1 is required for the assembly of the
Tip60 complex [63].

The role of Rvb1 is also linked to apoptosis through
Tip60. Tip60 is required for the acetylation of p53, and the
acetylation of p53 is required for its binding to promoters
of proapoptotic genes [66]. In another example, Feng et al
[44] showed that the stable expression of the Walker B
mutant of Rvb1 blocked the expression of endogenous β-cate-
nin/T-cell factor (TCF) target genes, which is because of
inhibition of histone acetylation of β-catenin/TCF target
gene sequences, thus suggesting that Rvb1 exerts its effect
through Tip60 [44]. Also, Rvb1, along with Tip60, binds to
and acetylates histones at the promoter of KAI1, which is a
metastasis suppressor gene, resulting in the induction of the
expression of KAI1 [67].

6. Role of Rvb1 and Rvb2 in box C/D snoRNP
biogenesis

In an attempt to identify the interactors of yeast Hsp90, which
is a ubiquitous molecular chaperone that is essential in many
 signalling pathways, our group conducted systematic genome-wide screens and found Rvb1 and Rvb2 to be compo-
nents of a complex interacting with Hsp90 that we termed the R2TP complex [68]. In yeast, this complex consists of
two Hsp90 interactors, which we identified and termed
Tah1 (tetrasricopeptide repeat (TPR)-containing protein
associated with Hsp90) and Pih1 (protein interacting with
Hsp90), and the two AAA+ helicases Rvb1 and Rvb2 [68], hence the name R2TP. Tah1, which was uncharacterized at the time and whose structure we solved recently [69], consists of two TPR motifs and a C-terminal helix. Tah1 was found to bind to the MEEVD peptide corresponding to the C-terminus of Hsp90, while the C-terminus of Tah1 binds to the C-terminus of Pih1 [69]. Pih1, also uncharacterized at the time, is a 40 kDa protein which was found to be unstable on its own, and stable upon binding to the C-terminus of Tah1 [69].

The R2TP complex is highly conserved in eukaryotes. In humans, R2TP contains Rvb1, Rvb2, RPAP3 (protein equivalent to Tah1 although not similar) and PIH1D1 (Pih1 orthologue) [26]. The R2TP complex has been implicated in small nucleolar ribonucleoprotein (snoRNP) assembly and
pre-ribosomal RNA processing in human and yeast cells [26,27]. The complex also plays essential roles in apoptosis, PIKK signalling [29] and RNA polymerase II assembly [70].

snORPs complexes are made up of either box C/D or box H/ACA small nuclear RNAs (snRNAs) complexes with proteins. snORPs are involved in cleavage and modification of small nuclear RNA (snRNA), ribosomal RNA (rRNA) and tRNAs [71]. Box C/D snoRNPs catalyse ribose 2’-O methylation of pre-ribosomal RNA (pre-rRNA), while box H/ACA snoRNPs mediate pseudo-uridylation of pre-rRNA [28]. Mature box C/D snoRNAs in eukaryotes are associated with four common core proteins: 15.5K (Snu13 in yeast), NOP56, NOP58 and the methyltransferase fibrillarin (Nop1 in yeast) [28]. The core box C/D proteins bind a conserved sequence termed the box C/D motif that folds into a stem-loop-stem structure known as a k-turn (figure 3b). 15.5K, an RNA binding protein, binds directly to the k-turn to recruit the other core proteins [72–74]. The assembly of the complete complex is essential for the nucleolar localization of the complex [74]. Several proteins are required for this assembly, including the R2TP complex, as well as, NUFIP, TAF9 and BCD1 [28,41,75,76] (figure 3b). It has been shown that Rvb1 and Rvb2 weakly interact with NOP56, NOP58 and fibrillarin, and that the presence of ATP stimulates the interaction of Rvb1 and Rvb2 with 15.5K [28]. Rvb1 and Rvb2 appear to bridge the interaction between 15.5K and both NOP56 and NOP58 proteins [28]. In both yeast [27,41] and mammalian cells [28], depletion of the Rvbs results in the mislocalization of the snoRNP core proteins. The data to date indicate that the Rvb proteins play an important role in the assembly and remodelling of the snoRNP complex during biogenesis (figure 3b) mainly as components of the R2TP complex.

7. Role of Rvb1 and Rvb2 in PIKK signalling

Recent studies revealed that Rvb1 and Rvb2 are common regulators of all phosphatidylinositol 3-kinase-related protein kinase (PIKK) members [77]. PIKKs are serine–threonine protein kinases with catalytic domains homologous to those of phosphatidylinositol 3-kinases. PIKKs regulate DNA damage responses, nutrient-dependent signalling, and nonsense-mediated mRNA decay (NMD) [77]. The PIKK family includes DNA-PKcs (DNA-dependent protein kinase catalytic subunit), ATM and ATR (ATM- and Rad3-related), which are collectively responsible for signalling the presence of DNA damage [77]. They phosphorylate proteins that have roles in regulation of cell cycle progression, DNA repair, apoptosis and cellular senescence [77]. The PIKK family also includes SMG-1 (suppressor with morphological effect on genitalia 1), mTOR (mammalian target of rapamycin) and TRRAP (transformation/transcription domain-associated protein) in mammals [77]. SMG-1 is an essential factor of NMD and TRRAP regulates transcription as a subunit of HAT complexes [78]. SMG-1 and TRRAP are also involved in DNA damage signalling and repair [78]. A multiprotein complex called SMG1C, which is composed of SMG-1, SMG-8 and SMG-9, is essential for NMD. SMG1C detects and degrades mRNAs to prevent the production of potentially harmful premature proteins [29]. mTOR regulates nutrient-dependent signalling.

Knockdown of human Rvb1 or Rvb2 has been shown to lead to decreased phosphorylation of direct downstream effectors of ATM, ATR, mTOR and SMG-1, and also to decreased abundance of mRNA and proteins for ATM, ATR, DNA-PKcs, TRRAP and mTOR but not the abundance of other kinases [29]. WT Rvb1 or Rvb2 were able to rescue the reduced PIKK abundance, however, ATPase-deficient mutants failed to rescue the reduced abundance, indicating that the ATPase activities of both Rvb1 and Rvb2 are required to control the abundance of PIKKs [29]. It was also revealed that human Rvb1 and Rvb2 are required for SMG-1-mediated Upf1 phosphorylation, which occurs on a spliced mRNP in the cytoplasm, and that the phosphorylation was dependent on the ATPase activity of Rvb1. This phosphorylation is induced by remodelling of the mRNA surveillance complex that involves first the formation of the SURF complex, which is composed of SMG1, UPF1, eRF1 and eRF3, on a ribosome recognizing premature termination codon(s) and then the formation of the decay-inducing (DECID) complex on an mRNP. Immunoprecipitation experiments suggested that the Rvb1/2 complex associates with SURF playing a role in the remodelling of the surveillance complex and, thus, in forming a DECID complex [29].

In addition, human Rvb1/2, as part of the R2TP complex, plays a role in the assembly and stabilization of the PIKKs. This stability and assembly is achieved when the R2TP-Hsp90/Prefoldin-like complex interacts with PIKKs via the Tel2 complex (also known as the TTT complex), which is composed of Tel2, Tt1 and Tt2 [78]. A recent study in yeast linked the Tel2 complex and Asl1p to PIKKs [79].

8. Role of Rvb1 and Rvb2 in telomerase complex assembly

Telomeres are repetitive nucleotide sequences located at the ends of chromosomes, capping and protecting them from degradation and recombinogenic activities. They are un-replicated and lost during cell division owing to the ‘end replication problem’ exhibited during DNA replication, and are replenished by the telomerase [80]. The end replication problem is a problem the DNA polymerase runs into because the leading strand in the double-stranded DNA can be replicated to the very end, but the lagging strand cannot. The polymerase needs RNA primers to replicate the lagging strand DNA; however, use of the RNA primer is not possible at the end of the DNA because there is nothing for the primer to bind to, therefore, the last section of the lagging strand cannot be synthesized. Thus, after several cycles of replication, the DNA would continue to get smaller.

Telomerase is a multisubunit RNP complex that adds DNA repeats to telomeres. The complex is composed of the catalytic subunit TERT (telomerase reverse transcriptase), TERC (telomerase RNA component) and the TERC-binding protein dyskerin. In humans, Rvb1 and Rvb2 were identified as subunits of the telomerase complex, and they were found to be required for telomerase assembly/biogenesis through maintaining the telomerase RNA stability. It was demonstrated that Rvb1 directly interacts with TERT, recruiting Rvb2 and bridging its interaction to the TERT complex. It was also shown that Rvb1 and Rvb2 interact with dyskerin. Depletion of Rvb1 and Rvb2 caused a loss of TERC and dyskerin from the complex suggesting that
dyskerin bridges the interaction between the Rvb proteins and TERC [33]. The Walker B mutant of Rvb1 could not rescue TERC and dyskerin loss from the complex, thus indicating that Rvb1 and Rvb2 are essential for telomerase activity and for TERC and dyskerin accumulation through a mechanism that requires ATPase activity [33]. Rvb proteins seem to help bring together TERT, dyskerin and TERC and remodel the TERT–Rvb1–Rvb2 complex into a mature TERT–TERC–dyskerin complex [33] (figure 3c).

In addition to their role in the assembly of the telomerase complex, Rvb1 and Rvb2 seem to be also involved in the transcrip- tion of TERT [81]. Knocking down Rvb1 or its partner Rvb2 using siRNA in gastric and cervical cancer cells led to significant decreases in TERT mRNA. In addition, human Rvb2 depletion resulted in a significant decrease in the activity of TERT promoter that is dependent on c-MYC [81]. Therefore, TERT transcription requires the constitutive expression of Rvb2 and its cooperation with c-MYC.

In yeast, the Rvbs are also subunits of what is called the ASTRA complex [79,82]. ASTRA (AStation of Tel, Rvb, and Atm-like kinase) complex is composed of Tra1 (TRRAP homolog), Rvb1, Rvb2, Tel2 (telomere binding protein), Tti1p, Tti2p and Asa1p (a WD-repeat-containing protein). The ASTRA complex is poorly studied, but it is proposed to play a role in telomeric maintenance and its components (Tti1p, Tti2p, Tel2 and Asa1p) have been shown to be linked to PIKKs as mentioned previously. The role of the Rvbs within this complex is not yet characterized.

9. Role of Rvb1 and Rvb2 in mitotic spindle assembly
Several studies reported the involvement of Rvb1 and Rvb2 in mitosis. Human Rvb1 was found to copurify with tubulin isolated from U937 cells [30]. Furthermore, human Rvb1 was found to colocalize with tubulin at the centrosome and at the mitotic spindle in addition to being present in the nucleus. Using an in vitro tubulin assembly assay, it was demonstrated that Rvb1 is involved in the formation of microtubules [30]. Subsequently, another study showed that Rvb2 associates with the centrosome and the mitotic spindle [31]. However, it was demonstrated that, unlike Rvb1, Rvb2 localizes to the midzone during telophase and to the midbody during cyto- kinesis [31]. In 2008, Ducat et al. [32] demonstrated that depletion of Rvb1 using siRNA causes a defect in spindle assembly in Drosophila and mammalian cell lines. The same result was observed when depleting Rvb1 in Xenopus egg extracts. Moreover, Rvb1 and Rvb2 were found to interact with the γ-tubulin ring complex in Xenopus, which is involved in nucleating spindle formation, suggesting that both Rvb proteins are involved in mitotic spindle assembly.

10. Role of Rvb1 and Rvb2 in cancer
In mammalian cells, Rvb1 and Rvb2, separately and together, were found to have a crucial role in pathways linked closely to cancer. Several studies have shown that both Rvb1 and Rvb2 are overexpressed in 80 per cent of colon cancer specimens. Rvb2 is found to be overexpressed in human hepatocellular carcinoma cells, while Rvb1 transcript levels are found to be increased in non-small cell lung cancer [83]. The transcription of both genes is deregulated in several cancers such as liver, bladder and melanoma. In addition, it has been demonstrated that decreasing the expression of Rvb1 or Rvb2 results in reduced tumor cell growth and increased apoptosis in vitro and that decreasing Rvb2 expression results in growth arrest of established tumours in xenograft experiments in mice [83].

The roles of the Rvbs associated with modulating cellular transformation, signalling, apoptosis and response to DNA damage is mediated through their interaction with a multitude of proteins such as the tumor suppressor protein Hint1 and the transcription factors β-catenin, c-Myc, E2F (only Rvb1) and ATF2 (only Rvb2) [6–9].

11. Role of Rvb1 and Rvb2 in transcription regulation
Rvb1 and Rvb2 can function together but in several cases have also been shown to function independently and to exhibit antagonistic effects on the regulation of transcription of several target genes. Rvb1 and Rvb2 interact with β-catenin, which is a major player in Wnt signalling that affects TCF-mediated tran- scription [8]. In the nucleus, stable unphosphorylated β-catenin binds to the TCF family of transcription factors and increases the expression of downstream genes (e.g., c-Myc, ITF-2 and Cox-2) [44]. Rvb1 and Rvb2 have opposing effects on β-cate- nin-TCF transcriptional activity. Rvb1 increases the transcriptional activation of target genes, while Rvb2 represses the β-catenin/TCF transactivation complex and thus decreases the transcription of downstream genes [8]. The Walker B mutant of Rvb1 was found to block β-catenin-mediated transcrip- tion of TCF-dependent genes owing to inhibition of acetylation of histones near β-catenin target gene sequences suggesting that Rvb1/Tip60 mediates the regulation of this transcription [44]. On the other hand, Rvb2 represses gene activa- tion mediated by β-catenin and TCF through its interaction with histone deacetylase HDAC1 and 2, and corepressor TLE (transducin-like enhancer) [8]. In another example, Rvb1/ Tip60 are recruited on the promoter of the KAI1 (a metastasis suppressor which inhibits metastasis by promoting cell adhesion) gene as a co-activator complex, while Rvb2/β-cate- nin act as a co-repressor of the transcription which recruits HDAC1 as well [84]. In addition, Hint1 (histidine triad nucleo- tide-binding protein 1), which acts as a co-regulator of β- catenin-TCF-mediated transcription, was shown to bind to the insert domain in Rvb1 and Rvb2 [85]. It was demonstrated that Hint1 prevents formation of hetero and homo complexes of the Rvbs, but not the interaction between the Rvb proteins with β-catenin. Hint1 was found to be a regulator of the Rvbs/Wnt-catenin signalling pathway since its overexpression was found to modulate Rvbs/β-catenin regulated genes. Rvb1 and Rvb2 were found to bind to and regulate the function of the transcription factor c-Myc [7] (table 1). c-Myc, which is involved in oncogenic transformation, apoptosis and stimulation of cell proliferation, contains two conserved regions: Myc homology box I (MBI) and MBII, with the latter being the region where both Rvb1 and Rvb2 bind. The Walker B mutant form of Rvb1 was found to inhibit c-Myc oncogenic activity but did not inhibit cellular growth indicating that Rvb1 is essential for c-Myc-mediated oncogenic transformation [7].
12. Concluding remarks

Rvb1 and Rvb2 are involved in various cellular complexes and processes in different organisms. They exhibit different roles and functions specific to the assembly, operation, and disassembly of protein complexes. Besides being ATPases that provide energy for several processes and helicases with potential DNA/RNA unwinding activity, many studies have shown that the Rvbs seem to act as chaperones. They have been found to recruit proteins/DNA/RNA to their respective complexes and to remodel these complexes by bridging the interactions between the different components within the complex. Hence, we propose that the Rvbs are potential chaperones for the assembly and maturation of protein–protein and protein–DNA/RNA complexes. However, further studies need to be conducted to determine the exact role of the Rvbs in the assembly of these complexes.

Note added in proof

While this review was in preparation for publication, the crystal structure of human Rvb2 with truncation in part of Domain II was published by Petukhov et al. [86] and the cryo-electron microscopy structures of human double-ring Rvb1-Rvb2 complexes were published by López-Ferrero et al. [87]. In addition, the role of human Rvbs (through the R2TP complex) in H/ACA RNP biogenesis was established by Machado-Pinilla et al. [88]

We thank Dr Yoshito Kikihara, Jennifer Huen and Liang Zhao for critical reading of the manuscript. This work was supported by a grant from the Canadian Institutes of Health Research (MOP-93778) to W.A.H.

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10. Putnam CD, Clancy SB, Tsuruta H, Gonzalez S, Weremowicz S, Panvin JD, Dutta A. 1998 Crystal structure of human Rvb2 with truncation in part of Domain II was published by Petukhov et al. [86] and the cryo-electron microscopy structures of human double-ring Rvb1-Rvb2 complexes were published by López-Ferrero et al. [87]. In addition, the role of human Rvbs (through the R2TP complex) in H/ACA RNP biogenesis was established by Machado-Pinilla et al. [88]

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Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders

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The family of the mammalian small heat-shock proteins consists of 10 members (sHSPs/HSPBs: HSPB1–HSPB10, see alternative names of each member in table 1), all of a relatively low molecular weight (MW, ranging from 15 to 45 kDa) and sharing some structural similarities, like a highly conserved C-terminal alpha-crystallin domain, important for the modulation of both their structural and functional properties. HSPB proteins are biochemically classified as molecular chaperones and participate in protein quality control, preventing the aggregation of unfolded or misfolded proteins and/or assisting in their degradation. Thus, several members of the HSPB family have been suggested to be protective in a number of neurodegenerative and neuromuscular diseases that are characterized by protein misfolding. However, the pro-refolding, anti-aggregation or pro-degradative properties of the various members of the HSPB family differ largely, thereby influencing their efficacy and protective functions. Such diversity depends on several factors, including biochemical and physical properties of the unfolded/misfolded client, the expression levels and the subcellular localization of both the chaperone and the client proteins. Furthermore, although some HSPB members are inefficient at inhibiting protein aggregation, they can still exert neuroprotective effects by other, as yet unidentified, manners; e.g. by maintaining the proper cellular redox state or/and by preventing the activation of the apoptotic cascade. Here, we will focus our attention on how the differences in the activities of the HSPB proteins can influence neurodegenerative and neuromuscular disorders characterized by accumulation of aggregate-prone proteins. Understanding their mechanism of action may allow us to target a specific member in a specific cell type/disease for therapeutic purposes.

1. Introduction

The family of the mammalian small heat-shock proteins, also called the HSPB proteins, consists of 10 members (HSPB1–HSPB10, see alternative names of each member in table 1), all of a relatively low molecular weight (MW, ranging from 15 to 45 kDa) and sharing some structural similarities, like a highly conserved C-terminal alpha-crystallin domain (ACD, [52]). This ACD plays an important role in the modulation of both structural and functional properties of the HSPBs. Indeed, monomers of the HSPB proteins associate (partially via their ACDs) into dimers that are thought to act as basic units/building blocks, capable of generating oligomers ranging from ca 200 to 600 kDa [49,53]. The various HSPB monomers can form both homo- and hetero-dimers as well as...
Table 1. The mammalian small heat-shock proteins. n.a., not analysed as far as we know; AD, Alzheimer’s disease; ALD, Alexander disease; CJD, Creutzfeldt–Jakob disease; DMPK, dystrophia myotonica-protein kinase; DRG, dorsal root ganglia; MNDs, motor neuron diseases; NPD, Neuman Pick’s disease; PD, Parkinson’s disease; RT qPCR, real-time quantitative polymerase chain reaction.

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<tr>
<th>HSPB name (alt. name)</th>
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<th>biochemical function (as detected in cells after upregulation)</th>
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<td>brain or sciatic nerves</td>
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<td>skeletal muscles</td>
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<td>HSPB4 (aA-cry stallin)</td>
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<td>HSPB9 (CT51)</td>
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<td>HSPB10 (ODFP, ODF1)</td>
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homoeo- and hetero-oligomeric complexes [54,55]. The dynamic association/dissociation of the oligomers has been suggested to be key to the function of the HSPB proteins and is often regulated by their phosphorylation state [49,56]. HSPB proteins are biochemically classified as molecular chaperones and participate in protein quality control; in fact, several HSPB family members have been shown to be able to bind to (partially) unfolded or to misfolded, aggregation-prone proteins [57] preventing their aggregation. In conjunction with ATP-dependent chaperones (e.g. HSP70s/HSPAs), the HSPB-bound clients can either be refolded or degraded; the mechanisms for either refolding or degradation is not fully understood, but may depend both on the state of the client and on the specific HSPB member that is bound to it (see later).

The chaperone activity of small HSPs has been discovered and explored mainly in cell-free experiments with purified proteins [58–60] and it accounts for, for example, the role that HSPB4 plays in maintaining eye transparency [26]. Whether this chaperone function is also underlying other cellular functions of HSPB members is less clear. For example, some HSPB members (e.g. HSPB1 and HSPB5) have the capability to modulate the assembly and stabilization of cytoskeletal components, such as actin and intermediate filaments [22,37,38,41,61–64], but how far these actions rely on their chaperone activity is unknown. It is also not clear whether other HSPB family members serve in cytoskeletal protection or whether different cytoskeletal and contractile elements may require different HSPB members. Other functional endpoints that have been shown to be affected by HSPB members include the maintenance of proper cellular redox state, protecting cells from oxidative stress conditions (HSPB1, [65]), a general anti-apoptotic function (HSPB1, [66,67]) and a role in skeletal muscle cell differentiation (HSPB2 and HSPB3, [25]). The biochemical mechanisms underlying these different cellular effects of the various HSPB members are often still elusive and not always directly linked to the in vitro-defined chaperone-like activities.

Several HSPB family members have been suggested to be protective in a number of neurodegenerative and neuromuscular diseases that are characterized by protein aggregation and axonal transport defects. This directly relates back to the two most postulated actions of these HSPBs: their chaperone action and cytoskeletal stabilizing function, respectively. On the other hand, mutations in some members of the HSPB family (namely, HSPB1, HSPB3, HSPB4, HSPB5 and HSPB8) have been associated with neurological and muscular alterations, suggesting that loss of their function as general chaperones or and cytoskeletal protectors is crucial for maintaining neuronal and muscular cell function and viability. Here, we will discuss the implication of HSPB proteins in neurodegenerative and neuromuscular diseases, focusing our attention on how the differences in the HSPB activities influence their protective functions.

2. The role of HSPBs in neurodegenerative and neuromuscular diseases

(a) Some HSPBs are upregulated in neurodegenerative diseases

The formation and accumulation of insoluble aggregates containing misfolded/mutated proteins is a pathological hallmark of many neurodegenerative and neuromuscular disorders with late onset, including amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD), Parkinson’s disease (PD), polyglutamine (polyQ) diseases (e.g. Huntington disease (HD), spinal and bulbar muscular atrophy (SBMA), etc.), and Creutzfeldt–Jacob disease (CJD). These diseases include both sporadic and genetically inherited forms. Some inherited forms are linked to mutations in a specific protein that misfolds and/or is prone to aggregation. Notably, often the proteinaceous aggregates are found in the corresponding degenerated tissues. Protein aggregation is a multi-step nucleation-dependent process starting from the oligomerization of the self-associating misfolded protein, which generates pre-fibrillar aggregates (detergent-soluble). Pre-fibrillar aggregates can subsequently generate fibrillar structures (detergent-insoluble). During this aggregation process, the mutated protein interacts with and entraps different intracellular components. These include several HSPs (including several HSPB members; [68]), components of the proteasome system, elements involved in vesicular transport and transcription factors. Sequestration of HSPs and proteasomal components may reflect their failure to degrade the misfolded proteins as well as their failure to prevent their aggregation or their unsuccessful attempts to disaggregate the inclusions. As a consequence, the HSPs might be diverted away from their normal functions. Similarly, entrapping of vesicular transport components and transcription factors may lead to their reduced activities in essential neuronal processes. Finally, aggregates might physically impair cellular processes (e.g. axonal transport) [69]. Probably, a combination of these events contributes to disease progression and, therefore, aggregation has long been considered the key pathogenic mechanism. Nevertheless, the precise role of aggregates in neuronal cell death and disease progression is still largely debated. Several studies have suggested that not all forms of aggregates may be toxic [70–72]. In fact, large (amyloidic) aggregates that entrap these various key cellular elements have been found to be protective in certain systems and under certain conditions [70–75]. It has thus been proposed that smaller (amorphic) oligomeric and/or heteromeric species are more toxic as they are capable of freely moving around in the cellular milieu and perturb various neuronal functions. Besides the aforementioned effects of large aggregates (e.g. impairment of cellular proteostasis, alterations of the degradative systems, disruption of axonal transport, dysregulation of the transcription of specific genes), this could also include, for instance direct effects on membrane integrity and/or synaptic functioning [70,73,76,77]. However, irrespective of the presumed differential toxicity attributed to these different aggregates, it is clear that any factor/approach able to decrease mutated protein accumulation, to prevent the initiation of their aggregation or to facilitate the clearance of (early) aggregates will greatly contribute to restore (or maintain) the normal neuronal proteostasis and function, thus slowing down disease progression. This potentially can be achieved by boosting specific molecular chaperones/HSPs (e.g. HSP70s/HSPAs and HSP40s/DNAJs and some sHSPs/HSPBs), which will avoid or attenuate protein aggregation, or by stimulating the degradative pathways (i.e. autophagy), which will help to clear the aggregates. The efficacy of both approaches has been experimentally demonstrated using cellular and/or animal models of aggregate diseases (e.g. polyQ diseases, AD and PD) [19,43,46,78–91].
Here, we will focus on boosting the activity of the HSPB proteins as a potential approach to counteract protein toxicity in neurodegenerative diseases, keeping in mind that not all HSPB family members share the same functional properties [1,92]. Therefore, the putative protective efficacy of the 10 members may largely differ depending on the specific protein causing the aggregation disease.

In addition, the various members present very different tissue/cell-specific distribution. In basal conditions, only some HSPB family members are expressed in the central nervous system (CNS) and very few in neurons (some are mainly confined in glial components of the CNS; table 1). However, misfolded protein expression and the consequent cell stress during neurodegeneration might trigger both the overexpression of some HSPBs already present in target cells or of HSPBs normally silent in neurons (table 1). Thus even the HSPBs normally absent in the brain might become players in the intracellular response to mutant misfolded protein neurotoxicity [92]. Regarding the specific pattern of expression of the HSPB family members, HSPB2 and HSPB3 are mainly expressed in the skeletal muscle cells [25], while HSPB9 and HSPB10 are only found in testis [52]. HSPB7 was originally termed cvHSP and indeed is highly expressed in cardiac tissue, although it is also expressed at lower levels in several other tissues, including the brain [1,92]. HSPB6 is constitutively and highly expressed in smooth, cardiac and skeletal muscles and plays a role in muscle function [93]. The other members (HSPB1, HSPB5 and HSPB8) are highly expressed in muscle tissues, but are also expressed in many other tissues, including in the CNS, with peculiar cell-type-dependent differences in expression (table 1). For example, while HSPB1 is highly expressed in peripheral sensory neurons [94], HSPB8 is highly expressed in motor neurons [17,33,43,44,95]. As it will be discussed later, some members of the HSPB family can prevent aggregation of (some) disease-associated mutant proteins and that is though to be protective in neuronal cells. Thus, HSPB upregulation (both at the level of neuronal and glial cells) might represent a protective cellular response to neuronal damage. However, it is also possible that this over-induction occurs as a consequence of neuronal stress, without an active participation to the protective processes. While the presence of HSPB1 and HSPB5 in ballooned neurons (e.g. in AD, CJD, etc.) could suggest that they interact with aggregates in neurons (either actively engaged in protein quality control here or reflecting failed function or being trapped here), the upregulation of the same proteins in astrocytes is more difficult to understand. But several lines of evidence suggest that misfolded proteins may act in a non-cell autonomous way. For example, the primary toxicity can be exerted on the glial (and microglia) cells surrounding affected neurons, which may thus also be indirectly affected via release of neurotoxic factors from glia or reduced removal of neurotoxic agents by the glia, i.e. through an altered dialogue between these cells [96,97]. Indeed, recent evidence suggests that astrocytes, together with other glial cells (especially microglia), participate in the maintenance of the extracellular milieu containing debris and aggregates from dying neurons. A comparable non-cell autonomous mechanism may play a role in motor neuron diseases, involving interactions between motor neurons and the target muscle cells [98] in which muscle denervation from motor neurons rather than being causative to muscle degeneration also may result as consequence of initial muscle damage/death [99,100]. It can be hypothesized that HSPB8, for example, somehow is involved in this process: in anterior horn spinal cord of ALS mice, HSPB8 is highly induced not only in motor neuron (at very high levels in this primary target of mutant protein toxicity), but also in the glial cells (at lower levels) of the affected regions [43], and in muscle tissues (at the highest levels; P. Rusmini, V. Crippa, E. Giorgetti, A. Boncoraglio, R. Cristofani, A. Poletti 2013, unpublished data). Studies should therefore not only focus on elucidating the role of HSPBs in individual populations of cells, but also look at cell non-autonomous and cell–cell interactions.

Finally, low or even a complete lack of constitutive and induced expression in target cells of individual HSPB members that did reveal protective power in cell models of disease should not be a reason to imply that these members cannot exert protective functions in disease. Obviously, such members are not part of the canonic intrinsic (neuronal/glial/muscular) response to the diseased protein, but could be still considered potential therapeutic targets if one were able to induce them by pharmacological means, with the specific aim to assist mutant neurotoxic protein clearance.

3. Refolding and anti-aggregation are distinct properties of the HSPBs

As previously mentioned, a number of neurodegenerative diseases are characterized by the progressive accumulation of aggregation-prone proteins. Upregulation of specific members of the HSPB family could slow down or completely inhibit the aggregation process in cell models [43,46–48,92,95,101], with significant differences among the 10 HSPB family members. In particular, using mutated polyQ-containing proteins (e.g. huntingtin and ataxin-3) we showed that HSPB6, HSPB7, HSPB8 and HSPB9 overexpression inhibited protein aggregation and protected against its mediated toxicity, while overexpression of all the other members had no effect [1]. Curiously, HSPB6, HSPB7, HSPB8 and HSPB9 could not efficiently facilitate the refolding of denatured substrates (e.g. heat-denatured firefly luciferase), but rather were linked to protein degradation by either the proteasomal or the autophagic systems (table 1; see also later, [1,46]). Conversely, the strongly heat-stress-regulated HSPB1, HSPB4 and HSPB5 members, which were found to be very efficient in facilitating the refolding of heat-denatured substrates, both in cells and in vitro, showed poor anti-aggregation effects against polyQ proteins (table 1) [1]. These data suggest that the different HSPB members may either have different client specificity and/or have a different impact on client processing.

Despite the lack of anti-polyQ aggregation activity, HSPB1 and HSPB5 showed some protective effects in some cell models of polyQ disease. As HSPB1 and HSPB5 can increase the resistance of the cytoskeletal network [22,37,38,41,62–64] and are upregulated both in ballooned neurons and astrocytes [9,27,28], one may speculate that they protect axonal transport and vesicular trafficking against disruption by the protein aggregates and thus delay the consequences of aggregation, without affecting aggregate formation as such. Yet, when directly comparing the cytoprotective effects of, for example, HSPB5 to that of HSPB7 in cell models, we found HSPB5 protection was marginal ([1], figure 1c).
Consistent with the cellular data, the three most potent suppressors of polyQ aggregation (HSPB7, HSPB8 and HSPB9) also reduced polyQ-mediated eye degeneration in a Drosophila melanogaster in vivo model [1,101] (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data; figure 1). These latter three HSPB members have been implied in polyQ degradation via the proteasome (HSPB9; M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data), by supporting autophagic clearance of polyQ aggregates [1] or by enhancing autophagic flux [43,46,95,101,102], see below).

4. Anti-aggregation activity of HSPBs: stimulating degradative pathways

As mentioned earlier, HSPB7, HSPB8 and HSPB9 seem to have the potential to trigger (or facilitate) proteasomal and/or autophagic degradation of certain misfolded proteins. Among these HSPB members, HSPB8 is probably the most studied in this context. Overexpression of HSPB8 efficiently prevents aggregation of different polyQ-containing proteins (huntingtin, ataxin-3 and androgen receptor (AR), responsible for HD, spinocerebellar ataxia type 3 (SCA3) and SBMA, respectively) [1,47,101], as well as SOD1 (protein responsible for ALS) and various truncated forms of TDP-43s (associated with both ALS and frontotemporal dementia (FTD; table 1)) [43,95].

Figure 2 illustrates this for the mutant ARpolyQ that causes SBMA. Overexpression of HSPB8 in SBMA motor neurons leads to a substantial decrease in mutant ARpolyQ aggregates (IF, figure 2a) and large insoluble species (filter retardation assay, FRA; figure 2b,c), which in this disease are triggered by the AR ligand testosterone. Several data indicate that this anti-aggregation activity of HSPB8 is caused by a facilitation of autophagy-mediated degradation of the mutated proteins or their (initial) aggregates. In this process, HSPB8 collaborates with BAG3 [46] and HSPA8 (Hsc70) and CHIP [43,104]. Indeed, as shown in figure 2, the anti-aggregation effects of HSPB8 were greatly reduced in the presence of the autophagy inhibitors 3-methyladenine (3-MA) or bafilomycin (figure 2c).

Differently from many other members of the HSPB family (e.g. HSPB1 and HSPB7), HSPB8 forms a stable complex with the HSPA8 (Hsc70) co-chaperone BAG3, which may explain why HSPB8 is so efficient in autophagy-mediated degradation of misfolded polyQ substrates. We previously showed that not only HSPB8 stability, but also its anti-aggregation and degradative functions, depend on its association with BAG3, whose knock-down prevented HSPB8 from exerting its protective role [46]. Also, within the complex, BAG3, but not HSPB8, is responsible for the stimulation of autophagy [46]. This suggests that HSPB8 might play a role in the recognition and delivery of the cargo, via BAG3, to the autophagosomes for degradation. In addition, we found that HSPB8 (in complex with BAG3), besides participating in autophagy-mediated degradation of misfolded proteins, is also involved in the translational shut-down mediated by the induced phosphorylation of eIF2 alpha that occurs during proteotoxic stress. Interestingly, induction of phospho-eIF2 alpha, which we observed upon overexpression of HSPB8, BAG3 or of the complex, leads to both protein synthesis inhibition (which decreases the total load of proteins to be refolded or degraded) and autophagy stimulation (which clears the aggregated proteins accumulating during proteotoxic stress) [48]. While in vitro translation experiments indeed have revealed that HSPB8 can cause translational shut-down, precise insight into how HSPB8 can modulate translation upon stress is still missing. Similarly, future studies will be needed to reveal the precise role of HSPB8 in the complex and if and how client recognition and targeting to autophagy is coupled to its action on translation.

5. The anti-aggregation power of HSPBs depends on several factors

The aggregation propensity of misfolded proteins depends on several factors, including the exposition of hydrophobic residues, the alternation of specific conformed domains, the capability to generate beta-plated sheets, and so on. These factors may affect the kinetics of aggregation, the biophysical nature of aggregates that are ultimately formed (e.g. insolubility and reversibility) and also the location inside the cells where these proteins may accumulate [105]. All of these may obviously also affect the possibilities of diverse HSPBs to interact and deal with these various proteins before or after they have aggregated. Even for apparently similar clients like the proteolytic polyQ-containing fragments, the length of the polyQ stretch and the kinetics of
aggregation (the longer the polyQ repeat, the faster the aggregation \cite{1}) were found to impede the possibility of the various HSPB members to suppress aggregation. While HSPB8 and HSPB9 were equally (or even a bit more) effective compared with HSPB7 on huntingtin fragments with relatively short expansions (43Q, figure 1 \cite{1}) only HSPB7 was efficient in suppressing aggregation of the longest expansions tested (119Q; see \cite{1}). For HSPB8, similar observations were made in our previous findings showing that HSPB8 efficiently decreases both soluble and insoluble levels of huntingtin fragments with relatively short expansions (43Q; \cite{46,47}), but has no effect on long expansions (Q119; \cite{1}). We also tested the selective effect of HSPB8 on ARpolyQ containing a stretch of different size (Q46 versus Q112), both being in the pathological range (even if no SBMA patients with Q112 have been described so far). The two types of ARpolyQs have a marked difference in their aggregation power, in response to the AR ligand \cite{72,73}. While HSPB8 almost completely counteracts AR.Q46 aggregation, induced by testosterone, its effects on AR.Q112 are much lower, since there is only a relatively small decrease in the total amount of insoluble ARpolyQ (figure 2b).

These differences in the efficiency of HSPB7, on the one hand, and HSPB8 and HSPB9, on the other hand, to prevent aggregation of polyQ proteins with different expansion sizes clearly suggests that their modes of action to deal with these disease-related proteins must be largely different. HSPB8 and also HSPB9 (M. P. Zijlstra, B. Kanon, H. H. Kampina 2011, unpublished data) seem to act by lowering the level of soluble species (non-aggregated or/and early aggregate intermediates) apparently maintaining these substrates in a state competent
for degradation, thus disposing of them before they form large, insoluble species. In the case of HSPB8, the lowered levels of soluble mutant polyQ proteins might be due, at least in part, to its effects on eIF2 alpha phosphorylation and translational attenuation [48]. Also, HSPB8, together with the co-chaperone BAG3, the chaperone HSP70 and the ubiquitine-ligase CHIP, works through stimulating autophagic degradation [43,46,48,106], thus also taking care of early nucleating species. Concerning HSPB9, it was found to stimulate proteasomal degradation (M. P. Zijlstra, B. Kanon, H. H. Kampinga 2011, unpublished data), which may lower the number of nucleating species of the polyQ proteins; however, when nucleation is initiated, which occurs more readily with longer polyQs, HSPB9 might become ineffective. Since HSPB9 is only expressed in testis, HSPB9 upregulation should not play a role in neurodegenerative diseases. However, as stated before, it is important to underline that pharmacologically induced upregulation of specific members of the HSPB family (normally not expressed or upregulated) in a specific neuronal cell type might still result in protection, therefore representing a good therapeutic approach. For those HSPB members that are already expressed in target cells, drugs may be designed that stimulate their (chaperone-like) activity, e.g. by acting on the phosphorylation or oligomeric status of the HSPB members. Alternatively, or in case HSPB members are not already expressed in the target cells, analysis on expression regulation of the various HSPB members combined with drug screens using reporter constructs may identify routes towards boosting or inducing expression of individual disease-ameliorating members.

HSPB6, which only effectively suppresses shorter polyQs [1,107], also plays a role in modulating autophagy [108]. By contrast, HSPB7 does not change the rate of proteasomal degradation and does not increase the autophagic flux; rather, it appears to prevent early aggregates from nucleating into inclusions with sizes that are too large to be handled by the autophagic machinery, probably by marking these early seeds, which enables their shuttling into the autolysosomes [1,57,109,110]. This HSPB7 action thus does not rely on the speed of seed formation but rather on the rate at which these seeds grow (and thus is less dependent on the size of the expansion).

Another aspect that might influence the efficacy of the diverse HSPBs to prevent mutated protein aggregation is represented by specific physical properties of the mutant protein and aggregate itself. Indeed, the effects of HSPB members on different aggregation-causing mutants seem to differ widely (table 1). It has been shown that, while polyQ proteins form aggregates with a core that is inaccessible to nascent proteins, mutated SOD1 (G85R/G93A), associated with ALS, forms a porous aggregate, through which nascent proteins can diffuse [110]. As stated above, we have already shown that overexpression of HSPB8 efficiently prevented the aggregation and facilitated the autophagy-mediated degradation of mutated SOD1 and of various mutated forms of TDP43, which is associated with both ALS and FTD [43] (figure 3b). When we compared the HSPB8 activity to that of other HSPB family members with anti-aggregation activity towards mutated polyQ proteins, namely HSPB6, HSPB7 and HSPB9, none of these blocked the aggregation of the truncated mutant form of TDP43 (TDP43 ΔC; figure 3b: HSPB1-5-6-7-9; table 1). HSPB1 and HSPB5, which mainly showed pro-refolding activity, were also unable to prevent aggregation of TDP43 ΔC (figure 3b). HSPB9 seems to have a mild effect on TDP-43 ΔC. Why HSPB6 and HSPB7 showed no anti-aggregation activity towards TDP43 ΔC, while being very active towards mutated polyQ proteins [1,107] (table 1), is still unclear and will be investigated in the future.

Finally, another factor that can explain the differential efficacy of the various members of the HSPB proteins in inhibiting protein aggregation is the stage at which they act. As previously mentioned, many neurodegenerative diseases, including AD, polyQ diseases and PD, are characterized by the accumulation of fibrillar proteinaceous aggregates in specific neuronal types [111–117]. The formation of these fibrillar aggregates consists of a multi-step process involving a nucleation step and the subsequent elongation of the fibrils [118]. Interestingly, it has been shown that some HSPBs can only prevent protein aggregation at a specific stage. For example, HSPB5, which showed very high refolding capacity in cells and in vitro but no anti-aggregation activity towards mutated polyQ proteins [1], inhibits fibrillar aggregate formation of mutated alpha-synuclein, associated with PD, only at early stages. HSPB5 binds to partially folded monomers of mutated alpha-synuclein, thereby preventing mature fibril formation and shifting the equilibrium to monomer fibrils, which can be easily disposed/degarded [35,119,120]. A similar mechanism has been shown for ataxin-3. HSPB5 can significantly inhibit the first stage of...
ataxin-3 aggregation [121], by directly interacting with the Josephin domain of ataxin-3, which has an intrinsic tendency to aggregate and form fibrils [122]; however, HSPB5 is ineffective on already formed SDS-insoluble fibrils of mutated ataxin-3. All together, these data strongly suggest that HSPB5 acts as a chaperone specifically towards growing fibrils at an early stage and may have only limited protective powers because it cannot block protein aggregation at later stages and does not seem to target its bound substrate to the proteolytic pathways. They also suggest that, in order to be efficient in inhibiting protein aggregation, upregulation of HSPB5 should either take place at a very early stage of disease or occur concomitantly with the upregulation of other chaperones able to bind to and target intermediate species and/or fibrils to degradation.

6. Subcellular localization of the aggregate-prone species and HSPBs

Finally, subcellular localization can influence the efficiency of the various members of the HSPB family in inhibiting the aggregation of a specific misfolded substrate involved in neurodegenerative diseases. In fact, while the ubiquitin proteasome system is present and active both in the nucleus and in the cytoplasm, autophagy (which can be facilitated by some HSPB members; see earlier) is confined to the cytoplasm [123,124]. HSPB8 and HSPB6, which mainly rely on autophagy, are expected to mainly act on cytoplasmic aggregating proteins. Indeed, HSPC8 could only efficiently block the aggregation and facilitate the autophagy-mediated clearance of ARQ46, which is located in the cytosol, but had very limited effect on ARQ112, which is aggregating inside the nucleus. Considering that the cytoplasmic retention of a mutated form of AR containing a Q112 tract ameliorates disease via autophagy [125], strategies that allow keeping the mutated proteins in the cytosol, where several HSPBs (and other molecular chaperones) can participate directly or indirectly in their targeting to the autophagosomes for degradation, will slow-down disease progression. Whether some HSPBs can also participate in modulating the shuttling of the mutated proteins from the nucleus to the cytoplasm and whether this may contribute to their protective effects is still unknown.

7. HSPB-mediated protection can also be independent of anti-aggregation/pro-refolding activities and/or autophagy facilitation

HSPB1, which shows good refolding capacity, had no anti-aggregation activity in cells against mutated huntingtin, both with short and long polyQ stretches [1] (table 1). However, overexpression of HSPB1 could inhibit the aggregation of mutated SOD1 [20] (table 1) and could also prevent the toxicity mediated by several polyQ-containing proteins [19]. This may depend on the different physical properties of the aggregating substrates (e.g. immobile versus mobile aggregates), as stated before, although so far no experimental proof supporting such a hypothesis exists. However, in the case of HSPB1, the protective effects may also be due to other specific functions, which are not related to refolding or inhibition of aggregation, but rather to its ability to prevent the activation of APAF-1 by the cytochrome C released from the mitochondria, that will trigger the apoptotic caspase cascade, as well as on its anti-oxidants effects [67]. In fact, overexpression of HSPB1 exerts a protective effect and significantly decreases cell death in cellular models of HD, characterized by high oxidative stress, by maintaining the redox state of the cell without showing effects on aggregation [19]. Moreover, in neuronal-like cells, HSPB1 could also protect against the toxicity mediated by mutated ataxin-3, associated with SCA3, where increased oxidative stress has been suggested to play a role in disease pathogenesis, again without effects on aggregates. Interestingly, overexpression of mutated ataxin-3 correlated with a reduction in the expression levels of HSPB1, suggesting that a decrease in the function of HSPB1 (not directly related to effects on the diseased protein as such) may play a role in disease progression. Such a decrease in HSPB1 expression has also been documented in other forms of SCA diseases, like SCA7 [126,127] and in transgenic mice models of SCA-17 [128]. This would imply that re-introduction of HSPB1 can compensate for such a loss of function of HSPB1 as a factor contributing to disease progression. However, data in HD mice did not reveal such an effect [129], suggesting that this may not apply to all polyQ diseases.

8. Conclusions and perspectives

Although similar in terms of primary sequence, the various members of the mammalian HSPB family are differentially expressed in tissues and cells, have different abilities to form homo- and hetero-oligomers, show other non-HSPB partner interactions and display different functions. The tissue-selective expression pattern of the members probably reflects a highly specific need of a given HSPB to assure proper function and viability of that cell type/tissue, and a particular HSPB may exert a protective function in a specific cell type. Some functional redundancy does exist between the various members, as evidenced by findings that several HSPB members can handle the same (un- or misfolded) protein equally (e.g. assist their (re)folding). However, HSPB members posses different affinity and specificity for clients and may handle the same client differently (e.g. routing them to proteasomal or autophagosomal degradation). Thus, depending on their client specificity and mechanism of action, only upregulation of specific HSPB members would exert protective functions against neurodegeneration. Moreover, impaired HSPB function may harm certain tissues more than others and explain why certain HSPB mutants have been linked to tissue-specific (e.g. motor neurons and muscle cells) degeneration. In addition, the same HSPB may have different biochemical activities: depending on their oligomeric status, they may independently function as chaperones for soluble proteins, as stabilizers/chaperones for cytoskeletal elements, or as modifiers of the cellular redox state. As a consequence of these properties, the same HSPB member may protect more or less efficiently and certain HSPB members may be better targets than others.

In general, a better understanding of the client specificity and functional diversity of the various HSPBs will be required in order to target a specific member or functionality thereof for therapeutic purposes.
References


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