General discussion & perspectives

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Screen & basic concept

Cells constantly have to deal with acute stresses caused for instance by changes in temperature, chemical or physical stress as well as chronic stresses such as the continuous expression of mutated proteins caused by heritable diseases. Life has evolved a protective mechanism to sense and deal with (stress-induced) protein unfolding called the heat shock response. This response consists of the induced expression of several proteins (heat shock proteins or molecular chaperones) whose role it is to assist in protein folding, stabilization and degradation (1;2). The simultaneous transcription of these various genes is brought about by the heat shock transcription factor 1 (HSF-1) (3). In general, higher eukaryotes are found to express multiple HSF genes which regulate gene expression of non-heat shock proteins as well. Regulation and alternative splicing of these HSFs introduces an even more elaborate signaling pathway, linking them to development, tissue-specific expression and reproduction (4-7).

Small heat shock proteins (small HSP) make up one node in the protein folding network. The ability of at least some members to de-oligomerize in response to stress allows them to instantly provide the cell with binding surfaces for non-native protein conformations without the need for (HSF-1 regulated) enhanced expression. Besides their normal level of expression, some of these members can in addition be elevated through HSF-1. In the preceding chapters several additional aspects of both human and Drosophila small heat shock protein families have been addressed. The described research offers a glimpse on the functional specialization and activities of small HSPs in these two species. Although separated by evolution, similar distributions in molecular activities can be observed. Both species contain members specialized in assisting refolding of (heat) denatured proteins (here referred to as “refolders”) while other members seem specialized in reducing aggregation of misfolded proteins, especially polyQ containing proteins (here referred to as “anti-Q”) (Figure 1a,b). Whereas some refolders can also exhibit (some) anti-Q activity (human HSPB4, Drosophila melanogaster (Dm) CG7409), the best anti-Q small HSP proteins did not show effects on refolding of heat-denatured substrates like luciferase (Figure 1a,b). Clearly, anti-Q small HSP members (human HSPB7, HSPB8, HSPB9; DmHSP67Bc) have evolved to specifically deal with non-refoldable substrates like polyQ proteins and maybe other amyloid forming substrates. When combining this data from both species, it is clear that small HSPs can be divided in three main groups based on their activities (Figure 1c, Chapters 4 & 5). Group one consists of highly active refolders (HSPB1, HSPB5, DmHSP27, DmCG14207, DmL2EFL) with no activity against polyQ aggregation, group two consists of highly active anti-Q proteins (HSPB7, HSPB9, HSPB8, DmHSP67Bc,) where some members are linked to protein degradation. Group two members show no activity on luciferase refolding and group three contains small HSPs with combined activities (HSPB4, HSPB6, DmHSP23, DmHSP26, DmCG7409). As this functional distribution is observed within two different species, this likely reflects involvement of small HSPs in conserved parts of the protein homeostasis network with specialization of individual members on proteins folding, anti-aggregation and protein degradation.

Whereas small HSPs appear to differ in their type of chaperone-like action, most of them have been reported to associate with cytoskeletal elements (Chapter 1, table 3). Intriguingly, several neuropathies and myopathies are linked to mutations in HSPB1, HSPB5 and HSPB8, affecting cell types and tissues that are highly dependent on cytoskeletal transport and functioning. In fact, neuronal and muscular tissues generally show a large diversity of HSPB expression, with relative high levels of HSPB5, HSPB6, HSPB7 and HSPB8 compared to other tissues like liver and lung (Figure 2). Apparently, neuronal and muscular tissues require a more elaborate small HSP subset, with devastating effects when this balance is disturbed by mutations in HSPB1, HSPB5 or HSPB8. For the other two HSPB members, HSPB6 and HSPB7, no link has yet been established between possible mutation and disease. To obtain data on HSPB6 and HSPB7, and
other less studied HSPB members, we analyzed their subcellular localization and consulted databases containing data on tissue expression and interactions between individual members. This revealed that especially HSPB7 has a remarkable localization pattern and is one of the most active HSPB members in preventing polyQ aggregation (Chapter 4).

**Different faces of HSPB7**

**HSPB7 and splicing speckles**

We identified HSPB7 as a cytoplasmic and nuclear protein with constitutive localization to SC35 splicing speckles. Within the nucleus, SC35 speckles are part of the highly organized nuclear space and together with promyelocytic leukaemia (PML) nuclear bodies (8) and Cajal bodies (CB) form the small nuclear domains (9). Speckles are rich in splicing-related proteins, linking them to RNA processing and splicing (10). In addition to HSPB7, also HSPB1 and HSPB5 have been reported to associate with SC35 speckles under either stressful conditions or in a constitutive manner respectively (11-14).

As splicing is extremely sensitive to heat shock (15;16), the involvement of heat shock proteins in safeguarding this cellular process seems obvious. Indeed, yeast HSP104 and HSP70 have been reported to enhance recovery of splicing after a heat shock (17). While HSPB1 has been directly linked to restoration of splicing activity after stress, a role of HSPB5 in splicing was not found (18). In addition to the faster recovery of splicing after a heat shock, HSPB1, yeast HSP104 and yeast HSP70 are reported to be efficient chaperones in preventing protein aggregation and confer refolding of non-native substrates (19-21). This suggests that these proteins support post-heat shock splicing activity via protection of other speckle-associated pro-
teins against heat-induced unfolding. Also HSPB1 and HSPB5 are highly active refolders in the cytoplasm and nucleus (Chapter 3, Chapter 4, Figure 1a), which might as well be extendable to proteins in SC35 speckles. HSPB7, however, is unable to assist in the refolding of heat-denatured luciferase in both cellular compartments (Chapter 3, Chapter 4, Figure 1a) which suggest that this HSPB protein functions in a different way than HSPB1 and HSPB5. These different molecular activities could suggest an intricate regulatory mechanism whereby HSPB1 is constitutively associated with protein folding in the cytoplasm and only during proteotoxic stress enters the nucleus to both restrict protein unfolding and support splicing activity. HSPB5, on the other hand, does not only seem to serve a constitutive role in protein folding in the cytoplasm but in addition may be involved in protein folding in the nucleus (Chapter 3 &4) and possibly at SC35 splicing speckles. HSPB7, not active in the process of protein folding in the cytoplasm or nucleus, seems to fulfill a constitutive role at splicing speckles unrelated to protein folding. Albeit not experimentally verified yet, HSPB7 could have a novel function in the splicing process as is discussed below.

The N-terminus of HSPB7 is required and sufficient for localization to SC35 speckles. Notably, the N-terminus contains a serine-rich stretch which could resemble an arginine-serine-rich (RS)-like domain. The RS domain is present in various SC35 speckle resident proteins and can play a role in protein-protein interaction as well as in RNA binding (22;23). The RS-domain is required for interaction with other splicing factors that also contain an RS domain. This leads to the formation of high molecular weight complexes in a phosphorylation-dependent manner (24). It is tempting to suggest that RS-domain containing proteins not only allow association of SC35 speckles with RNA but by binding also serve a chaperone-like function in preventing single stranded RNA from forming stable folds. Furthermore, the apparent similarity between SR-proteins and HSPB members concerning phosphorylation dependent oligomer formation is striking and as such, a fraction of HSPB7 might form mixed oligomers with other SR-proteins. Our data suggest, however, that HSPB7 does not form large oligomeric complexes like HSPB1 and HSPB5 nor forms mixed oligomers of HSPB7 with other HSPB proteins in living cells. This implies that at speckles, HSPB7 functionality is not determined by the association with either HSPB1 (during stress) or HSPB5.
**RNA chaperones**

So, what could be the functional relevance of the association of HSPB members with SC35 speckles. As stated above, speckles are linked to RNA processing and splicing (10) and in addition speckles have been reported to localize to genomic regions with high transcriptional activity (25;26) which was shown to be independent on the presence of introns (27). Thus, splicing speckles seem also involved in transcription where splicing activity is not required. Here, SC35 splicing speckles and their associated proteins, could facilitate additional biological activity concerning pre-mRNA synthesis, stabilization or transport. In view of this, HSPB1 might associate with speckles during heat shock to induce binding specificity of the splicing machinery for (heat shock induced) destabilization of RNAs. Sinsimer and colleagues showed that HSPB1 can bind destabilizing AU-rich elements present in RNA (28). Whether HSPB7 can bind RNA molecules as well has yet to be determined. However, not all pre-mRNAs associate with SC35 speckles (29) implying either structural requirements present in RNA to associate with speckles or specificity of SC35 speckles defined by its associated proteins.

Lin and colleagues recently found that SC35 might act as a single-strand RNA binding protein to facilitate transcriptional elongation (30). In addition, binding of speckle components to RNA might provide stabilization and prevent RNA misfolding which could potentially slow down the splicing- and translation process. In this way, the cell ensures proper trafficking from transcription to translation. This so called RNA chaperone activity has been described for several other proteins (31;32). They are able to promote RNA folding by resolving the complex tertiary structure of RNAs or prevent their formation (33). Such RNA chaperones include the stress-granules localized fragile X mental retardation protein FMRP (34;35) and hnRNP A1 (36;37), the DNA associated YB-1 (38;39) and DDX15 which localizes to nuclear speckles (40).

Furthermore, RNA misfolding and the formation of RNA aggregates could play a role in the development of disease. For instance, myotonic dystrophy (MD) has been shown to be caused by a CTG triplet expansion in the 3' untranslated region (3' UTR) of a gene encoding a serine-threonine protein kinase (DMPK) (41). Proposed mechanisms leading to MD pathogenesis include changes in mRNA metabolism and transport of DMPK mRNA and mRNA aggregation into foci (42-44). RNA foci are also observed for Huntington disease-like 2 (HDL2), which shares the pathological hallmarks of Huntington disease (HD) (45;46). In this case, a CTG/CAG extended repeat, is found in a variably spliced exon of the junctophilin-3 gene. In both cases, other mRNA species might co-aggregate in RNA-containing foci leading to a disruption of cellular homeostasis and a concurrent sensitivity for internal and external stressors. It is thus likely that SC35 speckles not only constitute the splicing machinery but in addition also contain components for RNA chaperoning needed to loosen tertiary RNA structures. The specific and constitutive localization of HSPB7 to SC35 speckles, as we demonstrated in Chapter 3, might reflect an additional biological function of this small heat shock protein involved in RNA chaperoning.

**HSPB7 and anti-Q activity**

Certain HSPB proteins have already been described before to be effective in preventing protein aggregation (47-49) probably by providing an interaction surface for hydrophobic protein stretches (48). Mutations in both HSPB5 and HSPB8 are causative for myopathy and neuropathy respectively (50-53). Furthermore, HSPB members have been reported to be upregulated in protein folding-related diseases (54;55). Thus it seems that the activity of these HSPB proteins is of key importance to guarantee a healthy cellular state and can be used to deal with high levels of proteotoxic stress. To identify the most potent protectors against misfolded proteins, we compared the human HSPB members for their (differential) capacities in anti-aggregation and renaturation using either amlyloid aggregated polyglutamine (polyQ) proteins or heat-denatured luciferase (Chapter 4). Using several polyglutamine lengths, which are correlated to aggregation rates (56), we found, HSPB6, HSPB8, HSPB9 and especially HSPB7 as the most active...
in preventing aggregation of polyQ proteins. At least HSPB6, HSPB7 and HSPB8 differ from the classical HSPB members HSPB1, HSPB4 and HSPB5 in that they do not form large oligomeric complexes in living cells (Chapter 1, Chapter 4). Oligomer dynamics have been suggested to be crucial for HSPB members to promote chaperone activity. This makes it puzzling why HSPB7, the most active HSPB member towards long polyQ stretches, apparently does not oligomerize in living cells. Whereas the precise mode of action remains to be elucidated, HSPB7 might prevent polyQ aggregation in both the cytoplasm and nucleus by shielding the polyQ stretch as has been described for yeast HSP70 and HSP40 resulting in detergent soluble inclusions (57). We were however unable to co-immunoprecipitate polyQ expanded Huntingtin and HSPB7 (data not shown) which could suggest an effect of HSPB7 on preventing polyQ aggregation independent from direct substrate interaction. Support for an indirect action of HSPB7 was further obtained by targeting HSPB7 to either the cytoplasm or nucleus using a nuclear export signal (NES) or nuclear localization signal (NLS) respectively (Figure 3a). Interestingly, targeting of HSPB7 to the nucleus resulted in anti-Q activity in both cellular compartments (Figure 3b). This would suggest that a biological activity of HSPB7 within the cells nucleus is sufficient to prevent polyQ aggregation in the cytoplasm.

As discussed in the previous paragraph, triplet extensions in mRNA can potentially form mRNA foci and deregulate normal mRNA processing. As HSPB7 is localized to SC35 speckles, and thus has a potential role in mRNA biology, it could protect triplet expanded mRNA species from entangling with other RNAs and thereby maintaining RNA and protein homeostasis. In this way, key cellular systems would be maintained which are able to deal with polyQ expanded proteins and accompanying proteotoxic stresses. As HSPB7 was effective in cells lacking a normal heat shock response (Chapter 4) these key systems seem not to involve HSF-1 dependent chaperones. Also, as HSPB7 did not lead to detectable changes in polyQ protein expression, its putative role in RNA homeostasis also did not seem due to reduced RNA processing of the CAG-repeat containing messengers. One option is that its action at the RNA level may somehow impact autophagy, as the effects of HSPB7 were significantly reduced in autophagy deficient cells. Clearly, much more biochemical evidence is needed to further unravel whether any of these speculations are valid.

**HSPB7, one function or moonlighting?**

Another question that arises from all our observation is whether the described activities of HSPB7 are functionally linked, or whether simply reflect different functions of HSPB7 depending on subcellular localization or/and available substrates. Multiple functions executed by one protein is a common phenomenon in biology known as gene sharing or moonlighting (58-61) which effectively extends the functional repertoire of the proteome. Protein function can change...
as a result of differences in sub-cellular localization. These include amongst others, oligomeric state or concentration of a substrate or co-factor and expression within a developmental context (58). For instance, localization-induced functional changes were reported for the Salmonella typhimurium PutA protein (62;63). Association with the plasma membrane enables proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase activity while PutA functions as a transcriptional repressor lacking enzymatic activity when present in the cytoplasm. It can thus not be ruled out that HSPB7 performs anti-aggregation functions in both the cytoplasm and the nucleus while a fraction of HSPB7 associates with SC35 speckles to perform other functions related to, for instance, RNA processing. This is currently under investigation.

**Small HSPs & protein homeostasis**

As stated before, small HSPs can be divided into refolders and anti-Q proteins which both feed into cellular protein homeostasis. Whereas refolders generally show the formation of oligomers and high molecular weight complexes containing bound substrate, anti-Q small HSPs seem to exist mainly as dimers. Based on this difference, two models can be extrapolated. As dimers, the anti-Q HSPB proteins indirectly affect polyQ aggregation by either RNA processing or translational repression. They thus act in a classical chaperone independent manner. Classical oligomeric HSPB proteins on the other hand act in a HSPA-dependent way in substrate transfer and release to refold denatured substrate. This latter function would allow efficient refolding activity but low anti-Q activity. In the other model, dimeric anti-Q HSPB proteins do associate with substrates (Figure 4), but in a high affinity manner. This makes HSPA transfer unlikely. HSPA refolding cycles thus do not occur, decreasing classical chaperone activity of these members. The oligomeric HSPB members on the other hand have a lower affinity to their substrates which allows transfer to the HSPA refolding system. In either case, chaperone activity towards either polyQ stretches, unfolded proteins or aggregation-prone RNA will result in protein homeostasis.

**Different chaperone pathways**

Whereas both refolders and anti-Q proteins are able to bind to the cytoskeleton (Figure 4), processing of non-native proteins appears to follow different pathways: refolders collaborate with HSPA members to refold substrate or target them for degradation through the proteasome while anti-Q small HSPs reduce polyQ aggregation followed by processing to the cellular degradation pathways either via the proteasome (HSPB9, Vos, Zijlstra et al. unpublished results) or via autophagy (HSPB8 and maybe HSPB7;64). Failure of direct processing of either substrate can result in transport along the cytoskeleton towards the microtubule organizing center to form aggresomes that later may be further processed by autophagic degradation. Whereas we can separate HSPB members functionally in this manner, this may not be the entire story and part of the processing of unfolded substrates may in fact depend on the substrates themselves (rather than the chaperone per se). HSPB5 for instance, is able to reduce amyloid fibril formation of certain substrates in vitro but was unable to reduce polyQ aggregation in vivo in our screen. Furthermore, via SC35 speckle association some HSPB members (HSPB1, HSPB5, HSPB7) may also affect protein homeostasis through additional biologic functionalities like RNA processing. The possibility of mixed small HSP oligomer formation could further modify substrate specificity and small HSP activity. Nevertheless, both pathways described above (refolding & anti-Q) can result in protein homeostasis and provide longevity on an organismal level.
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Figure 4. Model of small HSP activities based on the human HSPB family. Small HSPs can be divided into either refolders or anti-Q proteins with a main difference in oligomeric structure. Both types of small HSPs can however associate with the cytoskeleton for either stabilization, cytoskeletal dynamics or involvement in cytoskeletal transport. Deoligomerization (a) of refolders allows some small HSP members (HSPB1, HSPB5) to enter the nucleus and (b) associate, like anti-Q HSPB7, with SC35 splicing speckles. During protein unfolding (c), refolders bind the non-native substrate and associate into large substrate-HSPB complexes. Further processing can either result in proteosomal degradation or transfer to HSPA members (d) for substrate refolding. Anti-Q HSPB members likely also associate with the non-foldable substrate and subsequently target the substrate for degradation by using proteosomal or autophagic clearance. Failure of substrate refolding or clearance results in cytoskeletal transport of the non-native substrate to the microtule organizing center for subsequent degradation. Both single activities (refolding, anti-Q) or a combination of both can support protein homeostasis and provide longevity in Drosophila. See Appendix 4.
**From protein homeostasis to longevity**

The protective effect of small heat shock proteins in cells on protein aggregation and protein unfolding can be extended towards organisms as well. Here, support of the protein degradation and folding pathways can result in protective effects during aging, leading to an increase in lifespan.

Aging has long been attributed to damage caused by free radicals resulting in oxidative damage to the proteome, genome and other cellular components (65). This theory was further extended by including production of reactive oxygen species (ROS) by mitochondria as a major source of oxidative damage (66). Besides this more general insult, genome instability has also been proposed to contribute to the aging process (67). Genome instability is, in part, also the result of oxidative damage to DNA but is in addition burdened by mutations in proteins required for DNA replication, DNA repair and telomere shortening and replication stress (68). Although reducing oxidative stress by overexpression of Cu/Zn-superoxide dismutase resulted in an extended lifespan (69), little evidence has been provided that boosting DNA damage repair pathways can enhance longevity, except for one report in which overexpression of the ATM gene, that is mutated in Ataxia Telangiectasia, had a minor effect on life span (70). However, in the experiments it was not demonstrated that this was due to improved DNA repair. Furthermore, aging is paralleled by an increase in damaged and misfolded proteins leading to cellular dysfunction. Although cells are capable to suppress proteotoxic stress via asymmetrical division of protein damage during mitosis (71) and via cellular protein quality-control systems, including small HSPs, these pathways fail at saturating levels of protein damage (Figure 5, left panel).

Extending the lifespan of an organism can be achieved by a simple intervention: reducing food-intake which is referred to as calorific restriction (CR) (72-74). CR reduces metabolism and hereby ROS production is reduced, partially explaining the lifespan effects. CR leads to activation of insulin-like signaling pathway in which the chromatin silencing SirTuins (SIRT) (75;76) play a central role. (75;77). Intriguingly, whereas SIRT1 deacetylates and inhibits key transcription factors like nuclear factor-κB (NF-κB) (78), p53 (79) and FOXO (forkhead) transcription factors (80), CR induced longevity has also been associated with sustained activation of HSF-1 (81;82), the transcription factor regulating the stress-inducible heat shock response and is linked to overexpression of HSPs (83). In parallel, such manipulations reduced neurodegeneration as caused by oxidative stress.

**Figure 5. Aging, protein homeostasis and small HSPs.** During aging, protein damage increases with a concurrent decline of the efficiency of safeguarding protein homeostasis. In time, the amount of non-native proteins (proteotoxic stress) increases which can be divided into either refoldable substrates or non-refoldable substrates. Hereby, the healthy state of the cell slowly shifts to a condition favoring protein folding diseases, cellular damage and aging. Whereas reducing oxidative stress affects several cellular processes, leading to lower levels of proteotoxic stress, small HSPs overexpression leads to a specific decline of non-native proteins directly affecting protein homeostasis. Increasing either refolding capacity (CG14207) or anti-Q capacity (HSP76BC) directly reduces proteotoxic stress levels thereby delaying the onset of protein folding diseases and aging.
by protein misfolding diseases (84-87). These data suggest that the heat-shock response is an intrinsic system that cells can upregulate to provide the organism with a more robust state of protein homeostasis. Yet, with age these systems apparently fail and protein folding related problems arise. So, boosting the entire system or its essential components, restores protein homeostasis with no or little side effects on cellular functioning and promote healthy ageing.

In this thesis, we specifically focused on the role small HSPs may play in aging and asked what type of function (refolding or anti-Q) might be more relevant in boosting longevity. Hereto, we used Drosophila melanogaster and first determined whether, like in humans, members of the Drosophila small HSP family differ in molecular activity. Indeed, we were able to select a set of small HSPs which differ in their activities either being "refolders" or "antiQ" proteins (Figure 1b). Interestingly, both boosting of refolding activity (CG14207) or anti-Q activity (HSP67BC) provided longevity (Figure 5, right panel). Non-native proteins thus seem to be a major determinant of aging. Improvement of protein homeostasis does not seem to depend on a single cellular process, rather it can be safeguarded by both refolding and anti-aggregation activity on the pool of non-native proteins, effectively decreasing the total proteotoxic burden. This is especially important in view of HSP70 dependent refolding activity of small heat shock proteins such as DmCG14207. HSP70 induction has been suggested to deregulate signalling pathways (88) and correlates with the development of malignancies (89). Boosting protein homeostasis through HSP70 activity might thus result in adverse side effects. Boosting anti-aggregation activity in a non-HSP70 dependent manner (DmHSP67BC), on the other hand, would result in longevity without the risk of HSP70 associated side effects. Thus using HSPB6, HSPB7, HSPB9 or DmH-SP67BC (Figure 1c) to boost protein homeostasis seems a more safer choice.

**Perspectives**

The overall picture emerging from this thesis is the link between small heat shock proteins and key cellular functions related to protein homeostasis. Apart from the role of HSPB1, HSPB4, HSPB5 and *Drosophila* HSP23, HSP26, HSP27, CG7409 and CG14207 in refolding of denatured proteins, certain members are tailored for additional functions. While some members seemingly combine refolding and anti-aggregation, HSPB7, HSPB8, HSPB9 and *Drosophila* HSP67BC are specialized in clearance of protein aggregates depending on the length of the glutamine stretch.

The human anti-Q small HSPs generally show different mechanisms to prevent polyQ aggregation. One possible theory for the functionality of HSPB7 could be related to processing or chaperoning of RNA species in the presence of aggregation-prone triplet-expanded mRNA. Both DMPK and HD-2, which are caused by triplet expansion of the corresponding genes like HD, are also associated with nuclear RNA aggregates or foci. Both of these disease-related mRNA foci could serve as a tool to assess the role of HSPB7 in chaperoning RNA measured as a reduction in foci formation.

One functional implication of RNA foci formation is a negative effect on protein synthesis of the affected genes. Here, the luciferase reporter system could serve as an excellent tool to acquire data on protein synthesis levels in the absence or presence of HSPB7 expression. Hereto, a luciferase construct would be needed containing a triplet expansion in the 3' UTR, resembling expanded DMPK. After overexpression of HSPB7, reduction in foci formation and an increase of RNA chaperone activity could be directly linked to a functional improvement of cellular homeostasis measured as an increase in luciferase activity.

One other hypothesis on HSPB7 activity is a potential role in RNA splicing modification, leading to, for instance, exon skipping or active inclusion of otherwise skipped exons. This mechanism could result in changes in protein function, possibly generating yet to be identified anti-Q pro-
teins. A first step to confirm this hypothesis would be to test if HSPB7 is able to interact with RNA and if such attracts splicing factors. These questions could have a major impact on aging research as well. Whereas science has mainly focused on oxidative stress, DNA damage and proteotoxic stress in relation to aging, the role of a declining RNA modification system should also be taken into account. With the here described data on protein folding, anti-Q activity and aging, a possible role of RNA chaperoning in aging could make a worthwhile addition. In addition to HSPB7, several known RNA chaperones could be tested to determine their role in the network of cellular homeostasis and their contribution to aging.

References

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