Regulation of protein homeostasis in acute and chronic stress

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General discussion and perspectives
Maintaining a healthy proteome is crucial for cellular function and survival. In cells, protein homeostasis is constantly challenged by a variety of intrinsic and extrinsic stress factors that require adaptations of protein quality surveillance pathways. In response to acute forms of stress, such as environmental changes, but also cell intrinsic changes like differentiation, cells can rebalance protein homeostasis by activating highly inducible protein quality control pathways (1). To maintain basal protein control, relevant in more chronic forms of stress as, for example, the expression of disease-causing mutant proteins, other regulatory factors or pathways may become more important especially under conditions of aging where the acute stress responses have declined (2).

Whereas in studies on proteotoxic stress the differences between acute and chronic stress are often not specifically articulated, they do significantly differ in many aspects. Most forms of acute stresses involve a pleiotropic chain of events in which multiple proteins are affected at the same time and in high quantities and often cause destruction of the cellular ultrastructure (3). This suffices to trigger stress pathways (HSR/UPR) that upregulate the classical HSPs that have rather promiscuous client recognition characteristics (for example, HSP70s, certain J-domain proteins, and some small HSP like HSPB1) or that can stabilize cell structures (4). Indeed, e.g. the single elevations of proteins like Hsp70 (5) or HSPB1 (6) suffice to protect against heat induced toxicity and are associated with improved protein refolding (5, 7, 8) and reduced cytoskeletal collapse (6). In contrast, in chronic stress damage accumulates slowly, often involving (initially) only few or even single proteins and this is (initially) not associated with major structural changes. These chronic forms of stress (initially) also generally do not suffice to activate the stress pathways. Moreover, the substrates involved may require specific chaperones for recognition and handling (9) and handling may rather require client disposal than refolding. Indeed, in screens done in our lab, canonical chaperones regulated by the HSR were found to be rather ineffective in handling chronic stress as induced by many disease-related proteins (10). Instead, non-HSR regulated members of e.g. the J-domain protein family members like DNAJB6 (10) or small HSPB family members like HSPB7 (11) did handle such disease-associated clients such that they did not form pathological aggregates. What features specify such difference between canonical HSR-regulated and non-HSR regulated HSP is yet barely understood. Our data comparing HSPB1 (acute stress protector) and HSPB7 (chronic stress protector) (chapter 3) have provided some initial insights in this.

Another striking feature of acute proteotoxic stress in the transient pausing of protein synthesis (12). This will reduce substrate load for the protein quality control system and together with the (selective) expression of chaperones further help to rebalance protein homeostasis. Little was known about the translational control over misfolded proteins in chronic forms of stress. Our findings that the transcription factor FOXO1, at least partly, activates a program that controls translation of certain mRNA like those involved in polyglutamine disease, is one of the first in this field.

Finally, whereas as most emphasis on protein quality control is related to intracellular processes, we identified a process whereby cells seem to “solve” their inability to dispose accumulated chronic protein damage by excreting it. Whereas it is yet premature to conclude on the cell autonomous benefits of such an additional PQC level versus the putative non-cell autonomous threats of such events (e.g. prion like effects), these data add to the notion that maintaining protein homeostasis is key to cellular fitness and is regulated in multiple (backup) layers.

Aspirin (ASA): promising HSP inducer to ameliorate acute stress

In chapter 2, we show that ASA pre-treatment can protect cardiac cells against the toxicity of acute heat stress both in vitro and in vivo. These protective effects were related to the ability of ASA to activate
the HSR and elevate the expression level of HSF-1-regulated HSPs, including Hsp70 and the small HSP, HSPB1 (HSP25/27).

The most compelling effect of ASA pretreatment was the prevention of the cytoskeletal collapse of the cardiomyocytes. Temperature-induced collapse of the cytoskeletal structure has been reported since long ago as a major damage (3, 13). Such structural forms of damage will affect many cellular functions including e.g. cell motility and cytoskeleton related transport. The latter will also impede on several PQC-related functions like aggresome formation (14) or autophagy (15). For (cardio) muscular cells, cytoskeletal collapse is likely to be specifically detrimental it will affect cell contractility and viability ((16, 17), which eventually may cause tissue dysfunction either via direct loss of tissue contractility or via cell death-related disturbances in the electrical conduction system of the heart. Such may finally lead to e.g. sudden cardiac arrest (18, 19). It is thus not surprising that the major symptoms in heated poultry is in the heart, where a collapse of sarcomeres of myocardial cells will have major impact on contractile function (20) with ultimately will lead to organismal death.

It has been found HSPB1, HSPB5 and HSPB6 can stabilize microtubulies, filaments and actins. Of these, HSPB1 is most strongly up-regulated by HSF-1 (21). Indeed, when treating cells with the HFS-1 activator ASA, HSPB1 was clearly up-regulated and the animals were protected against a heat-induced collapse of the sarcomeres and maintained fit (Chapter 2 and (22)). The importance of HSPB1 in stress-resistance of cardiomyocytes has also been established in other proteotoxic stresses, e.g. during atrial fibrillation (23). Formally, however, it has yet to be proven that ASA treatment has cardioprotective effects that are at least partly, related to the elevated expression of HSPB1. Testing whether inhibiting the ASA-induced expression of HSPB1 (e.g. by specific siRNA treatment during ASA) eliminates the cytoprotective effects of ASA would be required. Inversely, it could be tested whether ectopic overexpression of HSPB1 alone suffices to protect chicken cardiomyocytes to heat damage.

Irrespective of the HSP involved, however, the promising data with ASA suggest that it could be used as a potential stress protective medicine in breeding industry. Comparing to other pharmacological HSR activators, short term and limited frequency of ASA treatment seems to have no to only limited side effects (22, 24), although more chronic applications may need more cautious considerations. ASA can induce cell death in multiple cell types in vitro (25). Whereas there are no long reports on long term usage in poultry, some toxicity has been found on upper gastrointestinal, liver and kidney after long-term usage of ASA in mammalian models (26-29). Such side-effects might affect food intake, meaning less productive. In addition, such side effects on liver and kidney may render the poultry sensitive to other medicines or additives in the fodder. Moreover, ASA is well known for its anti-coagulation effects (30). This might increase the symptom or death of some bleeding-prone disease, for example, coccidiosis and new castle disease. A more indirect concern is the presence of ASA after slaughter. ASA is stable up to 140 °C (31), meaning that it may not be decomposed after cooking. Therefore, it will be necessary to measure the pharmacokinetics of ASA in birds to determine when to stop ASA usage before slaughter.

Despite of above risks, ASA seems a promising anti-acute stress medicine with potential usage for poultry breeding, provided good dosing and timing of the application. The HSF-1 activated HSPB1 seems a possible main target for its action to preserve the cytoskeletal integrity of cardiomyocytes, which seems a likely critical determinant in determining the sensitivity to acute heat damage.

The non-HSR regulated DNAJB6 and HSPB7 that protect against chronic stress are not regulated by FOXO1 or IGF2

PolyQ aggregation is the result of slow accumulation of polyQ fragments that accumulate over time until critical conditions arise that initiates an amyloidogenic process that next rapidly progresses in which
the initial seeds act as sites of polymerization ends for the yet soluble polyQ (32). This is usually not sensed by the cells as a proteotoxic stress and thus does not activate the acute stress pathways like in the case of e.g. heat stress (33-35). Inversely, as stated above, most of the target genes activated by these pathways encode for proteins that are not very effective in suppressing polyQ aggregation (35). In particular, HSPB1 that plays a protective role in acute stresses (4, 8, 36) does not protect against the formation of polyQ aggregates (Chapter 3 and (11)). Rather, other HSP family members that are not regulated by these acute stress response pathways like e.g. DNAJB6 (37)or HSPB7 (Chapter 3 and (11)) are most active in dealing with such chronic forms of stress. In these theses, we tested whether longevity related pathways like those initiated by FOXO1 or IGF2 would act via these non-HSF1 regulated chaperones. However, whilst both pathways lead to suppression of polyQ aggregation we found no role for DNAJB6, HSPB7 nor any other of the small HSPs to play a role in this the effects of FOXO1 or IGF2-initiated pathways. Below, I will first discuss the mode of action of HSPB7 and its relevance to human diseases. Next, I will discuss the putative pathways induced by FOXO1 or IGF2 and how our findings related to what has been described for these pathways in the existing literature.

HSPB7, a peculiar member of the HSPB family

HSPB7 is a rather peculiar member of the HSPB family. Unlike most small HSPs, HSPB7 does not form large oligomers, has no capacity as ‘holdase’ for most classical substrates of small HSP like MDH, GAPDH, Rhodanese, CS, ADH and insulin (38) or e.g. firefly luciferase (II)(ref), and has not been associated with protection to acute stress. HSPB7 is not regulated by HSF-1 (11) and is low expressed in most tissues, except in the heart where its expression is high, which is why it originally was referred to as cardiovascular HSP22 (cvHSP27) (39).

It was therefore surprising that we found HSPB7 to be the most potent suppressor of polyglutamine aggregation within the family of human small HSPBs (11). And even here, HSPB7 showed peculiar features as its activity: unlike for most other small HSPs, its activity was found to be not dependent on Hsp70 (11). Instead, HSPB7 required active autophagy for full activity (23) where it seems to affect the (pre)aggregated structure and was no longer effective on pre-formed aggregates (40).

To get some insight in these peculiar functional characteristics of HSPB7, we addressed in chapter 3 which parts of HSPB7 are responsible in its protection against polyQ aggregation. We found that the NTD of HSPB7, predicted to be the most intrinsically disordered NTD of all HSPB, was crucial for its function and sufficient to turn HSPB1 into an effective anti-polyQ aggregation chaperone. Whereas the NTD also was linked to the non-oligomeric status of HSPB7, de-oligomerization alone was not sufficient to turn HSPB1 into an anti-polyQ aggregation chaperone. Rather the NTD also was required the guidance and/or binding to the polyQ proteins, likely via interactions to the regions flanking the polyglutamine stretch in the Huntington protein (Chapter 3). The highly disordered character of HSPB7 suggest that is likely to phase separate. Indeed, HSPB7 and its NTD has been shown to drive association with known membrane-less compartments (41). Our data show that, whereas this NTD may help to phase separate polyQ proteins, the ACD of HSPB7 was required to prevent the liquid to solid state transitions of the polyQ proteins. If fact, fusing the NTD alone directly to the polyQ protein enhanced aggregation, indicating that phase separation per se is not protective in preventing aggregation of proteins and even enhance the risk of such events most likely by enhancing the local protein concentration. The finding that a the same polyQ fragment fused to the entire HSPB7 has reduced aggregation propensity suggest that the presence of HSPB7 and likely also other small HSP in liquid droplets (REFs) may serve to prevent liquid to solid transitions in such compartments.

Why HSPB7 is expressed most highly in cardiac and fat tissues remains to be understood. It is
intriguing to note that dysregulated HSPB7 expression and gene polymorphisms (SNPs) have been repeated linked to disease. It was suggested that HSPB7 is associated with preserving contractile integrity by binding to and stabilizing sarcomeric proteins (42-44). In these same line, HSPB7 was also found to be able to protect atrial myocytes against tachycardia remodeling (45). How these activities are linked to the polyQ chaperone-related functions of phase separating potential of HSPB7 remains to be understood. However, the SNP data suggest that different HSPB7 variants may have different activities, and that lower-activity variants may even predispose to dilated cardiomyopathy (DCM) (46-48). Whilst more insight in the functional consequences of these SNP variants within the HSPB7-encoding gene are required, these data highlight the relevance of this chaperone for cardiac function.

**FOXO1 a multifacet transcription factor which yet mysterious links to PQC**

In order to test how non-HSF-1 regulated protein quality control networks like those involving DNAJb6 and HSPB7 related pathways were regulated by longevity-related pathways, we first turned to the FOXO1. Like HSF-1, FOXO1 is a transcription factor, negatively regulated by insulin signaling and has been associated with longevity likely via, yet mysterious, links to PQC networks. Elevated expression or activation of FOXO1 has been associated with increased autophagy, improved proteasome mediated protein degradation, as well as an elevated expression of some chaperones, including small HSPs. In chapter 4 we showed, for the first time in mammalian systems, that the transcriptional activity of FOXO1 can boost a PQC program capable to reduce polyQ aggregation. However, whilst we could confirm that such a program indeed activates autophagy (49) and slightly upregulates the expression of some small HSP (50, 51), we also found that none of these could explain the anti-aggregation activity elicited by the FOXO-1 activity (Chapter 4). Rather, we discovered a novel branch of PQC to be initiated that lead to a reduction in polyQ translation rates. This pathway was found to be associated with the up-regulation of a number of RNA binding proteins of which inhibiting STAU1 or DDX18 could negate the anti-aggregation effect of the FOXO1-induced network. Both of these two RNA binding proteins were identified when FOXO1 is overexpressed. This finding uncovered a corner of a gold mine. As a transcription factor FOXO1 is found the regulation function on translational level with a big network. In addition to these positive co-operators of FOXO1, we also found 29 proteins can be co-precipitated with mHtt mRNA only when FOXO1 is absent. Although further studies are not included in this thesis book, we have already uncovered a tip of an iceberg. The new nodes in this FOXO1 mediated translational network will provide more targets to cure protein homeostasis disease.

**IGF2 reduces polyQ aggregation**

Compared to IGF1, which is long known for its longevity and protein homeostasis regulating effects via both DAF-16/FOXO and HSF-1 (50, 51), IGF2 has been far less intensively studied. So far IGF2 regulated signaling was mainly connected to the development, cancer and cardiovascular diseases (52). In collaboration with the lab of Claudio Hetz, it was found that like IGF1, IGF2 also controls protein homeostasis such that its activated pathways lead to reduced polyQ aggregation. However, in this case restoration of protein homeostasis was not associated with preventing intracellular aggregation and increases polyQ degradation (like HSPB7 or DNAJb6) nor with or reduced polyQ synthesis (like for FOXO1). Rather, we found that IGF2 activation resulted in an unconventional secretion of aggregated polyQ proteins.

Appearance of intracellularly accumulated protein aggregates in the extracellular environment has been reported repeatedly and in fact has been proposed as leading to prion-like propagation of the
aggregation process into neighboring cells, hereby accelerating the degeneration process (53). Indeed, also polyQ aggregates may enter neighboring cells and seed aggregation herein (54). Whereas these extracellular aggregates have been most often suggested to be due to leakage from dead neuronal cells, our current data suggest that it may (also) be a more (IGF-2) regulated process, serving as an additional PQC level for cell autonomous protection. Similar suggestions for such a regulated, cell protective excretion have been recently proposed in data obtained with C. elegans (55). Here, it was suggested that the putative non-cell autonomous threats of such events (i.e. the prion-like propagation) may be counteracted by efficient clearance of the extracellular materials in young animals and that such may be lost upon ageing. In other words, since maintenance of cell autonomous protein homeostasis is key to cellular fitness, such a backup system may have evolved for pro-survival reasons early in life but may become a threat when we age.

**Perspectives**

The data from this thesis support the notion that is acute and chronic stress are quite different in both the damage they induce and the pathways required to survive them. Indeed, although acute stress and chronic stress both activate some members of HSPs for resistance or self-rescue, the chaperones for the two different stress are largely different. Even within one chaperone family, different members can mostly respond to only one type of stress. Therefore, currently, there is not a universal chaperone that can prevent damage related to both acute stress and chronic stress.

Our work on HSPB7 provokes the challenge of trying to generate a chimeric “super HSPB7” in which the IDR of the HSPB7 NTD is fused with any of the other HSPB-ACDs to test whether such fusion proteins could have even better anti-aggregation effects. On one hand, ACDs from other small HSPs may be more potent in suppressing polyQ-aggregation than the ACD of HSPB7 (although this was not found for HSPB1). On the other hand, other HSPBs bound to their specific substrates may more readily phase separate with their clients and hence prevent them for co-aggregation with other cellular proteins or structures. As HSPB7 is only expressed at very low levels in brain, it may be worthwhile to learn more about how its expression is regulated. Once a key transcription factor (or factors) would be identified, one could design drug screens to selectively upregulate HSPB7. It would be worthwhile to explore what are the endogenous “clients” of HSPB7, especially in cardiac cells. This may explain why specifically the heart is sensitive to single nucleotide polymorphisms (SNPs) in HSPB7. Finally, the precise functional consequence of these SNPs that link to cardiac disease susceptibility requires further studying.

Finally, uncovering the networks regulated by FOXO1 and IGF2 and the identification of their downstream targets that regulate protein homeostasis may provide various strategies for disease therapy. The regulation of polyQ translation rates by FOXO1 have shown that it requires STAU1 and DDX18. But are these activities sufficient? And how precisely do STAU1 and DDX18 work? Is this truly due to simple competitive binding to polyQ mRNA only? In fact, DDX18 is known as a RNA chaperone which can destabilize the RNA helices structure for refolding to specific structure (56) and hence, beside simple binding DDX18 may actually affect the polyQ mRNA structure such that it can no longer efficiently bind to the ribosome or translational regulators. STAU1 is known to bind to dsRNA and to affect RNA transport, which has been linked to RNA degradation (57-59). This could also be linked to the fact that we often noticed reduced polyQ RNA levels in case of FOXO1 overexpression although these effects were rather fluctuating (data not shown).
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