Stress response regulation and protein aggregate inheritance in *Caulobacter crescentus*

Frederic Dominique Schramm
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Abstract

Many stress conditions a cell encounters threaten the continuation of basic biological processes ultimately endangering its survival. Heat shock and antibiotic exposure can lead to a sudden surge of protein un- and misfolding, while nutrient starvation directly causes a lack of energy and molecular building blocks. Our understanding of how cells integrate environmental stress signals, execute protective functions and handle persistent damage is still far from comprehensive. In this thesis the model bacterium *Caulobacter crescentus* was used to answer basic questions about the regulation and execution of bacterial stress responses and damage clearance.

Persistent larger protein aggregates can be maintained as remnants of a past stress exposure and in all of the few bacteria studied to date these particles collect at the poles. In the symmetrically dividing bacterium *E. coli* this aggregate localization pattern was shown to lead to an old pole lineage-specific retention. In **paper I**, we studied aggregate formation and inheritance in an asymmetrically dividing bacterium. While aggregates are dissolved by molecular chaperones following moderate heat stress, intense stress induces the emergence of long-lived aggregates. Surprisingly, we find that the majority of persistent aggregates do not collect at the old poles but instead describe a mechanism by which they are constantly displaced towards the new pole. This causes inheritance of aggregates by old and new pole cells at a stable rate without lineage-specific retention, a previously unknown pattern of aggregate inheritance in bacteria.

While we found that deletion of most chaperones in *C. crescentus* does not affect viability in the absence of stress, the mechanistic basis for why DnaK, like in other bacteria, is also required in the absence of stress remains unclear. In **paper II**, we show that DnaK's function as a negative regulator of the heat shock sigma factor σ^{32} is essential for viability at physiological temperatures and uncover potential new layers of σ^{32} regulation. We find that the σ^{32}-dependent response comprises a reallocation of resources from proliferative to maintenance functions and in addition to its known function in blocking DNA replication also affects other processes like protein translation, a process vulnerable to proteotoxic stress. Prolonged unrestricted activity of this stress response induced by the absence of DnaK is lethal. We conclude that while DnaK is essential for protein folding at elevated temperatures, its evolutionarily newer function in balancing the cell's proliferative and maintenance programs is a requirement for survival.

Growth and cell cycle progression is also regulated in response to nutrient limitation. Like under heat shock conditions, we show in **paper III** that carbon starvation during entry into stationary phase leads to a block of DNA replication for which, in contrast to heat stress, the molecular basis was not yet understood. We find that downregulation of DnaA levels is achieved by an as yet unknown nutrient availability sensing process involving the 5' untranslated region, inhibiting translation of the *dnaA* mRNA, which combined with constant degradation of DnaA by the protease Lon results in its elimination. This study provided new mechanistic insight into nutrient-dependent control of DNA replication and shows that the same regulatory outcomes can be achieved through different means depending on the stress response. In conclusion this thesis describes the discovery of an unanticipated alternative way of protein aggregate inheritance with implications for our view on damage segregation in bacterial populations as well as new mechanistic insight into how cells balance proliferative with protective functions in response to heat shock and nutrient limitation.

**Keywords:** stress, protein aggregation, cellular aging, molecular chaperones, heat shock response, DnaK, sigma factor, suppressor genes, DNA replication, starvation, *Caulobacter crescentus*.

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STRESS RESPONSE REGULATION AND PROTEIN AGGREGATE INHERITANCE IN CAULOBACTER CRESCENTUS

Frederic Dominique Schramm
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Frederic Dominique Schramm
To my family
SUMMARY

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function in blocking DNA replication also affects other processes like protein translation, a process vulnerable to proteotoxic stress. Prolonged unrestricted activity of this stress response induced by the absence of DnaK is lethal. We conclude that while DnaK is essential for protein folding at elevated temperatures, its evolutionarily newer function in balancing the cell’s proliferative and maintenance programs is a requirement for survival.

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POPULAR SCIENCE SUMMARY

In their environment bacteria often face adverse conditions. Increases in temperature can damage a cell’s proteins and cause them to stick together in larger nonfunctional particles - so-called protein aggregates. The absence of nutrients will cause a lack of energy and cellular building blocks. As a countermeasure, bacteria engage in defensive responses, actively slowing down vulnerable growth and cell cycle processes and increase activities aimed at preventing and repairing damage. Furthermore, lasting damage in the form of protein aggregates is often asymmetrically distributed upon division. This allows for rapid damage clearance in one of the two daughter cells, possibly to the detriment of the aggregate-inheriting cell. Understanding bacterial stress response regulation and execution is important for controlling bacterial behavior in industrial processes and infectious disease treatment while studying bacterial aggregate inheritance could serve as a model system for human aging and protein aggregation diseases.

In this thesis the model bacterium *Caulobacter crescentus* was used to answer fundamental questions of bacterial stress response regulation and damage distribution. The results present mechanistic details about the regulation of growth and the cell cycle during stress and uncover a hitherto unknown pattern of bacterial protein aggregate inheritance.

I denna avhandling används modellbakterien *Caulobacter crescentus* för att besvara grundläggande frågor om bakteriell stressförsvarsreglering och skadefördelning. Resultaten visar verkningsmekanismen bakom regleringen av tillväxt och cellcykel under stress och avslöjar ett hittills okänt mönster i bakteriell nedärvning av proteinaggregat.
LIST OF PUBLICATIONS

This thesis is based on the following works:

**Schramm, F. D.***, Schroeder, K.*, Alvelid, J., Testa, I., & Jonas, K. (2019). Growth-driven displacement of protein aggregates along the cell length ensures partitioning to both daughter cells in *Caulobacter crescentus*. Accepted for publication in *Molecular Microbiology*.


*These authors contributed equally to the study.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>(p)ppGpp</td>
<td>guanosine penta- and tetraphosphate</td>
</tr>
<tr>
<td>5'UTR</td>
<td>5' untranslated region</td>
</tr>
<tr>
<td>AAA+</td>
<td>ATPases associated with various cellular activities</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>translation elongation factor Tu</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>NAC</td>
<td>nascent-chain-associated complex</td>
</tr>
<tr>
<td>PYE</td>
<td>peptone-yeast extract medium</td>
</tr>
<tr>
<td>RAC</td>
<td>ribosome-associated complex</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>sHSP</td>
<td>small heat shock proteins</td>
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<tr>
<td>SP</td>
<td>signal peptide</td>
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<tr>
<td>SRP</td>
<td>signal recognition particle</td>
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</tbody>
</table>
# TABLE OF CONTENTS

SUMMARY..........................................................................................................................................
i
POPULAR SCIENCE SUMMARY..................................................................................................iii
POPULÄRVETENSKAPLIG SAMMANFATTNING......................................................................v
LIST OF PUBLICATIONS..............................................................................................................vii
ABBREVIATIONS............................................................................................................................ix
INTRODUCTION..............................................................................................................................1
  1. Maintenance of protein homeostasis.......................................................................................1
    1.1. Protein folding...................................................................................................................1
    1.2. The molecular chaperone network..................................................................................3
    1.3. Protein degradation by AAA+ proteases.........................................................................5
  2. Proteotoxic stress and protein aggregation..........................................................................7
    2.1. Proteotoxic stress..............................................................................................................7
    2.2. Different types of protein aggregates.............................................................................10
    2.3. Protein aggregation – Friend or foe?.............................................................................11
  3. Aggregate inheritance.............................................................................................................13
    3.1. Replicative aging in unicellular organisms.....................................................................13
    3.2. Aggregation patterns and aggregate distribution.........................................................17
    3.3. Consequences of aggregate inheritance..........................................................................20
    3.4. Caulobacter crescentus – a model for aging and damage segregation in an asymmetrically dividing bacterium?...........................................................................21
  4. The heat shock response.........................................................................................................23
    4.1. The chaperone network in heat stressed cells.................................................................23
    4.2. AAA+ proteases during proteotoxic stress.......................................................................25
    4.3. Regulation of the heat shock response............................................................................26
    4.4. Molecular chaperones in heat shock response regulation.............................................27
    4.5. Regulation of σ^{32} in Escherichia coli...........................................................................28
  5. Chaperone and protease essentiality......................................................................................31
    5.1. Chaperone and protease essentiality under stress.........................................................32
    5.2. Chaperone and protease essentiality in the absence of stress....................................34
6. Bacterial growth and cell cycle control under stress.............................................37
   6.1. Regulated proteolysis in cell cycle progression and stress adaptation 38
   6.2. DnaK and Lon in proteotoxic stress-dependent cell cycle regulation of
       Caulobacter crescentus..........................................................................................38
   6.3. Cell cycle regulation in response to nutrient starvation............................40
AIMS OF THE THESIS.............................................................................................41
MAIN FINDINGS........................................................................................................43
DISCUSSION AND FUTURE PERSPECTIVES.........................................................55
ACKNOWLEDGEMENTS.........................................................................................61
REFERENCES..........................................................................................................65
INTRODUCTION

1. MAINTENANCE OF PROTEIN HOMEOSTASIS

Proteins are central effectors and building blocks of the cell encoded by genetic information. Beginning as a nascent peptide on the ribosome most mature by folding into a specific conformation in which they fulfill myriads of functions in the cell\textsuperscript{1–3}. Fulfilling these functions often requires protein-protein interactions or trafficking to specific destinations. At the end of their lifespans proteins are degraded in the course of many regulatory as well as quality control processes\textsuperscript{4}. The functional balance between the interconnected and competing processes of protein synthesis, folding and refolding, oligomerization, aggregation, trafficking and finally degradation make up the cell’s protein homeostasis (proteostasis)\textsuperscript{5} (Fig. 1). To effectively maintain protein homeostasis cells employ a plethora of machineries dedicated to these different processes in form of molecular chaperones and proteases\textsuperscript{4,6,7}.

1.1. PROTEIN FOLDING

Critical for their function, most proteins must fold into and maintain a defined three-dimensional structure. The blueprint lies in the amino acid sequence, also called the primary structure\textsuperscript{1–3}. While in some mature proteins regions will remain unstructured, the formation of hydrogen bonds between the peptide bonds of the peptide backbone will give rise to local secondary structures like $\alpha$-helices and $\beta$-sheets\textsuperscript{8,9}. The sequence of unstructured and structured regions along the peptide chain as well as their spatial arrangement determines the tertiary structure, the fold of a protein\textsuperscript{2,10–12}. Spatial arrangement is sometimes achieved through covalent bonds between amino acid side chains, like in the case of disulfide bridges formed by cysteines, but mainly through non-covalent interaction between the side chains of the amino acids that can
be far apart in the primary structure. Hydrophobic interactions are of especially high importance. The collapse of hydrophobic amino acids into the water un-exposed center of the structure is a major driving force of protein folding. Finally, proteins can function as subunits of larger protein complexes of which the number and arrangement is known as the quaternary structure.

Figure 1: Summary of major interconnected, reversible and irreversible processes governing protein homeostasis. After emergence at the ribosome a peptide chain will fold into its native conformation over several productive folding steps. Proteins can be transported to or through the membrane as folding intermediates or fully folded proteins. Especially under stresses but also under normal conditions proteins can un- and misfold. When these unproductive folding intermediates occur at high densities these tend to aggregate. Proteins are removed from the proteome by degradation. The presence of a single arrow between depicted processes signifies irreversibility while two arrows show that a process is reversible. Adapted from (6).

The process of protein folding is thought to follow a rugged multidimensional funnel shaped energy landscape in which a polypeptide moves towards its thermodynamically favorable functional fold or native state. Depending on the
protein, this is achieved through a number of possible folding paths. Along these, a polypeptide chain will go through intermediate unfolded and partially folded states coming closer to the native fold step by step. While the folding of small proteins is often fast and does not entail many intermediate conformations, bigger multidomain proteins will go through a number of folding states that sometimes have to cross considerable energetic barriers before assuming the next conformation. As a consequence, these proteins can linger at different folding steps. This puts them at risk of forming unproductive interactions within their own or with another protein’s peptide chain causing misfolding. Especially in the crowded environment inside the cell unproductive interactions between proteins can endanger protein homeostasis and lead to the formation of aggregates (Fig. 1). Protein aggregation will be discussed in detail in section 2.

1.2. THE MOLECULAR CHAPERONE NETWORK

To cope with physical constraints of protein folding and to prevent unproductive peptide chain interactions in vivo, cells harbor molecular chaperones. These are (mostly) proteins which interact with clients to promote their folding into or maintenance of their functional conformation, without remaining associated with the folded client. Many chaperones belong to one of the several highly conserved but phylogenetically unrelated groups of protein folding machineries. Different cellular compartments often have their own sets of paralog or unique chaperone machineries. While cells express an especially diverse arsenal of chaperones under various stress conditions, a number of abundant chaperones are constitutively produced and belong to a basic and often conserved set guiding the folding of a protein from as early on as its birth at the ribosome. Through interaction with each other and handing over of folding client proteins chaperones are organized in chaperone networks. Complex proteins especially are often handed down to several different chaperone types in a sequential hierarchical order.

The first cytosolic chaperones to bind to a nascent chain generally involved in the de novo folding of proteins are the non-homologous ATP-independent bacterial
ribosome-associated trigger factor and the eukaryotic ribosome-associated complex (RAC) as well as the nascent-chain-associated complex (NAC). These are followed by the ATP-dependent chaperones (bacterial/eukaryotic homolog) DnaK/Hsp70 (70-kDa heat shock protein) functioning together with the ATP-independent co-chaperone DnaJ/Hsp40 and nucleotide exchange factors, GroEL/ES (a complex composed of GroEL and GroES monomers)/TRiC as well as HtpG/Hsp90 systems\(^{17,20,22–24}\).

The bacterial cytosolic chaperone network has been most intensely studied in *Escherichia coli* (Fig. 2). Trigger factor is a ribosome-associated chaperone forming a cradle for the nascent chain\(^{22,25}\) and helps the folding of about 70% of the *E. coli* proteome\(^{6}\). For proteins requiring more assistance, the next chaperone to bind either directly to the nascent chain or after release from the ribosome is DnaK\(^{20,26}\). DnaK is a monomer forming a clamp cycling through an open and a substrate enclosing state in dependence of ATP-hydrolysis\(^{27–31}\). The co-chaperone DnaJ recognizes and transfers substrates to DnaK and thus modulates substrate specificity while, together with the nucleotide exchange factor GrpE, also regulating DnaK’s conformational changes by modulating ATP hydrolysis and nucleotide exchange\(^{31–33}\).

DnaK is involved in the folding of 20% of the *E. coli* proteome\(^{6}\). If protein folding is not completed or cannot be performed through interaction with DnaK, proteins are handed down to one of the two more specialized machineries\(^{19,20,34–37}\). While HtpG chaperones do not belong to the standard bacterial repertoire, the GroEL/ES machinery is almost ubiquitous\(^{38}\). The GroEL/ES machinery consists of two heptameric rings of GroEL forming two protein folding chambers oriented back to back that can each be capped by a smaller heptameric lid of GroES subunits. ATP- and cap-binding induced conformational changes of a GroEL folding chamber lead to the transfer of a bound protein into the chamber which provides a beneficial environment for folding\(^{39–43}\). In addition, GroEL/ES can also assist the folding of substrates too large to be transferred into the folding chamber\(^{44}\). In *E. coli* the GroEL/ES system is involved in the folding of 10% of the proteome\(^{6}\).
Figure 2: Chaperone network in the *E. coli* cytosol. The ribosome associated trigger factor is the first chaperone to interact with a nascent peptide chain. Downstream of trigger factor nascent chains and unfolded proteins interact with DnaK (DnaJ and GrpE are not shown). Proteins not achieving their final conformation through interaction with trigger factor and DnaK are passed on to the downstream chaperones GroEL/ES or HtpG. Percentages indicate the portion of the proteome folded by a given machinery. Adapted from (6, 17).

1.3. **Protein degradation by AAA+ proteases**

In addition to the folding and transport capacity provided by chaperones, the ability to process proteins by cleavage or to completely remove them is essential for a functional proteome\(^4,7,45,46\). To this end cells harbor peptidases, proteolytic proteins capable of cleaving a protein’s peptide backbone.

Peptidases can be ATP-independent or associated with an AAA+ (ATPases associated with various cellular activities) ATPase domain driving protein unfolding, in which case these are termed AAA+ proteases\(^4,7,45,47–49\). All AAA+ proteases form a barrel-shaped oligomeric complex composed of one or several multimeric rings of six to seven monomers. The proteolytic chamber consists of a ring of peptidase regions of which the active sites face the interior while the AAA+ ring is responsible for substrate recognition, unfolding and translocation into the proteolytic chamber by ATP-driven conformational changes. The peptidase chamber and AAA+ ring can either be made up of separate monomers or of one monomer with both activities\(^7,45,50\).

The 26S proteasome is the only member of the AAA+ protease family usually found in the eukaryotic cytosol. In bacteria and bacteria-derived eukaryotic organelles the AAA+ protease diversity is higher. Commonly occurring AAA+ proteases are the
single ring Lon and FtsH and the multiring HslUV and ClpP. The latter associates with one of the unfoldase rings ClpX, ClpA, ClpC or ClpE. The AAA+ proteases can have different localizations in the cell. FtsH is a membrane-bound protease which degrades inner membrane proteins but also proteins of other cellular compartments including the cytosol. The protease Lon is involved in the degradation of cytosolic proteins and can also localize to the nucleoid and degrade DNA-bound proteins. ClpP and HslUV are thought to mainly reside in the cytoplasm but can relocalize during stress or, in the case of ClpXP, cell cycle phases.

Regulated proteolysis by AAA+ proteases often follows two principles for attaining substrate recognition and specificity. Firstly, the substrates themselves contain certain motifs targeting them for degradation. These so-called degrons are more or less strict sequence motifs that can be located internally or at a protein’s termini. Appending degrons co- or post-translationally or exposing them through for example peptide cleavage, complex dissolution, and protein conformational changes are all important factors regulating proteolysis. Secondly, while some substrates can be recognized by the AAA+ ATPase ring directly, different adaptor proteins will either deliver certain substrates or prime the protease towards the degradation of certain proteins. A well-known example of degron and adaptor-mediated protein quality control can be found in the degradation of nonfunctional polypeptides originating from the rescue of stalled ribosomes. In the process of trans-translation these are C-terminally appended with an SsrA peptide tag targeting the polypeptides for degradation by the ClpXP protease, aided by the SspB adaptor, which recognized the SsrA tag. Protein quality control and bulk degradation of misfolded proteins can become particularly important when cells experience various stresses. Furthermore, single backbone cleavage events or complete degradation of proteins is important in a variety of processes including protein transport, many regulatory processes during cell-cycle, developmental growth-phase transitions and in stress responses. These aspects of protein degradation will be discussed in the sections 4.2 and 6.1.
2. **PROTEOTOXIC STRESS AND PROTEIN AGGREGATION**

Various stresses a cell encounters cause protein un- and misfolding. When the chaperoning and degradation machinery is oversaturated, un- and misfolded proteins separate from the phase of the soluble proteins by collecting into larger assemblies called protein aggregates\(^7^5\). Protein aggregates can tentatively be defined as a separate phenomenon from other phase separated structures like membrane-less organelles and stress granules by representing nonfunctional assemblies formed by non-native interaction of un- and misfolded proteins\(^7^5–^7^7\). However, this common definition is more and more challenged by findings suggesting that aggregates exhibit a spectrum of properties shared with other phase-separated structures\(^7^6,^7^7\). Further understanding of protein aggregation and other processes of protein phase separation is required to better evaluate the adequacy of commonly used definitions. While protein aggregation has traditionally been viewed as purely detrimental, it has become increasingly clear that collecting un- and misfolded proteins into larger assemblies can represent a stress adaptive response\(^7^8–^8^0\).

2.1. **PROTEOTOXIC STRESS**

Multiple stresses, whether inherent to the cell or environmental, can dysregulate the protein homeostasis by causing un- and misfolding\(^1^6\) promoting unproductive interactions between peptide chains – a condition termed proteotoxicity (Fig. 3A). Common intrinsic causes of proteotoxic stress are mutations and aging\(^5,^8^1–^8^5\) mostly known from eukaryotic cells and common interlinked drivers in the development of human proteinopathies. Here, mutations reducing the functionality of components of the protein homeostasis network or affecting the folding of certain proteins will be amplified by an age-dependent decline in the buffering capability of the proteostasis network\(^5,^8^1–^8^5\). In particular, proteins are damaged by reactive oxygen species that occur in the cell even in the absence of stress, which burdens the proteostasis network and is linked to aging\(^8^6\).
A multitude of environmental stresses induce proteotoxicity (Fig. 3A). Environmental exposure to substances like hydrogen peroxide and hypochlorous acid can drastically increase levels of reactive oxygen and chlorine species in the cell. Reactive oxygen and chlorine species cause carbonylation and chlorination of amino acid side chains, respectively, leading to a strong increase in irreversible un- or misfolding and can inactivate ATP-dependent chaperones. The often refolding-resistant covalently modified proteins sometimes cannot be proteolyzed and thus can accumulate in the cell and hamper the folding of other proteins. Protein carbonylation is also observed after exposure to mistranslation-inducing antibiotics. While these do not increase levels of reactive oxygen species per se, reducing translation fidelity produces peptides prone to oxidation by the natural background levels of reactive oxygen species in the cell.

Since protein folding is highly dependent on temperature, pH and ionic strength, changes in these parameters away from an organism’s optimal range will lead to protein un- and misfolding (Fig. 3A). Especially strong heat shocks induce bulk unfolding of the proteome. Thermal un- and misfolding of proteins caused by milder heat exposures in a cell is often temporary and can be reversed by increased chaperoning activity or after return to the optimal growth temperature.
Figure 3: Summary of proteotoxic stresses, aggregation causing interactions and protein aggregate types. A) Causes of proteotoxic stress can be extrinsic, intrinsic or both. Intrinsic factors like mutations (red stars) can reduce folding efficiency of an affected protein or reduce the chaperoning and degradational capacity of the cell. Accumulation of damaged proteins through cell-intrinsic reactive oxygen species can burden the proteostasis network and is observed in aging processes. Levels of reactive oxygen and chlorine (not shown) species can also strongly increase due to extrinsic causes like exposure to hydrogen peroxide and hypochlorous acid. The dashed line represents the dual nature of reactive oxygen species as intrinsic and extrinsic proteotoxic stress. Other extrinsic causes of proteotoxic stress are highly diverse and range from translation-affecting antibiotics, temperature increase, pH changes and ionic stress. B) The nature of a protein aggregate is determined by the aggregation-causing interactions and its constituents. Aggregates can form through the interactions of only one type of misfolded protein, the interaction of misfolded with unfolded proteins, interactions between mainly unfolded proteins or be driven by association of proteins with sHSPs. Proteins aggregate predominantly through β-sheet and hydrophobic interactions. Misfolded domains are labeled in red. C) Protein aggregates can be classified into different types. Amyloid fibrils are highly ordered structures often composed of a single type of misfolded protein interacting with one another through β-sheets. Amorphous aggregates can contain a multitude of un- and misfolded proteins interacting both through β-sheets and exposed hydrophobic regions. In turn, association with sHSPs maintains aggregated proteins in a near-native state.
2.2. **DIFFERENT TYPES OF PROTEIN AGGREGATES**

As variable as the stresses causing protein un- and misfolding, protein aggregates have very different sizes, compositions and structures and form by different processes (Fig. 3B, C). Aggregates can start out as small oligomers only detectable by biochemical methods and end up as large inclusions visible at cellular or even tissue scale\textsuperscript{75}. General processes causing aggregation are the formation of intermolecular $\beta$-sheet structures and hydrophobic interactions which have different levels of prevalence depending on the nature of the un- or misfolded protein species\textsuperscript{75}.

In amyloid proteinopathies, the detection of large intra- or extracellular protein aggregates is a hallmark of disease development. Aggregation is initially triggered by $\beta$-sheet associations of often only one misfolded aberrant protein species into smaller oligomers, seeding the formation of highly structured fibrils in which monomers contact each other through $\beta$-sheets running perpendicular to the fibril’s axis\textsuperscript{97,98} (Fig. 3B, C). Another type of fibril not belonging to the amyloid class are formed in serpinopathies. In a domain swapping event, a peptide loop of one serpin monomer reaches out into a $\beta$-sheet of another monomer\textsuperscript{99,100}. Thus, aggregates formed in these diseases are often composed of proteins assuming a largely or partially folded but non-native conformation. In contrast, stress induced bulk unfolding entails the presence of a large diversity of un- and misfolded proteins which are thought to mainly interact through now-exposed hydrophobic residues. The resulting amorphous aggregates are composed of diverse proteins in a much more unstructured state than in the fibrillar aggregates\textsuperscript{75}. However, it has become increasingly clear that also in less structured aggregates proteins can contact each other through $\beta$-sheet interactions\textsuperscript{16,101} (Fig. 3B, C).
2.3. PROTEIN AGGREGATION – FRIEND OR FOE?

Bulk protein un- and misfolding is likely to cause a significant fraction of the proteome to become nonfunctional\textsuperscript{75}. How does the downstream event of protein aggregation affect the cell? Large microscopically observable aggregate assemblies are seen as a hallmark of proteotoxic stress and many studies have used fluorescent reporter-tagged chaperones or model unfolding protein reporters to visualize large aggregates\textsuperscript{54,78,102,103}. These microscopically observable structures are often the final result of disturbances in the protein homeostasis. They represent the biggest particles in the aggregate size-range and require the preceding emergence of smaller aggregates and un- and misfolded proteins only detectable by biochemical methods\textsuperscript{75}. Thus, it can be difficult to uncouple whether microscopically observable aggregates are a symptom or cause of deterioration in the cell. In the case of amyloid diseases it was proposed that mainly non-aggregated or the smaller oligomeric states of the disease-causing misfolded proteins are associated with toxicity\textsuperscript{84,97}. Common mechanisms by which un- and misfolded proteins and aggregates of any size can interfere with the normal conformation of functional proteins is by trapping them with their sticky surfaces\textsuperscript{6,75}. Thus sequestering un- and misfolded proteins as well as smaller oligomers into bigger assemblies can effectively reduce the amount of these surfaces in the cell\textsuperscript{75}. Furthermore, concentrating un- and misfolded proteins in specific locations could facilitate the disaggregating work of chaperones\textsuperscript{16}. In eukaryotic cells deposition close to autophagosomal structures allows for the removal of irreversibly un- or misfolded proteins in bulk in the process of autophagy\textsuperscript{16}.

By now several mechanisms have been proposed arguing for protein phase separation during stress as an adaptive and beneficial event for the cell, not without sometimes blurring the line between which assemblies should be considered aggregates and which are not. It was shown in the baker's yeast \textit{Saccharomyces cerevisiae} that small heat shock proteins drive the site-specific deposition of proteins in a near-native state\textsuperscript{78} (Fig. 3B, C). Deletion of these chaperones leads to an inhibition or retardation in the formation of larger aggregate assemblies in response to heat stress and a loss in viability, showing that aggregation can be an active chaperone-driven
process beneficial to the cell\textsuperscript{78–80}. Furthermore, the assembly of specific proteins into larger structures was also proposed to be part of general regulatory mechanisms in eukaryotes\textsuperscript{104–106}. Translation factors were shown to assemble into hydrogels forming stress granules (a name for larger assemblies composed of translation factors and often also mRNAs) proposedly temporarily shifting translation towards mRNAs encoding stress-adaptive proteins. Importantly, it was suggested that some proteins contain specific domains evolved to drive stress-dependent phase separation while the majority of the protein remains folded\textsuperscript{106}.

Although collecting proteins into larger aggregates or other phase separated assemblies during proteotoxic stress can mean a fitness advantage to the cell, the consequences of maintaining these over a longer period can be different. Cells incapable of dissolving aggregate assemblies, for example when the disaggregation machinery is deleted\textsuperscript{54,107–110}, are very sensitive to proteotoxic stress. Furthermore, depending on the protein and the stress, some aggregate assemblies are disaggregation resistant\textsuperscript{102}. Continued growth of aggregate assemblies can mean a sequestration of essential proteins\textsuperscript{111} and particularly large aggregates can hinder cellular processes through their sheer size\textsuperscript{75}. Furthermore, in situations when the burden of un- and misfolded proteins or smaller oligomers is effectively reduced, persistent larger aggregate assemblies can still be proteotoxic through their remaining potentially disordered surfaces\textsuperscript{75} and can act as a source of proteotoxic species by shedding\textsuperscript{112}. 
3. **Aggregate inheritance**

If aggregates are not dissolved after a stress exposure or the condition of the protein homeostasis leads to continuous protein aggregation, aggregates will linger in the cell\(^{34,102,103,113-115}\). In a population of growing and dividing unicellular eukaryotes or bacteria, large persistent aggregates constitute discrete particles that can be asymmetrically inherited by daughter cells and represent a potential source of heterogeneity. Inheritable protein aggregates have been linked to fundamentally important topics such as replicative aging, where they are seen as “damage”, and cellular memory\(^{103,116,117}\). However, the impact of aggregates is controversial and principles governing their inheritance appear to vary between organisms. In the bacteria studied so far aggregates appear to collect at the poles and this was shown to ensure retention in the old pole lineage of *E. coli*\(^{54,102,118,119}\). Replicative aging has been shown in the asymmetrically dividing bacterium *C. crescentus*\(^{120}\) and assumed to be in part caused by accumulation of aggregates in the old pole lineage\(^{118,121}\), however without experimental validation. Studying protein aggregate inheritance in this organism promises to shed further light on the potential role of aggregates as aging factors and how they are managed in a bacterial cell.

3.1. **Replicative aging in unicellular organisms**

Aging is largely seen as a progressive loss of functionality associated with a decrease in growth, reproductive rates and survival caused by the accumulation of various damages\(^{122}\). While in many multicellular organisms aging appears mandatory for the individual, offspring will be born fully rejuvenated. This has been explained by asymmetry in damage repair or retention between parent and offspring and proposed to reflect a trade-off between reproduction and survival in a resource limited environment\(^{121,123,124}\).

First indication that aging also takes place in unicellular organisms came from the asymmetrically dividing baker’s yeast *S. cerevisiae*\(^{125}\). During the asymmetric division of this organism a smaller daughter cell buds off the larger mother cell\(^{126}\) (Fig. 4A).
While the number of consecutive divisions a cell undergoes (the replicative lifespan) is in principle already limited by a constant age-independent hazard rate, progression through consecutive division rounds in a baker's yeast mother cell leads to a continuous decrease in growth rate and increase in mortality\textsuperscript{125,127} – a phenomenon called replicative aging\textsuperscript{128–130}. In contrast, in daughter cells (with exception of late born cells) the replicative age is fully reset leading to a rejuvenation of the lineages\textsuperscript{127,131,132}. Importantly, this observation was correlated with accumulation of cellular damage that appears to be specifically retained in the aging mother cell\textsuperscript{133}. Taken together, the budding yeast’s morphological asymmetry is accompanied by a strong asymmetry in damage retention and aging (Fig. 4A).

Figure 4: Replicative aging in unicellular organisms. A) Schematic cell cycle of the budding yeast \textit{S. cerevisiae} indicating replicative age as the number of divisions a mother cell went through (number inside cells), and the accumulated damage associated with cells of different replicative ages (represented by absence or presence as well as size of weights underneath second generation cells). While the oldest mother cell has accumulated the most damage, daughter cells are age-reset and damage-free. B) Cell pole inheritance in \textit{E. coli}. Cells are born with an old pole (labeled in orange) that was already present in the mother cell and a new pole (labeled in green) that formed at the division plane. Cells consecutively inheriting the old pole will have the highest old pole age and potentially accumulate the most damage while cells inheriting the new pole evade damage. (B) adapted from (130).

Since this discovery, it has been strongly debated if replicative aging takes place in symmetrically dividing unicellular organisms lacking a distinction in size or morphology between daughter cells\textsuperscript{130}. With the fission yeast \textit{Schizosaccharomyces pombe} and the bacteria \textit{Bacillus subtilis} and \textit{E. coli} these questions have been addressed in both eu- and prokaryotes. Because of the lateral growth mode of \textit{B. subtilis}\textsuperscript{134} and \textit{E. coli}\textsuperscript{135},

14
in which new cell wall material is incorporated along the length of the cell but not at the poles, the old pole's cell wall has a slower turn-over compared to other parts of the cell. This region is generally thought of as a location for accumulation of older cell constituents and thus potential damage\textsuperscript{136} (Fig. 4B). The replicative age of a bacterial cell is generally defined by how many consecutive divisions the old pole versus the new pole was inherited (Fig. 4B). Initial microscopy-based studies in \textit{B. subtilis} and \textit{E. coli} showed that, after a division event, the old pole-inheriting daughter has a slower growth rate than the daughter inheriting the newly synthesized pole. Consecutive inheritance of the old pole was proposed to lead to slower and slower growth until death while the new pole-inheriting cells are rejuvenated, suggesting that aging might also take place in such organisms\textsuperscript{54,118,136} (Fig. 4B).

Aging in \textit{E. coli} has been addressed in numerous further, sometimes contradictory studies and the interpretation of observed growth rate differences between old pole and new pole-inheriting cells has evolved\textsuperscript{116,130,137–140}. Recently, using a microfluidics systems, the growth rates of continuously old pole and new pole-inheriting lineages were shown to converge towards two equilibria representing a stable slowest and fastest growth rate, respectively, in an unstressed population of cells\textsuperscript{140}. Exemplified, this means that a lineage having previously continuously inherited the new poles of its mother cells will first exhibit slower and slower growth rates when it now continuously inherits the old pole. After a certain number of continuous old pole inheritances the growth rate will however not decrease further, even when the lineage continues consecutively inheriting the old pole. The establishment of the two growth rate equilibria is proposedly achieved when the asymmetrical damage acquisition (through inheritance and cell intrinsic formation) and dilution (through growth and passing down to a sibling during division) are balanced and the same amount of damage is distributed between the daughter cells at every division\textsuperscript{140}. These results and the proposed mechanism behind them potentially reconcile conflicting findings of studies describing a growth decline in old pole-inheriting cells\textsuperscript{136} with those of studies observing a stable growth rate\textsuperscript{137}. The difference between the fastest and slowest growth rates was shown to increase as a function of stress intensity\textsuperscript{116,139}. Under severe stress, the damage dose present in the lineage continuously inheriting the old pole can...
lead to a steady growth rate decline culminating in death\textsuperscript{130}. Importantly, asymmetric damage segregation in the form of protein aggregates has been proposed to benefit an \textit{E. coli} population especially under high stress conditions during antibiotic treatment or thermal upshift\textsuperscript{116}.

In \textit{S. pombe} signs of replicative aging were reported early on\textsuperscript{141}, however, later studies showed that under physiological conditions the growth rate does not continuously decrease nor does the mortality increase from division to division\textsuperscript{142,143}. In contrast to \textit{E. coli} and \textit{B. subtilis}, \textit{S. pombe} exhibits polar growth and new cell wall structures are first predominantly integrated at the old cell pole and later also at the new pole\textsuperscript{144}. While \textit{S. pombe}, does not appear to show mandatory aging, it is currently debated if aging phenotypes can be observed after stress exposures but are likely to be absent at least in old pole daughter cells (the experimental set-ups of the microfluidics devices used did not allow the long-term tracking of new pole-inheriting cells)\textsuperscript{103,142,143,145}. Interestingly, in the asymmetrically dividing bacterium \textit{M. smegmatis} under physiological conditions, the old pole-inheriting daughter is longer and shows faster growth than the new pole-inheriting daughter. Here new cell material is also integrated first preferentially at the old pole and later also at the new pole\textsuperscript{146}.

Replicative aging is seen in the asymmetrically dividing yeast \textit{S. cerevisiae}. In contrast the results in the asymmetrically dividing \textit{M. smegmatis} and symmetrically dividing organisms studied until now present a variety of situations that in many cases need further research for clarification. While \textit{S. pombe} and \textit{E. coli} share the absence of a continuous growth decline in the absence of stress, only the latter appears to experience an increasing mortality rate with progression of replicative age\textsuperscript{137}. Thus \textit{S. pombe} does not seem to age under physiological conditions at all and \textit{E. coli} only shows some of its aspects under these conditions. It remains to be understood if and how aging might be affected by damage occurring during stress conditions.
Although its occurrence is still disputed in many organisms, aging has been associated with the accumulation of a variety of cellular damages. Amongst these, the accumulation of protein aggregates appears to be universal and belongs to the most studied potential aging factors in unicellular organisms. Importantly, in the model organisms in which aggregate inheritance has been tracked over many generations, aggregates are immediately or gradually retained in the cell type or lineage associated with aging (e.g. the *S. cerevisiae* mother cell and the old pole cell in *E. coli* and *S. pombe*), a process requiring asymmetric aggregate inheritance.

Asymmetric aggregate inheritance can be caused by various and often interlinked processes. The most simple is their numeric reduction by aggregate fusion or dilution through cell division which will make asymmetric distribution of the aggregate load very likely or even mandatory at the next division event. However, without aggregate localization-determining mechanisms in place the probability of aggregate inheritance will only strongly depend on how much of the mother cell’s volume is inherited by a given daughter. Thus, asymmetry caused by numeric reduction alone is not sufficient to cause cell type-specific retention and accumulation over generations but further requires active or passive retention mechanisms in one of the daughters or specific deposition sites the cell.

In the asymmetrically dividing *S. cerevisiae* aggregates are retained in the mother cell during division. The cells exhibit different general deposition sites depending on the type of stress, on the type of aggregating protein and on its subcellular localization. Aggregates emerging from the formation of amyloid fibrils are deposited in close proximity to the vacuole or preautophagosomal structures. Other protein classes are commonly deposited at sites inside the nucleus or in the cytoplasm. Different models exist explaining how aggregate retention in the mother cell is achieved ranging from active to passive mechanisms including retrograde transport from the daughter cell along actin filaments, attachment of aggregates to non-inherited organelles and the reduced likelihood of large protein deposits crossing the narrow bud neck.
In the symmetrically dividing fission yeast *S. pombe*, aggregates form randomly in two compartments with low exchange rates between one another: the space between the old pole and the nucleus (old pole compartment) or that between the new pole and the nucleus (new pole compartment).\(^{103,142,145}\) (Fig. 5). While the aggregate load thus has a high probability of being equally distributed between both daughter cells during the first division rounds, the irreversible differentiation of every new pole compartment into an old pole compartment leads to a preferential retention of aggregates in the cell inheriting the old pole.\(^{103,142,145}\) Aggregates can however escape this compartment at a low frequency eventually allowing inheritance to new pole cells.\(^{145}\) Interestingly, it was shown that especially during stress, sHSP-driven aggregate fusion facilitates asymmetric aggregate distribution.\(^{103}\)

In bacteria aggregate distribution amongst daughter cells has been studied in the symmetrically dividing *B. subtilis* and *E. coli* and the asymmetrically dividing *M. smegmatis*.\(^{146}\) (Fig. 5). In these organisms aggregates are either directly deposited at the poles, in the case of *B. subtilis*\(^{119}\) and *E. coli*\(^{54,117,118}\), or will gradually move towards the poles as described for *M. smegmatis*.\(^{102}\) While the reason for aggregate collection at the poles of *M. smegmatis* is unknown, it has been studied in more detail in *E. coli*. Most models favor a passive mechanism involving occlusion of larger particles from midcell by the densely packed nucleoid over active transport or the involvement of chaperones.\(^{54,154}\) The polar collection of aggregates which will usually not leave this location anymore has the consequence that aggregates will mostly be inherited by the old pole daughter lineages after the second division post aggregate formation in *E. coli*.\(^{54,118}\) Nucleoid occlusion has also been proposed to drive the polar aggregate formation observed in *B. subtilis*.\(^{119}\) It remains to be shown if aggregates are maintained in the old pole cell lineages over generations in *B. subtilis* and *M. smegmatis*.

Taken together, in all the unicellular organisms in which protein aggregate segregation patterns over many generations have been studied to date, passive or active mechanisms appear to drive retention in the cell with the higher replicative age.
Figure 5: Aggregate localization and inheritance pattern in symmetrically and asymmetrically dividing unicellular eukaryotes and bacteria. In the symmetrically dividing *S. pombe* multiple aggregates (green particles) form in the region between the old pole and the nucleus (blue region) or the new pole and the nucleus after stress exposure. These fuse in a sHSP-dependent way to form larger particles that can move around their respective compartment but only rarely cross the barrier imposed by the nucleus. When equal aggregate amounts are present on both sides of the nucleus the aggregate load will be symmetrically distributed between daughter cells. However, the resulting numeric reduction of aggregates and the differentiation of new cell pole compartments into old cell pole compartments will lead to an asymmetric preferential inheritance by the old pole cell after following division events. In the asymmetric division event of *S. cerevisiae* a smaller cell buds off a larger mother cell. Heat shock induced aggregates are collected and deposited at different sites in the cell. These will specifically be retained in the mother cell by active and passive mechanisms upon division causing strong asymmetry in aggregate inheritance between mother and daughter cells. In the symmetrically dividing bacteria *B. subtilis* and *E. coli* aggregates are quickly deposited at the poles upon formation. In *E. coli* this is caused by occlusion from the centrally positioned nucleoid (blue region) enforcing aggregate localization at the aggregate free poles and likely also takes place in *B. subtilis*. In *E. coli* the polar localization of aggregates will lead to a strong asymmetry of aggregate inheritance in the second division by retaining aggregates in the old pole lineages. In the asymmetrically dividing bacterium *M. smegmatis* aggregates were also shown to collect at the cell poles (only collection on one pole shown although bipolar collection possibly also takes place in this organism) causing asymmetric inheritance upon division. However, it is not known if these aggregates are specifically maintained in the old pole lineage over consecutive division events.
3.3. CONSEQUENCES OF AGGREGATE INHERITANCE

Separating correlation and causation between aging and aggregate accumulation in non-dividing terminally differentiated cells (e.g. neurons) has proven to be difficult. Studying the effect of unequal distribution of protein aggregates between cellular siblings that largely share the same history has the potential to facilitate understanding the impact of protein aggregates on aging.

It was shown that aggregates accumulating in the *S. cerevisiae* mother cell inhibit proteasome activity\textsuperscript{155} and that deficiency in this proteolytic machinery\textsuperscript{156} as well as in disaggregation\textsuperscript{114} shorten the replicative lifespan. In turn, providing higher proteasome activity was able to augment the replicative lifespan\textsuperscript{156}. It is however not clear if this is directly due to the reduction of damaged protein or factors not directly related to this function\textsuperscript{133}.

Several studies in *E. coli* showed a relative growth reduction in an aggregate-inheriting compared to an evading sibling\textsuperscript{54,116,118}. Although aggregates almost always localize at the old poles\textsuperscript{54,117,118} effects caused by aggregate inheritance were able to be separated from those caused by pole age\textsuperscript{54,118}. Thus, these studies proposed that aggregates are likely one but not the sole aging factor in *E. coli*\textsuperscript{54,118}. Complicating matters, a very recent study claims that protein aggregation does not take place under physiological conditions and that aggregate inheritance generally has less of an impact on growth rate than previously thought\textsuperscript{117}. While the authors could reproduce the old pole-new pole effect, they did not observe a correlation between inheriting a heat shock-induced aggregate and growth decline. Interestingly, it was shown that inheriting such aggregates post stress confers superior heat stress resistance in a following insult. Thus, aggregates were proposed to be particles of cellular memory conferring an advantage under certain conditions\textsuperscript{117}. In stressed fission yeast\textsuperscript{103,142} and *M. smegmatis*\textsuperscript{102}, a lower growth rate and higher mortality were observed in the sibling receiving the majority of the aggregate load from the mother while no differences could be observed when aggregates were distributed equally. However, as in *E. coli*, a more recent study in *S. pombe* reported neither growth reduction nor increased
mortality upon aggregate inheritance post stress, even in the case of large aggregates assemblies\textsuperscript{145}.

Taken together, to date studies about aggregate inheritance in the different unicellular systems do not allow us to draw a universal picture of the effects of aggregate inheritance. The effects, or lack thereof, of aggregates on the cells inheriting them might depend on the received amount, the nature of the aggregate, the environmental condition under which cells inherit an aggregate and the organism.

3.4. \textit{Caulobacter crescentus} – A Model for Aging and Damage Segregation in an Asymmetrically Dividing Bacterium?

In contrast to \textit{S. pombe} and the bacterial model organisms \textit{B. subtilis} and \textit{E. coli}, the freshwater \textit{\alpha}-proteobacterium \textit{Caulobacter crescentus} exhibits an asymmetric cell cycle\textsuperscript{157–159}. Because of its genetic accessibility and the ease of distinguishing cell types and cell cycle phases, this organism has mainly advanced as model for mechanistic studies on bacterial development. Each division results in a larger sessile stalked cell that is \textasciitilde17\% longer and \textasciitilde5\% thicker than the smaller motile swarmer cell\textsuperscript{160} (Fig. 6). Swarmer cells cannot initiate DNA replication and are thus locked in a stage reminiscent of the eukaryotic G1-phase\textsuperscript{159,161}. During a developmental program in which the single flagellum is shed from the old pole giving way to the emerging stalk, the swarmer cell accumulates cell mass and differentiates into a stalked cell. This stalked cell will initiate DNA replication once and only once per cell cycle, accumulate more cell mass and segregate into the two cell types\textsuperscript{158,159}.

\textbf{Figure 6:} Schematic cell cycle of \textit{Caulobacter crescentus}. The motile flagellated swarmer cell differentiates into a stalked cell capable of chromosome replication and increasing in mass until division. The asymmetric division yields a larger stalked cell inheriting the old pole and a smaller swarmer cell inheriting the new pole.
Interestingly, as for the *S. cerevisiae* mother cell, replicative aging in *C. crescentus* was reported for stalked cells albeit at a slower pace. Microfluidics experiments tracking up to ~130 consecutive divisions showed that for many cells the time intervals between divisions increased or reproduction stopped\(^{120}\). As in *E. coli* and *B. subtilis*\(^{134,135}\), incorporation of new cell wall material in *C. crescentus* takes place along the length of the cell and at the division septum but not at the poles making accumulation of damaged material at the old poles possible\(^{162,163}\). Because of the morphological asymmetry in *C. crescentus* the stalked old pole of a stalked cell always corresponds to the old pole of the mother cell while the swarmer cell’s flagellated old pole corresponds to the mother’s new pole.

Although not stated in the original work, later publications propose the asymmetric retention of damage in analogy to *S. cerevisiae* mother cells and *E. coli* old pole cells as a cause of aging for stalked cells\(^{118,121}\). However, until now no results have ever been reported to validate these assumptions. Especially in light of the many still open questions regarding the role of protein aggregation and its asymmetric distribution in cellular aging, expanding the study of these processes beyond the commonly used models promises new insight. *C. crescentus* as an asymmetrically dividing bacterium with a reported aging phenotype represents an excellent starting point.
4. **THE HEAT SHOCK RESPONSE**

In response to proteotoxic stress, cells will engage in a stress-adaptive program augmenting their repair and maintenance capacities while rewiring the physiology of the cell – a process comprehensively known as the heat shock response\(^{18}\). Depending on the organism this includes the induction of 50-200 heat shock protein encoding genes that can be subdivided into different classes according to the function they fulfill\(^{18}\). Molecular chaperones prevent further protein un- and misfolding and drive, manage and reverse protein aggregation\(^6,7^{5}\) while the proteolytic machinery removes damaged or other unwanted proteins\(^7,18\). Further classes of proteins are involved in maintaining other cellular components or processes like protecting and repairing nucleic acids, sustaining cytoskeletal structures and modulating protein transport, membrane transport and detoxification\(^{18}\). In addition to these repair and maintenance functions, certain metabolic enzymes are upregulated and although not fully understood, proposedly act in setting up and stabilizing energy supply under adverse conditions\(^{18}\). Finally, the expression of additional regulatory proteins can initiate other stress responses and fine-tune stress adaptation\(^{18}\).

While heat shock response activation and shut-down can be quickly regulated, its general dynamics depend on stress intensity and duration. Furthermore, significant differences can be seen in the relative induction levels as well as the timing of individual heat shock protein expression\(^{164,165}\). Generally, molecular chaperones and proteases are encoded by early-induced genes and belong to the most upregulated and abundant heat shock proteins.

4.1. **THE CHAPERONE NETWORK IN HEAT STRESSED CELLS**

While all organisms constantly express a specific suite of chaperones and proteases for the house keeping of the proteome, the quantitative and qualitative composition of the protein homeostasis network is strongly adapted in response to proteotoxic stress. Many chaperones involved in *de novo* protein synthesis as well as AAA+ proteases which are constitutively expressed as part of the basal proteostasis network
will be strongly upregulated\textsuperscript{17,18,166}. Furthermore, cells will start producing high levels of chaperones more specialized in aggregate handling and dissolution to facilitate refolding\textsuperscript{18,75,167}. These include small heat shock proteins (sHSPs) as well as the ClpB/Hsp104 disaggregation machineries.

sHSPs comprise the most diverse class of chaperones and are characterized by the presence of an \( \alpha \)-crystallin domain\textsuperscript{168,169}. These ATP-independent chaperones were shown to bind and stabilize substrates but not to catalyze folding\textsuperscript{17,170}. While under some conditions this prevents protein aggregation\textsuperscript{168,170}, under others the sHSPs specifically drive aggregation\textsuperscript{80,153,171}. As a result, sHSPs are present in many aggregates of which they can make up a significant fraction\textsuperscript{107,172–174}. Importantly, sHSPs bind early-unfolded proteins and maintain them in a near-native state\textsuperscript{171} (Fig. 7). The resulting aggregates composed of sHSP-substrate complexes are better shielded from the environment\textsuperscript{175}, have a different architecture facilitating later disaggregation and prime the protein content towards refolding instead of degradation\textsuperscript{75,175}.

In many organisms DnaK/Hsp70 is crucial for protein disaggregation. DnaK/Hsp70 chaperones bind to a broad variety of aggregate types\textsuperscript{176–178} and also bind to and help to dissolve aggregates formed in the absence of sHSPs\textsuperscript{107}. Furthermore, only these chaperones appear to be able to displace sHSPs from the aggregate surfaces to allow dissolution of sHSPs-containing aggregates\textsuperscript{175} (Fig. 7). While the DnaK/Hsp70 system is capable of dissolving smaller protein aggregates on its own\textsuperscript{179}, larger protein aggregates are dissolved in cooperation with additional factors. In metazoans Hsp70 is assisted by an unusual Hsp110 nucleotide exchange factor\textsuperscript{180,181} whereas many bacteria and non-metazoan eukaryotes make use of a DnaK/Hsp70-ClpB/Hsp104 bichaperone system to dissolve aggregates\textsuperscript{174,182} (Fig. 7). The highly conserved AAA+ disaggregase ClpB/Hsp104 forms an oligomeric ring capable of extracting proteins from aggregates by threading them through a central pore under ATP consumption\textsuperscript{183,184}. However, ClpB has no activity of its own and requires aggregate-bound DnaK for recruitment\textsuperscript{185–188}, substrate transfer\textsuperscript{189,190} and modulation of its ATPase activity\textsuperscript{190–192} (Fig. 7). While proteins disaggregated by this mechanism are often refolded, aggregate dissolution by other mechanisms can result
in their degradation\textsuperscript{75}. For instance the unfolding subunits of many AAA+ proteases have a disaggregating activity but will target the extracted proteins for degradation\textsuperscript{56}. This process could be more common in organisms like \textit{B. subtilis} that lack ClpB homologs and further do not strongly rely on DnaK for thermal stress adaptation\textsuperscript{96,193,194}.

![Diagram](image)

**Figure 7: Protein aggregation and disaggregation by heat shock-inducible chaperone machineries.** sHSPs bind early-unfolded proteins and can drive their aggregation in near-native states while being incorporated into the aggregate. In many bacteria and eukaryotes the resulting aggregates will be dissolved by a bi-chaperone system consisting of the DnaK/Hsp70 (DnaJ/Hsp40 co-chaperones and nucleotide exchange factors not shown) and ClpB/Hsp104 machineries. DnaK/Hsp70 binds to the surface of aggregates and displaces sHSPs. In a following step DnaK/Hsp70 recruits and activates the disaggregase ClpB/Hsp104 through which proteins will be threaded by ATP consumption leading to their extraction from the aggregate. The extracted protein can then be refolded into its native conformation by the cytoplasmic chaperones. Adapted from (175).

### 4.2. AAA+ PROTEASES DURING PROTEOTOXIC STRESS

If proteins are terminally damaged or remain un- or misfolded for a prolonged time, AAA+ proteases will take care of their removal\textsuperscript{7,96,194}. In \textit{E. coli} ClpP and HslUV at least partially relocate to protein aggregate assemblies in heat stressed cells\textsuperscript{37,54} and all of the cytosolic proteases have been suggested to be involved in the removal of cytosolic un- and misfolded proteins to varying degrees\textsuperscript{195}. Compared to the other AAA+ protease-encoding genes, those encoding HslUV are particularly heat shock-inducible\textsuperscript{196}. The membrane-bound FtsH also degrades damaged cytosolic proteins\textsuperscript{197} but is especially important for quality control in the inner membrane\textsuperscript{198,199}.

Amongst the cytosolic AAA+ proteases in \textit{E. coli}, Lon was shown to have a more important role in the removal of un- and misfolded proteins\textsuperscript{96,200}. Appropriately, Lon
has a large substrate repertoire, at least under proteotoxic stress. Under these circumstances, instead of strict sequence motifs, this protease is believed to recognize hydrophobic regions that would normally be buried in the native conformation of a protein. Importantly, Lon is allosterically activated by its substrates, meaning that with increasing concentrations of un- or misfolded proteins the proteolytic activity will increase. In that way protease activity can be regulated dependent on the level of proteotoxic stress.

4.3. Regulation of the heat shock response

The induction of heat shock proteins under thermal stress and other proteotoxic stress conditions as part of the heat shock response is a universal process. Heat shock-inducible proteins can generally be regulated on the level of transcription (by transcriptional activators or repressors), translation (involving RNA-thermometers) and activity (for example increasing protease activity).

While in eukaryotes the heat shock factors (HSFs) are conserved transcriptional activators of HSP expression, various factors are used in bacteria. Here, different phylogenetic groups have independently evolved fully or partially specialized heat shock alternative sigma factors priming the RNA polymerase to the transcription of heat shock genes. For this purpose many Proteobacteria like E. coli use the sigma factor (and in response to outer envelope stress also ) while the gram-positive B. subtilis, amongst other factors, employs the general stress response sigma factor to induce genes required for thermal stress adaption. In addition to these transcriptional activators many bacteria regulate heat shock gene expression by one or several transcriptional repressors. The widespread repressors HrcA, HspR and CtsR often bind to inverted or direct repeat DNA motifs in the operons of dnaKJ, grpEs and groESL under non-stress conditions and thereby prevent their induction in the absence of stress. While some bacteria base their heat shock response regulation on only activation or repression mechanisms, most species investigated show combinations of these with overlap between regulons.
4.4. MOLECULAR CHAPERONES IN HEAT SHOCK RESPONSE REGULATION

In addition to their conserved roles in counteracting protein unfolding and aggregation, different molecular chaperones are involved in the regulation of the heat shock response\textsuperscript{222–225}. This is generally achieved through chaperones functioning as positive regulators of heat shock gene repressors or as negative regulators of heat shock gene activators\textsuperscript{205,208,226}.

GroEL/ES is responsible for maintaining activity of the repressor HrcA by promoting its folding\textsuperscript{222,227,228} while DnaK functions as a co-repressor for HspR\textsuperscript{229,230}. A paradigm of chaperone-mediated regulation is the role of Hsp70 class chaperones in regulating the activity of heat shock transcription factors in eukaryotes and \textit{Proteobacteria}. In animals\textsuperscript{231}, fungi\textsuperscript{232} and plants\textsuperscript{233} Hsp70 chaperones were shown to bind to HSFs and inhibit HSF-driven gene expression\textsuperscript{226,231,234,235}. Furthermore Hsp90\textsuperscript{236–239} and the eukaryotic distantly GroEL-related chaperonin TRiC are also involved in regulating HSF\textsuperscript{240}. In \textit{Proteobacteria}, DnaK directly interacts with $\sigma^{32}$ and inhibits its association with the core RNA polymerase\textsuperscript{241–243}. GroEL/ES was proposed to also be involved in $\sigma^{32}$ regulation\textsuperscript{244,245}.

An increase in un- and misfolded proteins will titrate the chaperones away, alleviating heat shock gene repression by HrcA and CtsR while freeing transcriptional activators like $\sigma^{32}$ or the HSF to induce the expression of their heat shock regulon\textsuperscript{205,226}. Thus, intensity of heat shock gene expression will be regulated as a function of the folding demand. The regulating chaperones are usually under control of their respective activators or repressors which provides an effective means for feedback control of heat shock regulon expression\textsuperscript{205,246–249}. Once the new folding demands have been satisfied, freed-up chaperones can shut down heat shock regulon expression again\textsuperscript{205,226}. Importantly, this kind of feedback control on the heat shock response effector can be exerted by an entire array of factors as is well known for \textit{E. coli}.
The heat shock alternative sigma factor $\sigma^{32}$ is restricted to but widespread in the large phylum of the Proteobacteria of which some members harbor several paralogs. The majority of knowledge about $\sigma^{32}$-dependent heat shock regulon regulation comes from research in the $\gamma$-proteobacterium *E. coli* in which the single $\sigma^{32}$ controls transcription from an estimated 120-150 promoters.

In the absence of stress, $\sigma^{32}$ occurs in low numbers due to RNA-thermometer regulated low translation and constant proteolysis reducing the half-life to about 30-60 s. Under low stress conditions and in the heat shock response downshift phase, the GroEL/ES and the DnaK machinery will interact with and inhibit $\sigma^{32}$ activity. DnaK binding is promoted by DnaJ and both heat shock proteins were shown to bind to several and different regions in $\sigma^{32}$ (region C and a region including amino acids 198-201 for DnaK) leading to its destabilization and supposedly inhibiting its capability to interact with the RNA polymerase.

Interestingly, mutations in the 2.1 domain of $\sigma^{32}$ were found to greatly increase its stability and activity *in vivo* although chaperone binding was not affected. It was later shown that the signal recognition particle (SRP) binds to this region and targets the sigma factor to the inner membrane where it then can efficiently be degraded by the membrane-bound protease FtsH. While region 2.1 is important for FtsH-dependent degradation *in vivo*, efficient reduction of $\sigma^{32}$ levels also requires region C. In addition to FtsH, several studies indicate that the cytoplasmic proteases Lon, HslUV and ClpXP are also involved in the degradation of $\sigma^{32}$, albeit to a lesser degree. Although DnaK and SRP are both required for effective control of $\sigma^{32}$ by FtsH, it is not known if they can bind to the same sigma factor at the same time and if binding is sequential.

Taken together the regulation of $\sigma^{32}$ activity by multiple elements of the protein quality control and transport machinery allows for the induction of heat shock genes in response to proteotoxic stress in the cytosol and the membrane as well as when increased protein targeting capacity is required. (Fig. 8). As in other systems,
GroEL/ES, DnaK, SRP as well as FtsH and the other proteases involved in the control of $\sigma^{32}$ are all part of the sigma factor’s regulon\(^{248,249,273}\), thus allowing for tight feedback control. Although the regulation of $\sigma^{32}$ has been mainly studied in \textit{E. coli}, features like FtsH-dependent degradation\(^{267}\) and DnaK-dependent inhibition seem to be widespread and to also take place in \textit{C. crescentus}\(^{203,268,274,275}\).

\textbf{Figure 8: Interconnected processes regulating $\sigma^{32}$ activity in \textit{E. coli}.} In the absence of stress, induction of the $\sigma^{32}$ regulon is kept low by inactivation and degradation of the heat shock sigma factor. Under conditions of normal protein folding demands a portion of the DnaK (DnaJ and GrpE not shown) and GroEL/ES machinery will be free to bind to $\sigma^{32}$, impede its association with the RNA polymerase (RNAP) and promote its targeting to the protease FtsH by the SRP in a still unknown fashion. While many signal recognition particles (SRPs) are bound to ribosomes harboring nascent chains exposing signal peptides (SP), a fraction of free SRP will bind to $\sigma^{32}$ and target the sigma factor to the membrane by interaction with the SRP receptor and the Sec translocon. At the membrane $\sigma^{32}$ will be degraded by the membrane-bound protease FtsH. Under proteotoxic stress or situations of increased SRP-dependent transport demands, the majority of DnaK and GroEL/ES will be associated with un- or misfolded proteins while a larger fraction of SRP will be bound to the ribosomes. Furthermore, unfolding of membrane proteins increases the demand for FtsH-dependent degradation which could reduce the efficiency of $\sigma^{32}$ degradation. The reduced association of inhibiting factors with $\sigma^{32}$, reduced targeting of the sigma factor to the membrane and possibly reduced degradation because of a busy FtsH protease allows an increase of free $\sigma^{32}$ which can associate with the RNAP core and drive the increased expression of its regulon.
5. CHAPERONE AND PROTEASE ESSENTIALITY

A gene is considered essential when it is required for the reproductive success of an organism. While this requirement is sometimes unconditional, some genes might only be necessary for growth in certain environmental or genetic contexts. Unconditionally essential genes include those encoding the machinery involved in basic processes like DNA replication, transcription and translation which are universal to life. At the other extreme a gene involved in amino acid synthesis will often only be required when this amino acid is not obtainable from an external source and is thus conditionally essential. Furthermore, differences in gene essentiality can be observed in different strains of the same organism owing to variability of interaction networks and the degree of functional redundancy. Together with differences in life style, these reasons can be extrapolated to variability of gene essentiality between organisms in general. Conditionality of gene essentiality can also be judged by the capability of a cell to compensate for the loss by mutating another gene. This often takes place if a gene product is essential because it is required for prohibiting the potentially lethal activity of another factor. Screening for suppressing mutations has been and is a fundamental tool in the understanding of essentiality and how regulatory networks function.

Although the different chaperone and AAA+ protease classes are highly conserved throughout the bacterial tree of life, not all of them are present in every phylogenetic group. This shows that not all chaperone systems and proteases are obligatory for a functional protein homeostasis and that functions fulfilled by one machinery in a particular organism can be fulfilled by another in a different one. Furthermore, in most organisms the proteostasis network shows both high functional redundancy and specialization, which also strongly depends on the environmental conditions the organism experiences. On the one hand this will be reflected by the fact that chaperones and proteases can share substrate pools and that one can compensate for the other after deletion. On the other hand some chaperones and proteases are absolutely required for cellular survival or their loss has at least
severe debilitating consequences under stress or even under physiological conditions.107,290–294.

Because many chaperones and proteases, at least potentially, act on a multitude of proteins and in general protein quality control, defects in the absence of these machineries are often attributed to pleiotropy. However, numerous instances of suppressing mutations have revealed in many cases that even for these broadly acting machineries the essential function can be attributed to an activity towards a specific substrate.290,292,293,295. Understanding these specific and essential functions has important implications for unraveling the involvement of chaperones and AAA+ proteases in regulatory networks and how broader-acting factors can pass over to functional specialization.

5.1. CHAPERONE AND PROTEASE ESSENTIALITY UNDER STRESS

Given the increased need for protein folding and degradation capacity during proteotoxic stress, single or combined deletion of many chaperones and AAA+ proteases hinders stress adaptation to varying degrees96,107,275,294,296 while boosting their levels can increase resistance. For example E. coli mutants identified in a screen for increased heat resistance compared to the wild type showed augmented production of GroEL/ES297. In certain chaperone and protease deletion mutants the accumulation of a broad range of un- and misfolded proteins and increased formation of protein aggregate assemblies is observable under heat stress.96,107,193. Deletion of ClpB will often fully abolish the capability of a cell to dissolve larger protein aggregate assemblies and/or prevent thermal stress adaption.54,107–110.

While most bacteria (including C. crescentus) harbor only one DnaK homolog, some bacteria have several. In addition to DnaK, E. coli has two further DnaK homologs, HscA and HscB which are involved in the biogenesis of iron-sulfur clusters and the resistance to cadmium ions and UV radiation, respectively.298. Out of these three DnaK homologs only DnaK is induced upon heat stress, required for heat stress resistance and broadly involved in the maintenance of protein homoestasis.298. Similarly, also in other bacteria harboring several DnaK homologs, one homolog is
required for maintaining general protein homeostasis under heat stress as seems to be the case for most bacteria harboring only one DnaK homolog\textsuperscript{96,295,299–301} (Tab. 1). Thus, while some DnaK homologs exhibit functional specialization accompanied by a smaller substrate pool, in most bacteria one DnaK homolog is heavily involved in or required for the folding of a large pool of thermolabile substrates\textsuperscript{20,174}.

A noteworthy exception among studied bacteria is the gram-positive \textit{B. subtilis} in which the sole DnaK homolog can be deleted without pronounced consequences for heat shock resistance apart from very severe thermal upshifts\textsuperscript{302}. Here, heat-resistance seems to strongly depend on the ClpP protease system, which appears to be involved in maintenance of protein integrity and bulk degradation\textsuperscript{193,204}. Interestingly, \textit{B. subtilis} lacks a ClpB homolog and prevention of aggregate formation or aggregate removal is highly dependent on the ClpP protease\textsuperscript{193,194}. In contrast, in \textit{E. coli} deletion of all three cytosolic proteases ClpXP, Lon and HslUV does not entail strong accumulation of un- and misfolded proteins nor is cell viability affected upon exposure to 42°C\textsuperscript{96,195}. Only during heat stress under DnaK-limiting conditions could a substantial increase in un- and misfolded proteins be identified in the absence of the protease Lon\textsuperscript{96}. Together these findings show that at least under stress conditions the protein homeostasis networks can be set up very differently between organisms\textsuperscript{96,193,194}.

Thermosensitivity as well as bulk protein unfolding and the detection of protein aggregates is a useful phenotype for understanding the function and measuring the degree of involvement of specific chaperones and proteases in general protein quality control. However, it is difficult to judge what defects directly originate from the burden of un- and misfolded proteins and what amount is actually lethal. For example \textit{E. coli} cells in which an estimated total of 5-10% of the proteome is aggregated are still viable\textsuperscript{96}. Thus, evaluating general protein un- and misfolding only provides limited insight on the possibility that the failure of folding or interacting with a specific substrate causes death. Genetic screens have proven useful to identify these essential functions. For example, an overexpression screen showed that a \textit{Shewanella oneidensis} mutant lacking HtpG has a reduced resistance to heat stress because the chaperone is required for the folding of the essential tRNA-maturation factor TilS under heat stress\textsuperscript{290}. Overexpression of TilS was found to restore viability in heat-challenged cells.
Furthermore, since proteases and chaperones often have regulatory functions in stress responses as well as cell cycle and developmental processes, major impairments in viability can result from misregulation, which often also affects viability in the absence of stress.

Table 1: Summary of DnaK homolog essentiality among investigated species. When a bacterial species harbors several DnaK homologs all homologs are listed. A DnaK homolog is listed as essential in the absence of stress when depletion of the chaperone is lethal or no knockout strains could be obtained under standard laboratory or low temperature conditions. If a DnaK homolog is essential in the absence of stress its potential essentiality under heat stress was not tested (not available, n.a.). The presence or absence of heat-inducibility is shown for every homolog. DnaK in B. subtilis is not considered essential for heat shock survival, because a phenotype can only be observed during relatively severe stress. References are indicated next to the species name.

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5.2. CHAPERONE AND PROTEASE ESSENTIALITY IN THE ABSENCE OF STRESS

While some chaperones and proteases are essential for viability under stress conditions, loss of certain machineries has little consequence in the absence of stress. A prominent example of such a factor is the disaggregase ClpB³⁴,³⁰⁷–³¹³. However, a number of chaperones and AAA+ proteases are absolutely required for viability of the cell even in the absence of stress, phenotypes that again depend on the machinery and the organism investigated. While ClpP mutants in B. subtilis are very heat
sensitive, a high number of un- and misfolded proteins can be detected even in the absence of stress showing that the protease machinery is important for maintaining overall protein homeostasis. However, these cells have also a lot of other defects including in processes such as motility, cell separation upon division, sporulation and competence development. While these can in principle all be affected by the general lack of protein quality control, impaired competence development in *B. subtilis* lacking ClpP is largely attributable to the absence of regulated proteolysis of ComK or Spx. A suppressor mutation in Spx was found to restore competence in *B. subtilis* cells in which *clpP* was deleted.

Similar or even more severe defects after loss of AAA+ proteases resulting primarily or in part from misregulation are also known in other organisms. While not lethal, knockout of the protease Lon leads to slower growth and cell cycle defects including cell elongation in *E. coli* and *C. crescentus*. In *E. coli* Lon is responsible for the regulated proteolysis of the DNA-damage effector SulA. Elevated levels of this factor partially inhibit cell division. In *C. crescentus* Lon is involved in the temporal regulation of cell cycle factor levels. While loss of FtsH is highly detrimental for growth but not lethal in *C. crescentus*, loss of this protease is lethal in *E. coli*. Through identification of a suppressing mutation, the protease was found to be crucial for cell membrane/envelope synthesis by degrading the lipopolysaccharide biosynthesis key enzyme LpxC in *E. coli*. Interestingly, although degradation by ClpXP plays an important role in regulating the cell cycle in *C. crescentus*, loss of this protease is lethal because it is required for the degradation of the SocB toxin affecting the DNA sliding clamp. Suppressors mutations in the toxin-encoding gene restored viability in the absence of ClpXP.

The essentiality of the constitutively expressed basic chaperoning machineries is often variable between organisms but can also be highly conserved. In *E. coli* the chaperonin GroEL/ES is absolutely required for the maturation of 13 proteins. Depletion of the chaperone leads to filamentation and lysis in *E. coli*. Interestingly, GroEL/ES homologs (some bacteria have several homologs) is essential in a broad spectrum of phylogenetically unrelated bacteria. Thus, folding of certain
conserved substrates is likely to be a universal essential function of this machinery across bacteria. In the case of the heat-inducible DnaK homologs, requirements for growth in the absence of stress are more varied (Tab. 1). While deletion of the sole DnaK homolog in *B. subtilis* does not lead to reduced growth, homologs were found to be essential in representatives of such diverse bacterial groups as the *Actinobacteria*, *Cyanobacteria*, and *Proteobacteria*. In *M. smegmatis* DnaK is required to prevent strong protein aggregation even under physiological conditions. Furthermore, the chaperone is directly required for the folding of an essential large multimodular lipid synthase without which membrane integrity is severely impaired.

DnaK is not absolutely required for the viability of *E. coli* but its loss only permits cells to live at temperatures lower than the standard culture temperature of 37°C. Severe protein aggregation in cells lacking DnaK can only be observed after additional deletion of the trigger factor encoding gene or when cells are incubated at elevated temperatures. Interestingly, mutations in the *σ32* encoding gene were found to partially suppress the defects in cells lacking DnaK suggesting that although not essential the chaperone’s regulation of the heat shock sigma factor plays an important role for the viability at reduced temperatures.

Taken together, although AAA+ proteases and chaperones often have global functions in protein quality control, their essentiality can depend on discrete activities on specific factors. Importantly, understanding this essentiality often yields deeper insight into regulatory pathways. As in many organisms but in contrast to *E. coli*, DnaK is also essential for viability in the absence of stress in the model bacterium *C. crescentus*. Here DnaK controls DNA replication through *σ32* regulation, however, it is not clear if this is the only essential function controlled through this activity.
Progression through the cell cycle is a fundamental requirement for the multiplication of cells. To this end a cell has to increase in mass, replicate its genetic material, segregate its chromosomes and finally divide. However, under stress conditions cells can arrest growth processes or specific cell cycle steps in order to avoid potentially lethal consequences if they were carried out with errors or failed\textsuperscript{325}. Furthermore, arresting proliferative functions can allow for the reallocation of resources towards the expression of protective and reparative factors. For example, during heat shock the translation of a large subset of proliferative proteins can be reduced while translation of chaperones and proteases is not impaired or even favored\textsuperscript{106,326}.

The cell cycle model bacterium \textit{C. crescentus} has proven to be an excellent tool to study stress adaptive mechanisms targeting cell cycle and growth and by now several mechanisms affecting different cell cycle steps are known\textsuperscript{325}. For instance, in an analogous way to \textit{E. coli}, damage to the chromosome elicits the production of small proteins inhibiting cell division but still allowing growth, leading to filamentous cells\textsuperscript{327–329}. Regulated proteolysis of cell cycle factors causing the inhibition of specific cell cycle steps has recently been shown to take place under various conditions like osmotic and ethanol stress\textsuperscript{330} as well as proteotoxicity induced by heat shock or DnaK depletion\textsuperscript{203}. In addition to a block in DNA replication other yet unknown processes seem to be affected by the heat shock regulon. In \textit{C. crescentus}, DNA replication initiator levels are strongly reduced in response to stationary phase entry and nutrient starvation by mechanisms currently not understood. Taken together the study of these still poorly understood processes in \textit{C. crescentus} promises more insight into how bacteria adjust growth and their cell cycle in response to stress.
6.1. REGULATED PROTEOLYSIS IN CELL CYCLE PROGRESSION AND STRESS ADAPTATION

Regulated proteolysis plays an important role in regulation of cell cycle processes by ensuring their correct sequence and their arrest and modulation during stress by acting in initiating, effecting and shutting down stress responses. Regulated proteolysis by Lon and ClpXP are crucial for proper cell cycle progression in *C. crescentus*. Here, periodical degradation of the cell cycle master regulator CtrA by ClpXP regulates the swarmer to stalk cell transition and DNA replication. Compared to transcriptional and translational activation or shut-down, degradational control allows for faster regulation of protein levels. Interestingly, it was recently shown that the degradational control over CtrA extends to conditions of high osmolarity and ethanol stress. These stresses induce rapid degradation of the cell cycle regulator CtrA culminating in a cell division block. The resulting filamentous cells were shown to have a growth advantage over those not degrading CtrA under the same conditions. Similar to heat shock response regulation through $\sigma^{32}$ degradation by FtsH, many transcriptional programs of stress responses are under degradational control. For example, in *E. coli* ClpXP regulates the transcription of genes which are part of the stationary phase and envelope stress response by degrading $\sigma^{E}$ while promoting $\sigma^{E}$ activity by degrading the antisigma factor RseA.

6.2. DNAK AND LON IN PROTEOTOXIC STRESS-DEPENDENT CELL CYCLE REGULATION OF CAULOBACTER CRESCENTUS

It was recently shown that inhibition of DNA replication by regulated proteolysis represents a discrete activity of the *C. crescentus* heat shock response. In a screen for mutants surviving otherwise lethal overreplication-inducing overexpression of the replication initiator DnaA, mutations reducing but not abolishing the functionality of the DnaK machinery were found to restore viability.

DnaK indirectly controls DNA replication through its function as a negative regulator of the $\sigma^{32}$-dependent heat shock regulon (Fig. 9). DnaK depletion or proteotoxic stress-dependent activation of $\sigma^{32}$ induces transcription of the heat shock
regulon, leading to increased expression of the protease Lon. Elevated levels of Lon as well as its allosteric activation by unfolded proteins increase the rate of DnaA degradation and prevent DNA replication\textsuperscript{203}. Reduction of Lon levels through attenuating mutations in $\sigma^{32}$ were found to restore DNA replication and viability in the absence of DnaK\textsuperscript{203}. However, it is not clear if maintaining DnaA levels is the only essential function mediated by DnaK’s regulation of $\sigma^{32}$. For example, in a similar fashion to the increase in Lon levels, higher levels of other proteases or heat shock proteins induced as part of the $\sigma^{32}$ regulon could inhibit other proliferative functions. Further investigation of DnaK essentiality and cellular changes occurring during $\sigma^{32}$ regulon activation in \textit{C. crescentus} will shed light on chaperone essentiality and if and how the heat shock response protects the cell from proteotoxic stress by inhibiting proliferative processes.

\textbf{Figure 9: Model of DnaK’s role in controlling DNA replication in \textit{C. crescentus}.} In the absence of proteotoxic stress DnaK inhibits the induction of the $\sigma^{32}$ regulon by destabilizing $\sigma^{32}$. DnaA can accumulate and DNA replication is initiated. Under strong proteotoxic stress, un- and misfolded proteins titrate DnaK away from $\sigma^{32}$. The liberated sigma factor induces expression of Lon and other heat shock proteins. Elevated Lon levels, together with an allosterically regulated increase in degradation efficiency by unfolded proteins (symbolized by unfolded proteins associated with Lon), lead to the removal of DnaA and thus the inhibition of DNA replication. Adapted from (203).
6.3. CELL CYCLE REGULATION IN RESPONSE TO NUTRIENT STARVATION

In their natural environment bacteria often face fluctuating nutrient availability resulting in periods of starvation. In the absence of nutrients cells stop cell cycle progression and increasing in cell mass. Guanosine penta- and tetraphosphate ((p)ppGpp) are signaling molecules strongly produced during carbon and amino acid starvation and have been shown to globally reduce protein synthesis and the growth rate in general as part of the so-called stringent response\(^\text{333}\).

In many organisms, starvation also leads to cessation of DNA replication. High production of (p)ppGpp inhibits the initiation of DNA replication in *E. coli*\(^\text{334,335}\) and *C. crescentus*\(^\text{336,337}\) while replication fork progression is affected in *B. subtilis*\(^\text{338}\). Previous work in *C. crescentus* has shown that DnaA abundance is greatly decreased during nutrient limitation\(^\text{337,339,340}\). It was proposed that starvation stimulates DnaA degradation\(^\text{337}\) and that (p)ppGpp is involved in regulating the stability of the replication initiator under this condition\(^\text{339}\). Interestingly, in *E. coli* (p)ppGpp and polyphosphate, production of which increases in response to elevated levels of (p)ppGpp, were shown to activate Lon to degrade ribosomal proteins\(^\text{64}\). While the mechanism controlling DnaA abundance in response to heat shock has recently been revealed\(^\text{203}\), it still remains largely unclear how DnaA levels are regulated in response to nutrient starvation and what direct roles, if any, the protease Lon and (p)ppGpp play in this process.
AIMS OF THE THESIS

In this thesis I aimed at deepening our understanding of basic molecular mechanisms governing chaperone essentiality as well as bacterial stress responses and recovery using proteotoxicity and nutrient starvation as model stresses in *Caulobacter crescentus*. Specifically, I addressed the following aims:

**Aim I:** Tracking and uncovering the dynamics of protein aggregate formation and inheritance in an asymmetrically dividing bacterium (paper I).

**Aim II:** Deepening our understanding of the essential role of the highly conserved chaperone DnaK in controlling heat shock regulon-dependent functions (paper II).

**Aim III:** Elucidating the mechanisms by which replication initiator levels are regulated in response to stationary phase entry and nutrient starvation (paper III).
MAIN FINDINGS

Paper I

Growth-driven displacement of protein aggregates along the cell length ensures partitioning of aggregates to both daughter cells in Caulobacter crescentus

In all bacteria in which protein aggregate localization was studied to date, these particles collect at the cell poles\textsuperscript{54,102,118,119}. In \textit{E. coli} this aggregation pattern was shown to lead to an almost obligatory retention in old pole cell lineages\textsuperscript{54,118}. Replicative aging of the old pole-inheriting stalked cells in the strongly asymmetrically dividing bacterium \textit{C. crescentus} was tentatively explained by a similar preferential retention of aggregates in these cells versus the new pole-inheriting swimmers\textsuperscript{118,121}. In this study we have investigated the dynamics and composition of protein aggregates, chaperone and protease requirements for aggregate dissolution as well as aggregate inheritance in \textit{C. crescentus}. We describe a new pattern of bacterial aggregate localization and inheritance preventing cell type-specific enrichment of the majority of aggregates.

Aggregates form as multiple DnaK-attended foci throughout the cell volume of Caulobacter crescentus

To monitor the dynamics of protein aggregate formation and dissolution as well as heat shock response induction in \textit{C. crescentus} by microscopy, we natively tagged the major chaperone DnaK with the monomeric fluorescent protein mVenus. Analysis of aggregation patterns and dynamics in Lon, sHSP and ClpB deletion backgrounds revealed similar requirements for aggregate dissolution as in \textit{E. coli}. Heat shock treatments as well as exposures to sublethal concentrations of the antibiotic kanamycin induced relocalization of DnaK-mVenus from a diffuse pattern into mainly two to four foci. These did not preferentially collect at the poles but were equally distributed over the cell length. Furthermore, the presence of aggregates...
induced a change in the spatial organization of the chromosome, with DNA staining less intense in regions occupied by aggregates. We conclude that in contrast to previously studied bacteria, in *C. crescentus* aggregate formation is not restricted to the cell poles which could be explained by *Caulobacter*'s less compact chromosome.

**The stress severity determines aggregate longevity and thus damage clearance through dissolution or dilution**

After establishing the basics of aggregate formation and dissolution in *C. crescentus*, we sought to track the dynamics of protein aggregation and aggregate clearance in developing microcolonies under different heat stress regimes by fluorescence time-lapse microscopy. While cells recovering from a one hour exposure to 40 or 42°C showed normal cell morphology and dissolved all aggregates within one cell cycle, cells stressed more severely were often elongated and exhibited heterogeneous aggregate lifespans. Both short-lived aggregates which are dissolved within a time-span shorter than a cell cycle as well as long-lived aggregates persisting over multiple generations could be found in cells continuously exposed to 44°C or recovering from one hour at 44 or 46°C. In turn, aggregates were largely stable in living but non-growing cells observed after temporary exposure to 46°C or continuous exposure to 44°C. Thus, we find that aggregate lifespan strongly depends on stress severity and the growth rate of the cells. Lower stress intensities allow for aggregate removal from individual cells by dissolution while during more severe stress persistent aggregates are handed down to the progeny over generations.

**Aggregates are handed down to both cell types at a stable frequency in *C. crescentus***

Aggregates were shown to be specifically retained in the old pole-inheriting cell in *E. coli* and *S. pombe*. We tracked the subcellular localization and inheritance of persistent aggregates arising under different conditions over several generations. We found that aggregates are relatively static particles that do not change their position relative to the cell poles during the longitudinal growth of a cell. However, the growth along the length of the cell and setting of new cell boundaries will cause most aggregates to assume a position closer to the new pole after each division event until
they are inherited by a swarmer cell (Fig. 10). Only a minor fraction (30%) of aggregates originally formed at the poles will remain in this location presumably due to the absence of cell growth in this area. The pattern of aggregate displacement and inheritance prevents polar deposition of aggregates that did not originally form there and results in these aggregates being partitioned to the old pole/stalked daughter cell and the new pole/swarmer cell at a constant rate. Taken together, we describe the mechanistic basis for a new pattern of bacterial aggregate inheritance in which most aggregates are not retained in the old pole-inheriting lineage.

Figure 10: Inheritance pattern of persistent protein aggregates in the asymmetrically dividing bacterium C. crescentus. Schematic showing how aggregate positional changes and inheritance are determined by growth along the length of the cell, the pole regions being excluded, and cell division. Numbers represent relative cell positions between the old (0) and the new pole (1). Lines allow tracking these positions along the growth and division events of the cell. Reproduced from (paper I).
Paper II

An essential regulatory function of the DnaK chaperone dictates the decision between proliferation and maintenance in Caulobacter crescentus

While DnaK’s global chaperoning activity is absolutely required for withstanding thermal upshifts in many bacteria, it is not clear why the chaperone is also often essential in the absence of stress. In *C. crescentus* DnaK is required for maintaining DnaA levels through inhibiting $\sigma^{32}$, however, their restoration is not sufficient to rescue DnaK-lacking cells. We provide new insight into potential further mechanisms involved in $\sigma^{32}$ regulation and show that in addition to causing the degradation of DnaA, strong $\sigma^{32}$ regulon induction leads to reallocation of cellular resources from proliferative to maintenance functions. We conclude that in *C. crescentus* preventing the inappropriate induction of the $\sigma^{32}$-dependent stress response which affects multiple cellular processes is the only essential function of DnaK in the absence of stress.

DnaK’s chaperoning function is dispensable in the absence of stress

Since DnaK is well known for its global chaperoning function as well as for being an essential partner for the disaggregase ClpB we hypothesized that its loss might induce strong protein aggregation affecting viability. However, *in vivo* aggregation assays revealed that the depletion of DnaK under non-stress conditions does not induce a significant increase in protein aggregation. We conclude that while loss of DnaK exacerbates protein aggregation during heat shock the chaperone is not required for maintaining global protein homeostasis in the absence of stress.

Mutations reducing $\sigma^{32}$ regulon induction restore viability in the absence of DnaK and provide new insight into the mechanisms controlling $\sigma^{32}$ activity

To identify causes for DnaK essentiality other than its role in maintaining DnaA levels, we performed a screen for mutations restoring viability in the absence of DnaK under DnaA overproduction conditions. Similar to the previously identified mutations suppressing the strong degradation of DnaA, also in the presence of DnaA most mutations were localized in the gene encoding $\sigma^{32}$, leading to a reduction of the
sigma factor’s activity. Importantly, the suppressor mutations could not rescue viability of DnaK-lacking cells at higher temperatures where cells sustained strong protein aggregation indicating a requirement for DnaK’s chaperoning activity during heat shock.

We also found other mutations negatively affecting the heat shock sigma factor less directly. An amino acid exchange in the C-terminus of the sigma factor-contacting constituent of the RNA polymerase RpoB reduced $\sigma^{32}$ promotor occupancy and thus regulon induction likely by lowering the affinity of the RNA polymerase for $\sigma^{32}$. Another mutation affecting availability of the RNA polymerase led to an increase in house-keeping sigma factor $\sigma^{70}$ levels resulting in increased competition between $\sigma^{32}$ and $\sigma^{70}$ for the formation of a transcriptional complex. Finally, we found a mutation in the protease HslUV rendering the protease capable of degrading $\sigma^{32}$ and strongly lowering sigma factor levels in the absence of DnaK.

Taken together our genetic screen and the analysis of the identified mutants provided new insight into the regulatory mechanisms controlling $\sigma^{32}$ activity and showed that DnaK’s regulatory function on $\sigma^{32}$ is essential not only to maintain DnaA replication but also other proliferative processes.

**$\sigma^{32}$ activity induces reprogramming of gene expression, reallocates resources from proliferative towards protective functions and inhibits growth**

The fact that most identified mutations circumvent strong $\sigma^{32}$ regulon induction in the absence of DnaK even when DNA replication is enabled indicates that $\sigma^{32}$ activity globally affects the cell or at least several processes in addition to DnaA degradation.

To understand mechanisms underlying the heat shock sigma factor’s growth-inhibitory effect we sought to identify the genes under its control as well as proteomic changes upon sustained $\sigma^{32}$ regulon induction. We identified 338 genes to be upregulated due to $\sigma^{32}$ activity of which 181 represent probable direct targets of the sigma factor. Importantly, the conserved heat shock-inducible chaperones and proteases only comprised a small fraction of these genes, while many were involved in metabolism, small molecule membrane transport and other functions. These results suggest that the $\sigma^{32}$-dependent transcriptional response in *C. crescentus* has the potential to rewire the cell in a profound way. Finally, we compared whole proteome fractions
of DnaK-harboring and -depleted cells by mass spectrometry to see how sustained $\sigma^{32}$ regulon induction affects the proteome. While, as expected, the total mass fraction of the part of the proteome containing highly abundant chaperones and proteases involved in repair and maintenance was strongly upregulated, proteins involved in DNA replication, certain metabolic processes and generally the sub-proteome involved in protein translation was strongly downregulated. Especially, levels of the translation elongation factor Tu (EF-Tu) and the small ribosomal protein RpsF were highly reduced.

In summary our data show that the $\sigma^{32}$-dependent stress response in $C.~crescentus$ represents a global reprogramming of the cell away from proliferative functions and towards cell maintenance. Importantly, in $C.~crescentus$, keeping this program suppressed is the sole essential function of DnaK in the absence of stress (Fig. 11).
Figure 11: Temperature-dependent essential function of DnaK and the decision between cell proliferation and maintenance in response to heat stress. A) Model depicting the temperature-dependent change of DnaK’s essentiality and how it dictates the decision between proliferation and maintenance behaviors. The wedge-shaped color-gradients symbolize how the essentiality of DnaK changes depending on the temperature. Temperatures at which experiments were performed are indicated. At low temperatures DnaK’s essential function is to inactivate $\sigma^{32}$ and in this way to promote proliferation while repressing maintenance functions. The higher the temperature, the more DnaK’s chaperone activity is required to prevent cytotoxic protein aggregation until, at the highest temperatures, it becomes essential for surviving heat shock. Under these conditions $\sigma^{32}$-dependent gene expression is de-repressed leading to the upregulation of maintenance functions while downregulating proliferative processes. B) Scheme of how activation of the $\sigma^{32}$ regulon reprograms the cell by inhibiting cell cycle progression and growth generally while upregulating functions required for cell maintenance.
Paper III

Nutritional control of DNA replication initiation through the proteolysis and regulated translation of DnaA

In response to nutrient starvation and stationary phase entry bacteria actively arrest their growth and cell cycle. It was shown that in various organisms DNA replication is often targeted at different levels. In this study we uncovered a mechanism by which levels of the DNA replication initiator DnaA are regulated as a function of nutrient availability in *C. crescentus*. While constant degradation by the protease Lon keeps DnaA turnover at an unchanged rate between exponential phase and nutrient-depleted conditions, the 5’ untranslated region (5’UTR) of the *dnaA* mRNA causes downregulation of translation under the latter. We show that by combining proteolytic and translational regulation, cells can achieve fast regulation of DnaA levels and thus DNA replication in response to nutrient availability.

DnaA levels correlate with nutrient availability in Caulobacter crescentus

Similar to heat shock conditions, entry into stationary phase in the standard peptone and yeast extract based rich PYE (peptone-yeast extract) growth medium leads to a strong downregulation of DnaA levels and thus a block of DNA replication in *C. crescentus*. Multiple stress factors like the accumulation of toxic products or exhaustion of resources could affect DnaA levels during entry to stationary phase. However, we found that nutrient depletion is the driving factor causing DnaA depletion and that DnaA levels are generally highly correlated with nutrient availability and growth rate. Stepwise reconstitution of PYE by adding different amounts of its components to minimal medium increased DnaA levels in a dose-dependent manner. Similarly, readdition of concentrated PYE components to a stationary phase culture quickly restored growth and DnaA levels. Together, these results excluded the possibility of an accumulation of waste products as a driver of DnaA downregulation. Furthermore, *C. crescentus* cells starved of glucose when provided as the sole carbon source in minimal medium experienced a drastic reduction of DnaA levels that can be restored by nutrient readdition. Taken together we identify the decline of nutrient availability and cellular growth rate at entry to stationary phase in PYE as the cause of the observed reduction of DnaA levels.
DnaA elimination during entry into stationary phase and carbon source depletion is not caused by (p)ppGpp

Nutrient starvation conditions like entry into stationary phase or carbon source exhaustion induce the stringent response mediated through (p)ppGpp. Alongside functions in downregulating protein translation and modulating transcription, this small signaling molecule has also been proposed to affect DnaA levels in *C. crescentus* and *E. coli*. To test if (p)ppGpp is involved in the downregulation of DnaA upon stationary phase entry or carbon source depletion we compared DnaA levels at different time points along the growth curve in the wild type and a mutant lacking SpoT, the only (p)ppGpp synthase in *C. crescentus*. While ΔspoT cells were deficient in arresting growth, we found that DnaA levels are downregulated and DNA replication arrested with similar timing as in the wild type. Thus, we conclude that (p)ppGpp is not required to downregulate DnaA during entry to stationary phase or carbon source depletion.

Growth phase-dependent reduction of DnaA requires degradation by Lon and inhibition of dnaA mRNA translation through the 5'UTR

It was previously shown that DnaA is constantly degraded at a stable rate by the protease Lon even in the absence of stress. Proteotoxic stress leads to quick depletion of DnaA levels by increasing the rate at which Lon degrades DnaA, while DnaA synthesis remains largely unaffected ([Fig. 12](#)). By monitoring DnaA levels in Δlon cells entering stationary phase or starving for a carbon source, we found that degradation by Lon is also required to reduce DnaA levels under these conditions. However, *in vivo* degradation assays revealed that in contrast to heat stress, the degradation rate is not significantly increased in comparison to non-stress conditions, indicating that DnaA levels are regulated on the level of synthesis. While we found that dnaA transcript levels do not change significantly enough to account for the observed reduction in DnaA levels, the relatively long 5'UTR of the dnaA mRNA turned out to be of great importance in controlling DnaA synthesis. Removing this sequence stretch from transcripts expressed from either the native dnaA or a lac promoter completely abolished DnaA downregulation.
In summary, these results show that the reduction of DnaA levels during nutrient depletion is achieved through unchanged constant degradation by Lon outpacing the now strongly reduced *dnaA* mRNA translation. Translation of the *dnaA* mRNA is regulated by a yet to be elucidated mechanism involving a directly or indirectly nutrient availability-sensing 5’UTR (Fig. 12).

**Figure 12: Regulation of DnaA levels in response to stress.** During exponential growth DnaA levels allowing DNA replication initiation are maintained despite constant degradation by the protease Lon through high translation rates of the *dnaA* mRNA. During stationary phase and carbon starvation, the reduced nutrient availability causes the inhibition of translation of the *dnaA* mRNA by a yet unresolved mechanism involving the 5’UTR. Under these conditions of reduced DnaA synthesis the constant degradation rate of Lon is sufficient to clear DnaA and cause a block of DNA replication initiation. In contrast, under proteotoxic stress, synthesis rates are largely maintained while the degradation of DnaA by Lon is strongly increased as a result of higher levels of Lon and allosteric activation of Lon leading to a G1-arrest. Adapted from (paper III).
DISCUSSION AND FUTURE PERSPECTIVES

This thesis provided new insight into bacterial aggregate inheritance, the molecular principles causing chaperone essentiality and the regulatory mechanisms affecting growth and cell cycle progression in response to proteotoxic stress and nutrient starvation. Our findings have implications for the general view of aggregates as aging factors as well as on the general idea of pleiotropy behind chaperone essentiality. Furthermore, they build a fundament for future research on bacterial growth and cell cycle adjustment in response to stress.

A new pattern of protein aggregate inheritance and its relevance for questions of cellular damage segregation

Larger aggregates, as discrete particles unequally distributed over a cell population have been proposed to be driving factors for heterogeneity in cellular aging and stress resistance. We find that in *C. crescentus* aggregates form throughout the cell volume, do not collect at the poles and are partitioned between daughter cells over generations at a stable rate. This represents an aggregation pattern very different from the polar collection observed in previously studied bacteria and the concomitant retention in the old pole daughter cell in *E. coli*. In *E. coli*, spatial occlusion by the compact nucleoid in the center of the cell enforces aggregate formation at the poles. In agreement with this, we suggest that the comparably more relaxed chromosome which extends through the entire cell body in *C. crescentus* does not produce high enough differences in molecular crowding to drive polar deposition.

Similarly as for the old pole cell in *E. coli, S. pombe* and the mother cell in *S. cerevisiae*, previous works proposed that aging in *C. crescentus* could be caused by the accumulation of protein aggregates in the old pole/stalked cell. Our finding that most persistent aggregates do not accumulate at the poles could refute this hypothesis and calls for other explanations, for example the retention of older membrane...
components. First results of an analysis of the growth rate of aggregate containing versus aggregate-lacking cells indicate that only inheritance of very large aggregates might reduce a cell’s growth rate in *C. crescentus*. It remains to be investigated in detail, including different aggregate types and environmental conditions, if inheriting an aggregate impacts cell growth in this organism. Since most aggregates eventually leave an old pole/stalked cell to be inherited by a swarmer cell long after stress exposure, we propose *C. crescentus* as an excellent model system to study potential age-related old pole and aggregate effects in isolation. This might be of even higher importance in light of recent studies performed in *E. coli* and *S. pombe* in which the long established negative impact of the presence of aggregates was relativized.

By studying aggregate formation and inheritance in the asymmetrically dividing bacterium *C. crescentus* we have uncovered a new mechanism of aggregate inheritance. We expect that the study of further species, especially those belonging to groups with unusual morphology or intracellular structures like *Streptomycetes*, *Planctomycetes* or *Cyanobacteria*, will lead to important new insight on aggregate distribution and inheritance in bacteria and help understand processes of damage segregation and heterogeneity in aging and stress resistance.

**Heat shock protein essentiality and single cause behind pleiotropy**

In this work we have tested the requirement of different chaperones and AAA+ proteases for the viability of *C. crescentus* in the absence of stress and find that the disaggregase ClpB, the two small heat shock proteins sHSP1 and sHSP2 and the protease Lon are dispensable under these conditions. In stark contrast, the highly conserved chaperone DnaK is essential even in the absence of stress. DnaK is a major hub for protein homeostasis in *E. coli* and loss of this chaperone in mycobacteria leads to a lethal protein homeostasis collapse. Thus defects in the cell cycle, growth or in general viability after the loss of major chaperones are often tentatively explained as being of pleiotropic origin due to the lack of folding of several or many clients.

Importantly, we find that DnaK’s chaperoning function is dispensable at physiological temperatures in *C. crescentus* and that its regulatory function as an inhibitor of the σ32 regulon is not only required to allow DNA replication but also
essential for other proliferative processes. Interestingly, although loss of DnaK is not lethal in *E. coli*, reducing induction of the $\sigma^{32}$ regulon alleviates defects in the cells\(^{295}\). We thus propose that DnaK essentiality in other *Proteobacteria* might be explainable by the chaperone’s evolutionary new function as a regulator of the $\sigma^{32}$ regulon in this bacterial group.

Taken together, this study exemplifies that the essentiality of such a broad acting factor as DnaK can at least in some cases be broken down to one specific function. Furthermore, we have shown that identifying the reason for essentiality under one condition is not always sufficient to extrapolate to others. While DnaK’s chaperoning function is essential under heat stress in *C. crescentus* it is its regulatory function that is required in the absence of stress. The reason for the essentiality of other chaperones in the absence of stress still remains unclear. For example, depletion of GroEL/ES leads to distinct cell cycle defect phenotypes in *C. crescentus*\(^{275}\). Future studies thus promise to deepen our knowledge about chaperone function in growth and cell cycle control.

**Additional regulatory layers of $\sigma^{32}$ control and the HslU mutation as a tool for studying protease function**

In our suppressor screen we identified a number of mutations reducing heat shock response induction that were located outside of the $\sigma^{32}$ encoding gene *rpoH*. These affected the association of $\sigma^{32}$ with the RNA polymerase or caused non-canonical degradation of the sigma factor. In principle, all these processes could also be relevant in wild type cell $\sigma^{32}$ regulation. It is intriguing to find a mutation in the sigma factor-contacting domain of the RNA polymerase $\beta$-subunit which reduces $\sigma^{32}$ promoter occupancy. While regions relevant for house-keeping sigma factor binding have been identified in this protein\(^{344}\), knowledge about alternative sigma factor binding determinants is still limited. It is attractive to speculate that different regions of the $\beta$-subunit could be involved in stress-dependent regulation of the RNA polymerase’s affinity for alternative sigma factors. Similarly, regulation of the abundance or availability of sigma factors competing with $\sigma^{32}$ could be an important mechanism regulating the heat shock response in wild type cells as proposed previously\(^{274,345}\).
In addition to these alterations in the transcriptional machinery, we found that a small amino acid deletion in the unfoldase ring of HslUV causes the protease to degrade \( \sigma^{32} \). Amongst the highly conserved AAA+ proteases in bacteria, the biological function of HslUV is least understood. This is particularly surprising in light of the fact that it is the most strongly heat shock response induced AAA+ protease, both in *E. coli* and in *C. crescentus*. It was suggested that in addition to FtsH, HslUV is also involved in the degradation of \( \sigma^{32} \) *E. coli* and it was later shown that *E. coli* HslUV degrades \( \sigma^{32} \) in vitro with increasing efficiency as temperature increases. We did not observe that *C. crescentus* cells overproducing wild type HslUV exhibit a shorter half-life of \( \sigma^{32} \), neither under normal nor under elevated temperatures. It remains to be understood if in *C. crescentus* under certain conditions an adaptor or other processes modulate HslUV-dependent degradation of \( \sigma^{32} \) or if it takes place with the wild type protein at all. Independent of this question, finding a mutation so drastically affecting substrate recognition or degradation efficiency of HslUV could provide an important tool for gaining further insight into the machinery’s mode of action and biological function.

**Arresting growth and cell cycle progression as a potential function of the \( \sigma^{32} \) regulon**

We show that in *C. crescentus* strong induction of the \( \sigma^{32} \)-dependent regulon leads to the reallocation of resources by the proteome involved in translation towards the synthesis of heat shock proteins. While we as of yet do not know how drastic this effect is under heat stress or other proteotoxic stress conditions, we suggest that a lower or temporary inhibition of translation could be beneficial during stress. Especially nascent peptide chains are vulnerable to these stresses under which they are thought to be one of the main sources of protein aggregation\(^{346}\). Furthermore, slowing down translation can increase viability in cells experiencing proteotoxic stress which was among other conditions shown for EF-Tu depletion\(^{347}\). Importantly, our data show that this translation factor is strongly downregulated after strong heat shock response induction.

Regulated proteolysis is an integral part of many stress adaptive mechanisms\(^7\). Under proteotoxic stress conditions, Lon-dependent proteolysis of DnaA is strongly
increased leading to a DNA replication block and thereby protection of chromosome integrity. We show that all of the highly conserved AAA+ proteases are upregulated as part of the \( \sigma^{32} \) regulon. This provides the possibility that at least a number of the downregulated proteins are subject to regulated proteolysis with the possible purpose of inhibiting a vulnerable proliferative process or redirecting the cellular program towards maintenance.

By studying the essential function of DnaK in the absence of stress we have found that the \( \sigma^{32} \)-dependent regulon has a growth-inhibiting activity. While we have many indications of how \( \sigma^{32} \) activity might inhibit growth and cell cycle progression, it remains to be shown if it does so under stress conditions and what specific mechanisms may underlie this inhibition. Specific experimental inhibition of identified processes without a concomitant upregulation of heat shock proteins could allow to test if the cell benefits from the inhibition of certain processes under stress in an independent way.

**Targeting of DNA replication as specific cell cycle control mechanism under nutrient starvation**

Conceptually similar to the \( \sigma^{32} \) activity-dependent inhibition of DNA replication initiation, different examples of how cells block proliferative processes in response to nutrient starvation are known in various bacteria. Here we have identified a discrete (p)ppGpp-independent mechanism by which DNA replication is specifically regulated in response to nutrients. While the replication initiator DnaA is constantly degraded by Lon at a stable rate, entry to stationary phase and glucose exhaustion leads to a strong reduction of \( dnaA \) mRNA translation which is sensed through the 5'UTR. We suggest that the combination of the short half-life of DnaA together with its translational control allows for faster and more precise regulation than possible through transcriptional regulation alone.

While we have identified the long 5'UTR as being responsible for nutrient-dependent regulation of \( dnaA \) translation, the nature of the signal and how it is sensed remains to be uncovered. Importantly, in addition to carbon source exhaustion, starvation for nitrogen as well as fatty acids was shown to lead to the downregulation of DnaA and we found that to also be the case for starvation for...
phosphate and sulfur (unpublished data, Michele Felletti). It remains to be investigated if the downregulation under these conditions also involves the 5'UTR. Depletion of various nutrients influencing translation in this way suggests that changes in the central metabolism or the growth rate control the signal sensed by the leader. We envisage a 5'UTR-interacting metabolite, a regulatory protein or a small non-coding RNA as signals transmitting nutritional state potentially by changing the mRNA’s secondary structure.

Our study has unveiled a new mechanism by which bacteria regulate the essential and sensitive process of DNA replication initiation in response to nutrient availability. Future work identifying the discrete signal transferring the information to the dnaA leader will have significant impact on our understanding of how bacteria balance cell cycle progression and protection of vulnerable processes.
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