

Mechanisms of chaperones and proteases

Introduction

Our research aims at understanding the molecular basis of the intricate functional network of chaperones and proteases that controls protein folding processes in the cytosol. This quality control network is responsible for maintaining protein homeostasis in cells and thereby is directly linked to various diseases and aging. Using bacteria, yeast and mammalian cells as model systems, and a methodological spectrum ranging from genetics, cell biology, biochemistry to biophysics, we are currently focusing on the cell biology and molecular mechanisms of (i) folding of newly synthesized proteins, (ii) quality control and regulation of proteins by the Hsp70 chaperone network, (iii) the bacterial Hsp90 chaperone, (iv) AAA+ chaperones involved in regulated proteolysis and bacterial pathogenesis, (v) cellular strategies fighting protein aggregates.

Folding of newly synthesized proteins

G. Kramer and B. Bukau, project leaders
A. Becker, A. Hoffmann, D. Huber, F. Merz, S. Preißler, A. Rutkowska, B. Zachmann-Brand, A. Sandikci, F. Gloge, M. Rocco

How newly synthesized proteins fold into their correct tertiary structures, within the crowded cellular environment, is one of the most fundamental yet complicated problems in molecular biology. We are particularly interested in dissecting the first folding events in the life of proteins that are mediated by ribosome-associated chaperones, targeting factors and processing enzymes.

1. Trigger Factor

In bacteria, Trigger Factor (TF) is the first chaperone that associates with nascent polypeptides as they emerge at the peptide exit tunnel of the large ribosomal subunit. N. Ban (ETH Zuerich), in collaborative effort with us and the Deuerling lab (now at the University of Konstanz), solved the crystal structure of TF at 2.7 Å resolution. TF has an extended shape composed of an N-terminal ribosome binding domain, a C-terminal domain that builds the center of the molecule with two protruding “arms”, and a peptidyl prolyl isomerase (PPIase) domain located at the distal end of the protein. The three domains form a long cavity



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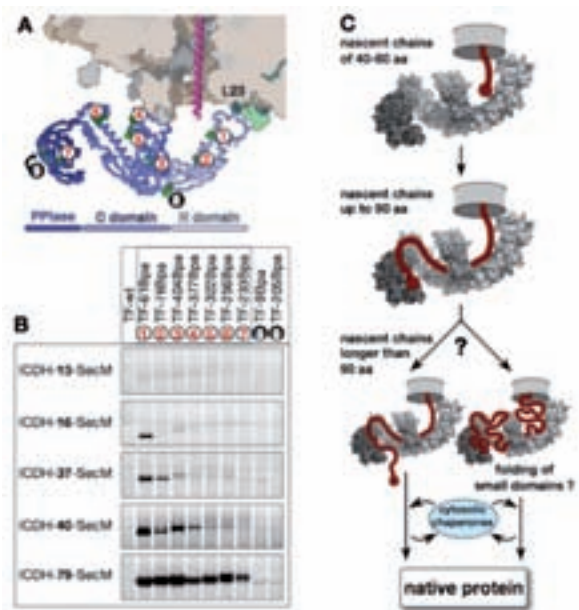


Fig. 1: Mechanism of action of Trigger Factor on nascent chains during translation. A. Structural model of ribosome-bound TF showing the crosslinker positions within TF. B. Nascent chains traverse the interior of TF in a length dependent fashion C. Model of the passage of a nascent chain through the interior of TF.

which starts at the ribosome binding domain and extends towards the distal PPIase domain. Based on the structure of the N-terminal domain of TF crystallized on the ribosome, and a recent cryo-EM structure of full-length TF stabilized in association with ribosome-nascent chain complexes (collaboration with the Ban lab), we propose that TF hunches over the exit tunnel of the ribosome leaving enough space to accommodate small folded domains of nascent chains. TF seems to adopt also other conformations, thereby being able to flexibly adopt to different nascent chains. We performed a detailed crosslinking analysis of the path of nascent polypeptides as they emerge at the ribosomal exit tunnel (Figure 1). Dependent on their length and folding states, the nascent chains contact the entire interior of the cavity of TF until they reach the PPIase domain. These interactions allow TF to protect unfolded nascent polypeptides from degradation, thereby providing one mechanism by which TF supports productive *de novo* folding of proteins into their native states.

We also investigated the kinetics of the binding/release cycle of TF with translating ribosomes. Both association and dissociation rates of TF-ribosome complexes are modulated by the presence of nascent chains, whereby their length, sequence and folding status are major influencing parameters. Overall the nascent chains can stabilize TF binding to the ribosomes in such a way

that the half-life of these complexes increases from 15 seconds up to 50 seconds. How exactly TF affects the folding process during this time frame remains enigmatic.

2. Peptide Deformylase

Bacteria, plastids and mitochondria initiate translation with N-formyl methionine. This formyl group is removed co-translationally by the enzyme peptide deformylase (PDF), often followed by further cleavage of the N-terminal methionine by methionine aminopeptidase (MAP). In collaboration with the Ban lab, we showed that PDF interacts directly with the ribosome via a C-terminal helical extension, an interaction which contributes to the functionality of PDF *in vivo*. Crystallographic analysis of the complex between the ribosome-interacting helix of PDF and the ribosome revealed that the enzyme orients its active site towards the ribosomal tunnel exit for efficient co-translational processing of emerging nascent chains. PDF and TF seem to be able to bind to the ribosome in close vicinity, so that PDF positions its active site towards one of the lateral openings of TF between the arms and the ribosome-binding tail.

Quality control and regulation of proteins by the Hsp70 chaperone network

B. Bukau, project leader

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1. Mechanism of the Hsp70 machine

Hsp70 chaperones with their plethora of co-chaperones are key components of the cellular chaperone system. Central to the Hsp70 mechanism is the allosteric control of substrate interaction by nucleotide. To elucidate this coupling mechanism we performed a structure-function analysis of the DnaK homolog in collaboration with the Mayer lab (ZMBH). We identified a proline switch, which controls the structural transition between the ATP- and ADP-bound states of the ATPase domain, and an arginine that relays the conformational change onto the domain surface. The coupling mechanism is critically dependent on the linker that connects the ATPase and substrate binding domains. By extending these studies, including an analysis of the action of members of the DnaJ family, we are heading towards a comprehensive model of the Hsp70 mechanism.

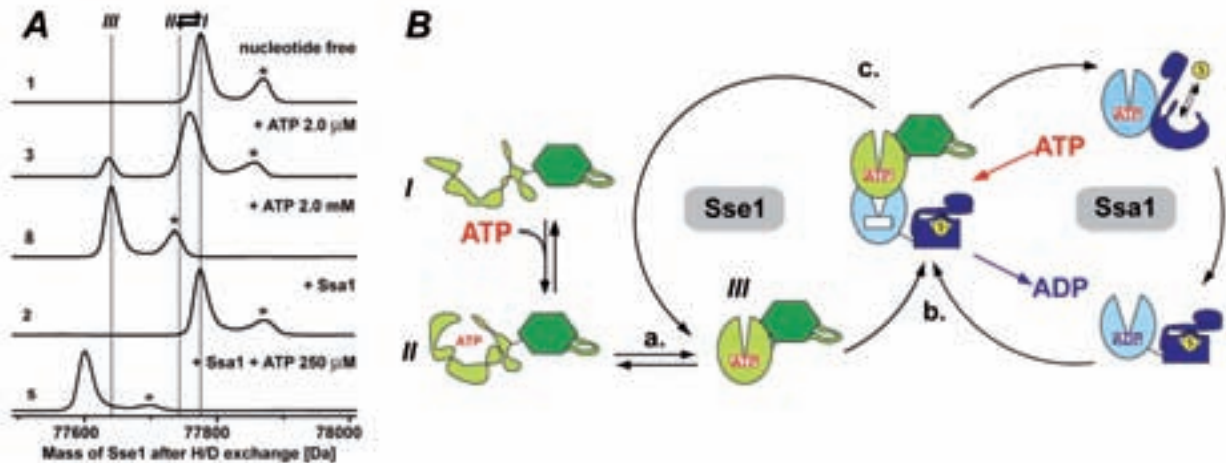


Fig. 2: Nucleotide binding to Sse1 controls its action as nucleotide exchange factor for Ssa1.

A. The different conformations induced by ATP and Ssa1 binding are resolved by monitoring their respective amide hydrogen exchange properties in H/D exchange experiments.

B. Model for the Sse1-catalyzed nucleotide-exchange cycle. Sse1 (dark grey) requires stabilization through ATP binding (a.) to interact with Ssa1 (light grey) and catalyze nucleotide release (b.). Rebinding of ATP to Ssa1 triggers dissociation of the Sse1-Ssa1 complex and completes the cycle of nucleotide exchange (c.).

2. Molecular basis of Hsp70-substrate interactions: regulation of the heat shock response by Hsp70

Several observations led us to hypothesize that Hsp70 binding may induce conformational changes within protein substrates so that (re)folding, disassembly or even degradation is facilitated. To test this hypothesis we analyzed the effects of DnaK binding to the heat shock transcription factor sigma32. DnaK binding inhibits sigma32 activity and promotes its proteolytic degradation, thereby providing a negative feedback regulation of the heat shock response. DnaK binds to an unfolded segment of sigma32, whereas the DnaJ co-chaperone binds to a separate site where it induces structural changes which facilitate DnaK association. DnaK association destabilizes parts of the N-terminal domain, which may allow the regulated degradation of sigma32. These findings provide the first clear example of an Hsp70-induced conformational alteration within a substrate.

3. Role of Hsp110 chaperones in the Hsp70 network

Our interest in the cellular network of Hsp70 chaperones has led us to study the poorly characterized Hsp110 proteins that represent an evolutionary diverged branch of the Hsp70 superfamily. The yeast *Saccharomyces cerevisiae* possesses two highly similar Hsp110 encoding genes, *SSE1* and *SSE2*. We showed that Sse1 acts as potent nucleotide exchange factor (NEF) for the two major Hsp70 proteins of the yeast cytosol, Ssa1 and Ssb1. Hsp110 proteins appear to represent

the major nucleotide exchange factors for Hsp70 chaperones in eukaryotic cells. We found that Sse1 itself is controlled by nucleotide. Nucleotide binding results in formation of a stabilized conformation of Sse1 that is required for association with Ssa1 (Figure 2). The interaction triggers release of bound ADP from Ssa1. Rebinding of ATP to Ssa1 prompts the dissociation of the complex. Sse1 represents the first NEF that requires nucleotide for its own activity. Together these findings demonstrate that different Hsp70 chaperones, through physical interaction and cross-regulation, constitute a functional network in the cytosol.

To obtain further insights into the role of Hsp110 in the cytosolic chaperone network, we have evaluated what role Sse1 plays in yeast prion biology. Formation and propagation of the yeast prion $[PSI^+]$ critically depends on the activities of the disaggregase Hsp104 and Ssa1. Our *in vivo* studies reveal that the nucleotide exchange activity of Sse1 on Ssa1 is central to the establishment and maintenance of $[PSI^+]$. Intriguingly, Sse1 possesses a chaperone-like activity that directly stabilizes early folding intermediates of the Sup35 prion conformation.

Currently, we are focusing on dissecting the mechanistic and structural features of the Hsp70-NEF interaction and on elucidating the functional role of Hsp110 in the Hsp70 network.

The bacterial Hsp90 chaperone

G. Kramer, project leader

In contrast to our understanding of the function of Hsp90 in eukaryotes, little is known about the function of Hsp90 in bacteria. In *E. coli* deletion of the gene encoding the bacterial Hsp90 homolog, HtpG, is not lethal, but results in a phenotype that is slightly temperature sensitive. We are pursuing roles for HtpG in physiology and adaptation to changing environmental conditions. Furthermore, we are directly testing the hypothesis that HtpG buffers genetic diversity by stabilizing mutant proteins in a similar manner to eukaryotic Hsp90 and thereby increases the fitness of this bacterium under particular conditions.

Experimental approaches include the identification of specific substrates of HtpG by chemical crosslinking and subsequent identification by mass spectrometry. Initial experiments lead to the identification of proteins involved in transcription, translation and amino acid catabolism. Further *in vivo* experiments using a dominant negative allele of HtpG strongly hint towards the central gene regulator CRP in *E. coli* as a potential client of HtpG chaperone function. In an ongoing collaboration with the laboratory of Susan Lindquist (Whitehead Institute, MIT, Cambridge, USA), we are analyzing the consequences of loss of HtpG activity on the fitness of mutated bacterial cells under a variety of environmental conditions where signal transduction is key to survival.

AAA+ chaperones involved in regulated proteolysis and bacterial pathogenesis

1. Regulated proteolysis by the N-end rule pathway

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R. Schmidt, R. Zahn

Protease systems of the bacterial cytosol are composed of ring-shaped oligomeric AAA chaperones with ATPase activity (e.g. ClpA, ClpX) and an associated peptidase (e.g. ClpP). These proteolytic machineries are engaged in protein quality control as well as regulated proteolysis. We recently found that *E. coli* ClpS, a ClpA-specific adaptor protein, is essential for the degradation of N-end rule model substrates. The N-end rule pathway was defined earlier by Varshavsky and coworkers to mediate the degradation of regulatory proteins

via recognition of destabilizing residues at the N-termini of proteins, termed N-degrons. ClpS recognizes N-degrons and through direct association transfers them to the interacting ClpA/ClpP protease, thereby compensating for the absence of ubiquitin in prokaryotes. ClpS exhibits homology to N-recogin, an E3-Ligase that targets N-end rule substrates for ubiquitin-dependent proteasomal degradation in eukaryotes, showing the evolutionary conservation of the N-end rule pathway at the level of substrate recognition. In an attempt to elucidate the biological role of the N-end rule pathway we are currently searching for *in vivo* N-end rule substrates.

2. Role of the ClpV chaperone in bacterial pathogenesis

A. Mogk, project leader

G. Bönemann, A. Pietrosiuk

We recently identified a novel AAA+ protein family member, ClpV, which is present in many pathogenic proteobacteria. ClpV is always co-organized with a conserved gene cluster that encodes for the novel type VI secretion system (T6SS). We are studying ClpV function in T6SS and virulence by using the human pathogen *Vibrio cholerae* as model organism and *Dictyostelium discoideum* as model host. We could demonstrate that ClpV is strictly required for the secretion of Hcp and VgrG, the secretory proteins of T6SS. A ClpV pore mutant cannot restore type VI protein secretion implying that ClpV-mediated substrate threading is an integral part of protein export. Using a biochemical approach we identified VipA and VipB (ClpV interacting protein) as interacting proteins that associate with the N-terminal domain of ClpV. VipA and VipB represent conserved members of all T6SS and form a complex. Analysis of *V. cholerae* *vipA* and *vipB* mutants revealed that both are crucial for type VI protein secretion. We are currently investigating whether the VipA/VipB complex serves as an adaptor protein that directs the flow of secretory proteins to ClpV or whether one component of the complex itself acts as ClpV substrate.

Cellular strategies fighting protein aggregates

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1. Mechanism of protein disaggregation by the ClpB chaperone

The bacterial AAA+ protein ClpB and its Hsp104 homolog in yeast are ring-shaped chaperones that play a central role in protein quality control by mediating the solubilization and refolding of aggregated proteins in cooperation with the DnaK (Hsp70) system. We showed earlier that DnaK acts at initial stages of the disaggregation process, enabling ClpB to extract single unfolded polypeptides from aggregates via substrate threading through its central channel. We now demonstrated that the middle domain of ClpB, which is an unusual domain insertion into the first of the two ATPase domains of ClpB, acts as a regulatory device in the disaggregation process by coupling the threading motor of ClpB with the DnaK chaperone activity.

In a recent study we analyzed the disaggregation mechanism of aggregates consisting of protein fusions comprising misfolded and native domains, to mimic the aggregation of multidomain proteins. ClpB/DnaK reactivated all fusion proteins with similar efficiencies, demonstrating that the presence of folded domains does not affect protein disaggregation. Native domains were not unfolded during the disaggregation process and resisted ClpB-mediated threading indicating that partial threading of the misfolded moiety is sufficient for aggregate solubilization. ClpB/DnaK reactivated aggregated proteins even when two stably folded domains flank the aggregated moiety, demonstrating threading of internal substrate segments by loop formation. ClpB rings are highly unstable which may facilitate dissociation of trapped substrates during threading. The information for complete or partial threading through the ClpB pore is provided by the thermodynamic properties of the substrate and allows a case-by-case decision to ensure optimal reactivation yields.

2. Mechanism and cellular activities of yeast Hsp104

Hsp104, the ClpB homolog of *S. cerevisiae*, is not only involved in protein disaggregation and thermotolerance but also in the establishment and propagation of the amyloid fiber state of prion-like proteins, e.g. the PSI+ state of Sup35. It remained unclear whether the Hsp104 activity on Sup35 prions depends on the threading of amyloidogenic Sup35 through the central pore of Hsp104. By analyzing a genetically engineered Hsp104 variant, HAP, which physically interacts and cooperates with the bacterial peptidase ClpP, we were able to show that similar to ClpB, threading of aggregated protein through the central pore also holds true for Hsp104. Furthermore, expression of the HAP variant allowed the isolation of Sup35 from PSI+ cells as encapsulated substrate. Prion propagation was no longer supported by a translocation-deficient Hsp104 mutant indicating that the fragmentation of Sup35 fibrils depends on an Hsp104-mediated threading activity.

Another study we started recently is based on the hypothesis that similar to other chaperones, Hsp104 might not only be involved in protein quality control but also in cellular housekeeping activities. In order to identify potential physiological substrates of Hsp104 in yeast cells, we expressed HAP/ClpP that degrade Hsp104 substrates instead of remodelling them. The rationale of this approach was that the irreversible degradation of substrate proteins might exert a dominant negative effect and reveal Hsp104-dependent processes. Indeed, expression of this variant led to growth arrest and changes in cell morphology and the actin cytoskeleton at physiological temperatures. Using a candidate approach we identified components of the polarisome complex and the septin ring as substrates for Hsp104, implying a function of Hsp104 in polarity formation during the yeast cell cycle (Figure 3). As an alternative, unbiased approach, we performed a synthetic lethality screen and identified a protein involved in cytokinesis to be synthetically lethal with *hsp104*. In promoter shut-off experiments we could demonstrate that double deletions of *hsp104* and the identified protein cannot establish a polarized actin cytoskeleton, adding further genetic evidence for a role of Hsp104 during polarity formation. The involvement of Hsp104 in bud formation is intriguing, as it might provide a direct link between cell division and protein damage repair. Its role during polarity formation may enable Hsp104 to link protein quality control with the yeast cell cycle.

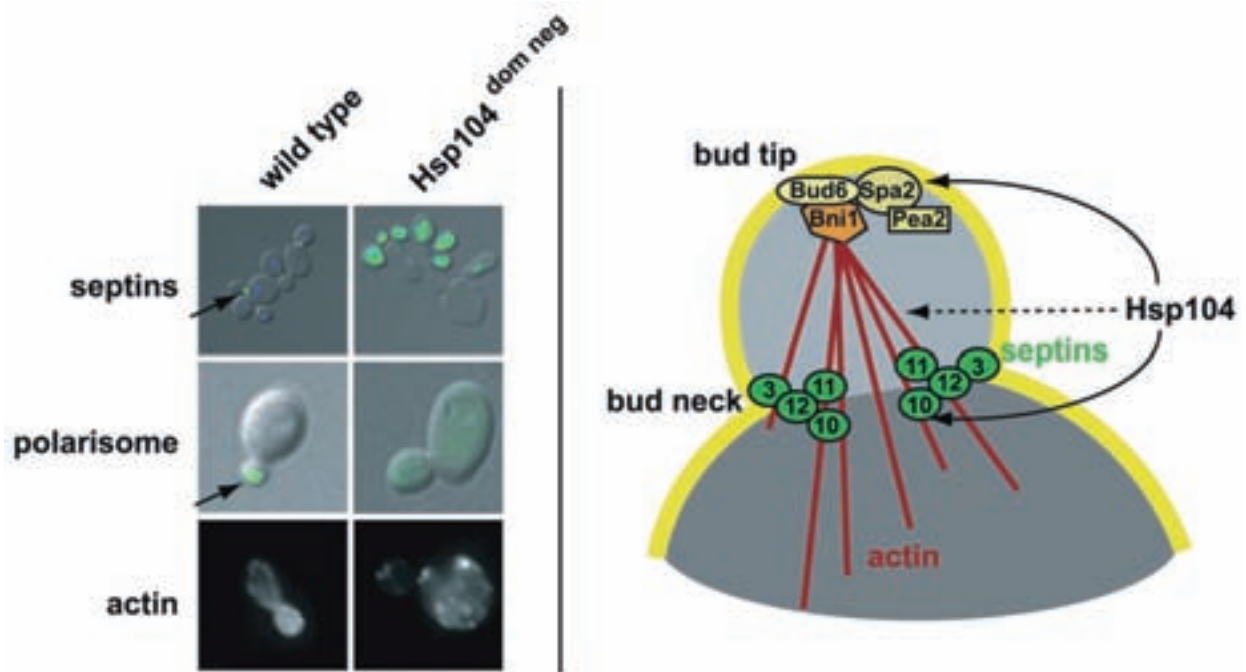


Fig. 3: Hsp104 influences polarity formation in *S. cerevisiae*. We used a genetically engineered dominant negative variant of Hsp104 (HAP) that selectively degrades Hsp104-dependent substrates through association with a peptidase as experimental tool to search for Hsp104 substrates in yeast. Expression of HAP/ClpP causes loss of polarity formation as shown by mislocalization and misassembly of GFP-tagged components of the septin ring, the polarisome and the actin ring (for wild-type localizations see arrow). The right panel summarizes our findings as described in the text.

Collaborations

We acknowledge our collaborators N. Ban (ETH Zurich), A. Clarke (Goteborg Univ.), P. Dersch (Univ. Braunschweig), E. Deuerling (Universität Konstanz), D. Dougan (LaTrobe Univ.), S. Lindquist (Whitehead, MIT), M. P. Mayer (ZMBH), T. Ruppert (ZMBH), H. Saibil (Birbeck College, London), J. Schneider-Mergener (Humboldt Univ. Berlin), V. Sourjik (ZMBH), F. Tsai (Baylor School of Medicine), K. Turgay (FU Berlin).

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- Merz, F., D. Boehringer, C. Schaffitzel, S. Preissler, A. Hoffmann, T. Maier, A. Rutkowska, J. Lozza, N. Ban, B. Bukau and E. Deuerling (2008). Molecular mechanism and structure of trigger factor bound to the translating ribosome. *Embo J*, in press.
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THESES

Dissertations

- Thomas Rauch (2005): The role of ribosome-associated chaperones in *de novo* protein folding
- Holger Raviol (2006): Functional characterization of the Hsp110 family of molecular chaperones
- Rainer Nikolay (2006) Investigations of structure, function and regulation of the chaperone-associated ubiquitin E3 protein ligase CHIP. (jointly with M. Mayer)
- Jimena Weibezahn (2006): Investigation of the mechanism of protein disaggregation by the AAA+ protein ClpB
- Fernanda M. Rodriguez (2007): Study of the interaction between the DnaK chaperone and its substrates. (jointly with M. Mayer)
- Markus Vogel (2007) Investigations on the molecular basis of interdomain communication in Hsp70 chaperones. (jointly with M. Mayer)
- Frieder W. Merz (2008): The mechanism of action of the ribosome-associated chaperone trigger factor
- Peter Tessarz (2008): Studies on the mechanism and physiological role of the AAA+ chaperone ClpB/Hsp104

Masters and Diploma Theses

- Ingo Brand (2005): Untersuchungen zur Expression und Funktion von ClpV und des assoziierten CVAC-Genclusters
- Jan Kerschgens (2005): Untersuchungen zum Mechanismus des Substrattransfers innerhalb des Hsp70 Systems von *E. coli*
- Karina Wagner (2005): Untersuchungen zur Funktion des C-Terminus des AAA+ Chaperons Hsp104
- Steffen Preißler (2006): Characterization of the functional interplay of ribosome associated Trigger Factor & Signal recognition Particle in *Escherichia coli*
- Agnieszka Zdanowicz (2006): Dissection of ClpB-mediated protein disaggregation by using aggregated model substrates that retain functional domains

Lars M. König (2007): Membrane protein aggregation: Establishment of a model system to study aggregation, disaggregation and degradation of membrane proteins

Aleksandra Pietrosiuk (2007): Identification of interaction partners of Hep – the major secretory protein of the Type VI Secretion System of *Vibrio cholerae* V52

Habilitation

Axel Mogk (2005): Mechanism of protein disaggregation by the AAA+ chaperone ClpB

AWARDS

- Bernd Bukau (2005): Leopoldina Forschungspreis
- Christian Schlieker (2005): Ruprecht-Karls-Award of the University of Heidelberg for his PhD thesis on "Substrate recognition and processing by the AAA+ chaperone ClpB"
- Thomas Rauch (2006): Ruprecht-Karls-Award of the University of Heidelberg for his PhD thesis on "The role of ribosome-associated chaperones in *de novo* protein folding"
- Bernd Bukau (2008): Bijvoet Medal of the University of Utrecht

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