Meeting Review

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Molecular chaperones and the heat shock response at Cold Spring Harbor

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The fifth Cold Spring Harbor heat shock meeting, organized by Costa Georgopoulos, Susan Lindquist and Richard Morimoto, was held 1–5 May, 1996. Like the 1994 meeting, which had 408 participants, the 1996 meeting was filled to capacity with an exciting mix of students, postdocs, newcomers and veteran heat shockers. The sizes of the abstract books for these international meetings which began in 1982 are indicative of the growth of this field. If you feel buried in a deluge of journal articles, Figure 1 shows why. The field has been growing exponentially, and although our meeting meter saturated in 1994, the number of articles published annually continues to increase. Costa Georgopoulos, organizer of four of these meetings, and Rick Morimoto, organizer of the last three meetings, will be succeeded by Carol Gross and Arthur Horwich, respectively. They will join Susan Lindquist, an organizer of the last two meetings, to plan the 1998 meeting.

In addition to the plenary sessions and posters, two other scientific events occurred at the 1996 meeting. First, the inaugural meeting of the *Cell Stress & Chaperones* Editorial Board was held. The meeting, organized by our Managing Editor Gillian Griffith, was attended by 31 members of the board. A number of useful suggestions for future development of the journal and its associated Web page were collected. And second, a session was organized by Lutz Nover and Dieter Scharf to discuss a unified nomenclature for plant heat stress transcription factors and their functional modules. An agreement was reached among the participants which included J. Bonner, W. B. Gurley, R. Morimoto, H. Nelson, R. Voellmy, C. Wu and V. Zimarino, and a mini-review entitled 'The Hsf world: classification and properties of plant heat stress transcription factors' introducing the new terminology was published in a previous issue of *Cell Stress & Chaperones* (Nover et al 1996).

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WHAT HAPPENS IN THE ANFINSEN CAGE?

Two years ago, the chaperonin sessions featured a barrage of verbal bullets and footballs whizzing past the

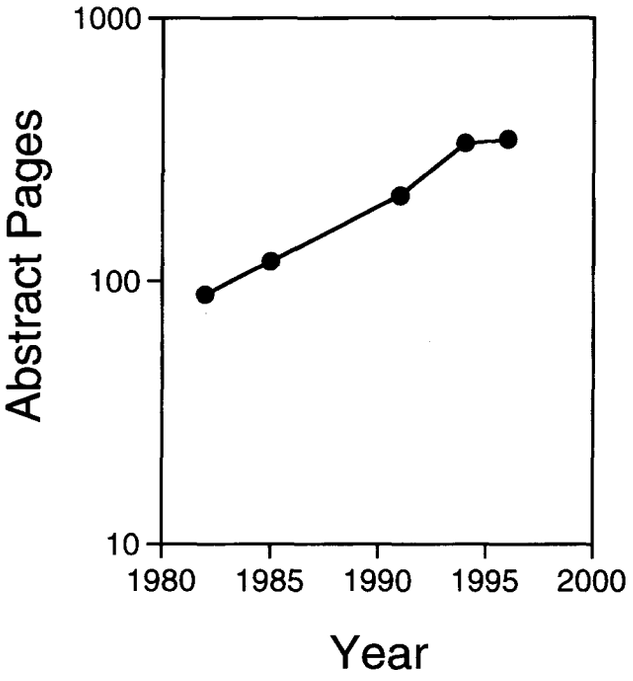

Fig. 1 Growth curve of the Cold Spring Harbor meetings.

audience as the participants debated the importance of asymmetric (one GroES cap) and symmetric (two GroES caps) intermediates in the GroEL/ES folding cycle. Another big issue was whether proteins folded inside or outside of the chamber (for background see Ellis 1996). This year's session saw substantial agreement and Colonel Craig did not actually need the UN peacekeeper's blue helmet she was given by A. Horwich to chair the first session. The structure of GroEL bound protein folding intermediates was a major theme. When the GroES cap binds to GroEL, a chamber capable of holding up to about 50–60 kDa of expanded folding intermediate is created. R. J. Ellis has termed this vault the Anfinsen cage in honor of the discoveries in protein self-folding and assembly by Christian Anfinsen (Ellis 1994). A. Horwich reported that bound intermediates of human dihydrofolate reductase are loosely structured, as assayed by deuterium exchange and NMR spectroscopy. S. Radford reached the same conclusion based on hydrogen exhange labeling studies and electrospray ionization mass spectrometry. She also presented an interesting comparison of lysozyme folding with and without GroEL assistance. Surprisingly, GroEL actually increased the rate of folding. How can a binding reaction speed up folding? Possibilities include destabilization of folding intermediates, stabilization of the transition state and changing the pathway. S. Walter found that protein *unfolding* was not catalyzed by GroEL, at least for the RNase T1 variant without disulfide bonds used as a model.

J. S. Weissman summarized a model for GroEL/ES mediated protein folding that featured a molecular timer and the use of the cage as both a folding and an unfolding chamber. In this model an asymmetric GroEL/ES complex is the polypeptide acceptor. Polypeptide binds to the uncapped GroEL chamber, and a single round of ATP hydrolysis in this same ring drives conformational changes in GroEL resulting in the release of GroES. A GroES cap then rebinds to the GroEL chamber containing the polypeptide. Seven ATP molecules bind to the unoccupied GroEL chamber on the opposite end, initiating the polypeptide folding reaction and starting the timer. After about 15–30 seconds, the timer goes off, i.e. ATP hydrolysis drives the release of the GroES cap. There was general agreement that the main function of ATP hydrolysis is to drive conformational changes in GroEL that allow binding and release of the GroES cap (M. Hayer-Hartl). This is also the timer for polypeptide release and the time elapsed is too short to allow folding of many proteins. The authors propose that those polypeptides that have gained sufficient native structure upon release can complete folding in solution, i.e. they are committed to fold. Molecules that do not fold successfully may be rebound to GroEL and then the chamber serves to unfold kinetically-trapped intermediates. F.-U. Hartl (1996) described a combination of kinetic and EM studies which indicated that symmetrical particles of GroEL capped at both ends by GroES are not an obligatory intermediate in the reaction cycle (see Torok et al 1996 for the case for symmetrical intermediates). Hartl's model also featured folding in the capped chamber with the binding of GroES actually driving the release of the polypeptide from hydrophobic binding sites on the walls of GroEL. He emphasized the importance of multiple rounds of ATP dependent release of polypeptides from the chamber walls, partial folding and rebinding of unproductive intermediates until the polypeptide is either completely folded or committed to fold once released from the chamber. To highlight his thinking that either folded or committed molecules are primarily what is released from the chamber, Hartl reintroduced the 'jack-in-the-box' label for his model (Hartl 1996). To more closely mimic in vivo folding conditions, macromolecular crowding agents were added to a rhodanese-folding reaction. Under these conditions release of unfolded rhodanese from GroEL/ES was suppressed even further (J. Martin).

Obviously the molecular details of the GroEL and GroES interaction are keys to understanding the reaction cycle. S. Landry summarized previous studies based on proton NMR, limited proteolysis and bacterial mutants that GroES and bacteriophage T4-encoded Gp31 cochaperonin use a mobile segment to bind GroEL. The crystal structure of GroES has a loop extending from the bottom rim of the cap which appears to be a mobile binding segment. Actually, seven mobile loops are predicted but only one was captured in the crystal structure. TrNOE spectroscopy and molecular modeling were used to determine the structure of a GroES loop bound to GroEL. Its structure is different from that found in the crystal structure, indicating that the loops have a considerable range of conformational flexibility. Using a mutant Cpn10 with a point mutation in the sequence encoding a putative mobile loop, Y. Dubaquié showed that the mobile loop is involved in both binding to GroEL and productive release of substrate proteins.

The binding of α-lactalbumin to GroEL is entropy driven (negative change in heat capacity), indicating hydrophobic interactions (K. Kuwajima). The model presented by Landry suggests that the hydrophobic edges of the mobile loops of GroES may compete with polypeptides for binding to GroEL, aiding in the release of the polypeptide. Staphylococcal nuclease, which is positively charged at neutral pH, has higher affinity for the highly negatively-charged GroEL than α-lactalbumin does, indicating that electrostatic interactions are also important (K. Kuwajima).

Another way to ask the question 'How do chaperonins recognize their protein substrates?' is to ask 'What are the proteins that are assisted by chaperonins in cells?' N.

Cowan thinks that the target range of eukaryotic cytoplasmic chaperonins (cyt Cpn60) is highly restricted to actin, tubulin and closely related proteins. And at least for tubulins, the chaperonin has plenty of help from at least four additional cofactors. Interaction of denatured tubulins with cyt Cpn60 results in ATP dependent folding to a quasi-folded form capable of binding GTP. Then cofactors take over to finish folding to native tubulin by ATP independent steps. J. Frydman presented evidence that actin does not cycle among cyt Cpn60 molecules during folding. Additional experiments were presented from the Hartl lab comparing chaperone interactions of chemically denatured actin added to reticulocyte lysates and vectorially translated polypeptides. Denatured actin formed complexes with Hsc70 and Hsp40 (DnaJ homolog). Mutant GroEL was used to trap actin folding intermediates. Nascent actin chains also interacted with Hsc70 and Hsp40 during translation but GroEL was excluded from ribosome-bound nascent chains. GroEL traps could bind these nascent polypeptides after puromycin release, suggesting a tightly coupled chaperoning pathway.

HSP70 FAMILY CHAPERONES IN THE CYTOPLASM

Hsp70 proteins are encoded by multigene families, and in the *Saccharomyces cerevisiae* genome there are at least 14 Hsp70 related genes, based on the genome sequence. Understanding the functional significance of this genetic diversity is another facet of chaperone biology. E. Craig reported on the use of chimeric ssa/ssb genes to rescue yeast Hsp70 mutants. Differences in peptide binding specificity were not responsible for the functional differences between the Ssa and Ssb classes of cytoplasmic Hsp70. It was suggested that protein–protein interactions as with accessory proteins may be the difference (James et al 1997).

Hsc70 proteins self-associate in vitro to form dimers, trimers and larger oligomers. Either binding of ATP or peptides/unfolded proteins causes dissociation of multimers and a shift in equilibrium favoring monomers, respectively. Additional data presented by N. Benaroudj and M. Ladjimi suggested that self-association involves the peptide binding domain and may mimic Hsc70-peptide interactions. The carboxy terminal EEVD sequence of Hsp70 family proteins, previously implicated in regulation, possibly as an Hdj1 (an Hsp40 member) interaction site, is required for maintenance of β-galactosidase in a refoldable state but not for binding to reduced carboxymethylated lactalbumin (D. Bimston). Therefore, binding unfolded proteins and maintaining a folding competent state are separable properties of Hsp70.

A new regulatory protein p16 for vertebrate Hsc70 was introduced by S.-M. Leung. The p16 accessory protein modulates Hsc70 function by maintaining Hsc70 in a monomeric state and by dissociating unfolded proteins. The ability of p16 to dissociate multimers of Hsc70 suggests that it may carry out the reverse reaction to DnaJ which catalyzes oligomerization of Hsc70 in the presence of ATP (C. King). P16 carried out its effects on Hsc70 in the absence of exogenously added ATP. P16 is a member of the nucleoside diphosphate kinase family, a highly conserved and phylogenetically broadly distributed family. Until recently, NDP kinases were considered to be housekeeping enzymes that maintained nucleotide pools by transferring the gamma phosphate of ATP to any other nucleoside diphosphate. An additional family member was recently identified as a *c-myc* transcription factor and possible negative regulator in tumor metastasis (Leung and Hightower 1997).

G. Richarme reported that the *Escherichia coli* cochaperones DnaJ and GroES reduce the specificity of DnaK and GroEL, respectively, for hydrophobic amino acids and increase their affinity for hydrophilic ones, particularly Arg/Lys for DnaK. A role was suggested in protein folding/renaturation for these alternating binding preferences. This may also explain the dual selectivity of mammalian Hsc70 for hydrophobic and basic peptide sequences as well (Takenaka et al 1995). A protein disulfide isomerase activity was also identified on DnaJ by the same laboratory. A. Fink provided further evidence that DnaJ is a chaperone in its own right capable of binding short peptides such as CALLQSR and unfolded Staphylococcus nuclease mutants. Hsc70/DnaK in association with DnaJ family proteins and other accessory proteins appear to have the necessary activities to facilitate the folding of some proteins in vivo on their own. A key issue to be resolved is how closely coupled cyt Cpn60/GroEL-ES is to the initial chaperoning machine formed on nascent polypeptides. If polypeptides are released first, they would have the opportunity to fold and the chaperonin complex may then be used primarily to restart folding of kinetically-trapped folding intermediates picked up from the cytoplasm. The second possibility is that the chaperonins are tightly coupled to the first machine which only releases polypeptides directly to them and almost all folding would occur in association with chaperonins.

J. Glover (Lindquist lab) described experiments in which yeast Hsp104 dramatically enhanced refolding of denatured luciferase by Ydj1 and Hsp70 in vitro. Ydj1 was physically associated with His-tagged Hsp104. Together Hsp104 and Ydj1 appeared to prevent irreversible aggregation of luciferase. Luciferase that aggregated in the presence of Hsp104 and Ydj1 could be resolubilized with additional Hsp70. The concept that certain chaperones such as yeast Hsp104 and mammalian Hsp90 as described below may provide an unfolding/holding function for denatured proteins in order for the Hsp70 and

DnaJ families to work efficiently fits well with the observations that the latter chaperones prefer to bind to linear sequences.

HSP90 CHAPERONES

B. Freeman reported on work from the Morimoto lab using folding of β-galactosidase to evaluate the roles of Hsp90, Hsp70, Hsc70 and Hdj1 in protein refolding (Freeman et al 1996). Both Hsp70 and Hsp90 could maintain β-galactosidase in a refoldable state for long periods, but Hsp90 was more efficient at this function. In contrast, Hdj1 could not do this. The refolding activity of Hsp70 and Hdj1 was temperature-dependent. Between 22°C and 41°C folding occurred, but at 4°C and above 41°C, denatured β-galactosidase was held in a folding competent state without folding. Using thermal denaturation of firefly luciferase as a model, Y. Minami showed that the luciferase was denatured in the presence of Hsp90, but that Hsp90 bound denatured luciferase and held it in a renaturable form. Addition of Hsp70 and Hsp40 or Hdj2 was not sufficient to fold luciferase. Another unidentified factor in reticulocyte lysates was needed. R. Schumacker also studies firefly luciferase renaturation using reticulocyte lysates and purified proteins. In his hands, luciferase can be renatured with Hsp70 and DnaJ. Hsp90 enhanced the process but was not required, and its activity could be blocked by geldanamycin. Geldanamycin, which binds to Hsp90 and reduces its affinity for substrates, has emerged as a useful inhibitor of Hsp90 for dissecting its interactions. S. Hartson showed that Hsp90 has substrate specificity since geldanamycin had no effect on firefly luciferase folding but blocked production of cellular src-kinase $p56^{lck}$. Even within the folding pathway of luciferase, geldanamycin blocked renaturation of thermally denatured luciferase but not folding of nascent luciferease in reticulocyte lysates. D. F. Smith examined the exchange of proteins within the mature progesterone receptor complex. Hsp70 interacted with p60 and Hip whereas p60 had interactions with Hsp70 and Hsp90. The complex of Hsp90-p60-Hsp70 assembled without ATP and was highly dynamic. Hip's interaction with this complex was nucleotide-dependent.

A requirement for Hsp90 in the formation of a ribonucleoprotein was found during a study of hepatitis B virus reverse transcriptase (J. Hu). This RNP is used to prime RNA-directed DNA synthesis and is also essential in the assembly of subviral core particles. Geldanamycin blocked RNP formation in vitro and viral replication in vivo but did not affect binding of either Hsp90 or p23 to reverse transcriptase.

A current debate centers on the role of ATP in the activities of Hsp90. D. Toft presented studies showing that ATP is required for both stages of assembly of the progesterone receptor complex. Part of the ATP requirement is understandable due to the involvement of Hsc70. However, binding of Hsp90 to p23 was dependent on both temperature and presence of ATP/Mg^{++}, and was blocked by geldanamycin. It was suggested that Hsp90 cycles between a p23-binding and non-binding conformation, and ATP is required to convert Hsp90 to a p23-binding form.

α,β CRYSTALLIN, A CYTOPLASMIC STRESS PROTEIN

α,β Crystallin belongs to the small Hsp family. Although first identified as a major protein of the vertebrate lens, it is broadly distributed in tissues and has been implicated in human pathologies including Alexanders' disease where it accumulates in brain astrocytes. α,β Crystallin was induced in C6 glioma cells treated with the anti-microtubule drugs colchicine, colcemid, vinblastine and nocodazole, which cause microtubule disassembly (K. Kato). Staurosporine, a protein kinase inhibitor, blocked the induction by these drugs. Taxol, a microtubule stabilizer, was not an inducer. Interestingly, α,β crystallin, but not Hsp27, is induced by osmotic stress in cultured brain astrocytes. K. Kegel reported that overproduction of α,β crystallin in astrocytes resulted in increased uptake of the organic osmolytes taurine and myo-inositol, a physiological defense against osmotic injury usually associated with kidney tubule cells. Interestingly, both brain astrocytes and kidney epithelial cells accumulate α,β crystallin in response to osmotic stress and develop resistance. In vitro studies of the polymerization/depolymerization dynamics of actin indicated that α,β crystallin stabilizes actin filaments by binding to F actin and prevents cytochalasin-induced depolymerization, consistent with a role in cytoskeleton protection during stress (K. Wang). Overexpression of α,β crystallin was protective against ischemic damage in both neonatal and adult cardiomyocytes (J. Martin).

CHAPERONING THE MITOCHONDRION

W. Neupert summarized data in support of the molecular or Brownian ratchet model of movement of polypeptides into the mitochondrial matrix. Tim44, which is peripherally associated with the inner membrane, interacts with mt Hsp70 and preprotein during translocation. Cycles of ATP binding, hydrolysis and product release switch mt Hsp70 between high and low affinity binding for Tim44 and the preprotein, creating the ratchet-like movement. This contributes to unfolding of the preprotein outside of the mitochondrion and insures unidirectional protein translocation. Mdj1 is not required for translocation but appears to recruit mt Hsp70 to polypeptides to initiate

folding. The nucleotide exchange factor Mge1p is required for both protein import and protein folding. B. Glick prefers a more active role for the mt Hsp70–Tim44 complex in translocation embodied in the ATP dependent translocation motor model. In his opinion, the ratchet model does not account for the rapid rate of unfolding of preproteins on the mitochondrial surface. In Glick's model mt Hsp70 initially hydrolyzes bound ATP and binds tightly to Tim44 and a precursor protein. Mt Hsp70 then undergoes a conformational change to drive translocation, reminiscent of a myosin-like power stroke. This force would accelerate unfolding of the preprotein. Evidence was presented that the import rate constant depends on the length of the presequence attached to DHFR. Presequences longer than 65 residues could traverse the membrane and bind mt Hsp70 without unfolding DHFR. These longer fusion proteins were imported 40 times faster than shorter ones, the former following the motor model and the latter following the ratchet model according to Glick.

A novel ATP-dependent protease complex belonging to the AAA family (ATPases associated with a variety of cellular activities) located in the inner mitochondrial membrane was described (T. Langer). This complex appears to serve the dual roles of both a molecular chaperone in the absence of ATP hydrolysis and a protease that degrades aberrant proteins in association with the mitochondrial inner membrane. These proteases have high sequence similarity to *E. coli*'s FtsH. A potential source of damaged proteins would be mitochondrial toxicants such as a metabolite of the nephrotoxic gas tetrafluoroethylene which acylates ε-amino groups of lysines (S. Asmellash). Targets include mt Cpn60 and mt Hsp70 along with mitochondrial dehydrogenases.

Yeast Hsp78 is the mitochondrial Clp homologue and a relative of cytoplasmic Hsp104. M. Schmitt showed that Hsp78 is essential for recovery of mitochondrial protein synthesis following a heat shock; however, it could not block thermal inhibition of translation even when overexpressed. Mutations in mtHsp70 and Mge1p do not result in increased aggregation of imported luciferase upon heat shock. However, mutations in Mdj1p do, giving this co-chaperone a major role in protection from protein aggregation in mitochondria (E. Schwarz).

CHAPERONING THE ENDOPLASMIC RETICULUM

New Hsp70 family members were identified in the endoplasmic reticulum (ER) of both yeast and mammalian cells. Completion of the yeast genome sequencing project revealed a novel ER Hsp70 (Ssi1) that is predicted to be somewhat larger than Kar2p (yeast BiP). Studies from E. Craig's group demonstrated that Ssi1 is not essential, will not complement Kar2p mutants, but interacts genetically with Kar2p class II mutants to cause a more severe phenotype. Cloning by J. Subjeck's lab of mammalian Grp170, which binds transiently to assembling immunoglobulin molecules, revealed it to be an Hsp70 family member with a high degree of homology to other Hsp70s in the N-terminal ATP binding domain.

The mammalian ER Hsp70 member, BiP/Grp78, was shown to be directly involved in the folding of nascent chains. In vivo folding studies showed that wild-type BiP binds to completely unfolded and partially folded Ig λ-light chains (LC) but not to completely folded λLC. Expression of BiP ATP binding and hydrolysis mutants that are incapable of release blocked complete folding of LC and prevented their secretion. Studies with combinations of BiP mutants and LC mutants suggested that cycles of BiP binding and release normally provide the opportunity for a LC to fold and prevent aggregation (Y. Argon, L. Hendershot). Grp94 also associates with λLC but is preferentially found with the more fully oxidized form (Y. Argon). Whether this implies a hand-off mechanism or some sort of cooperation between these chaperones is not currently understood. An algorithm to predict linear sequences that are likely to bind BiP has been produced based on an extensive BiP: peptide binding study (Fourie et al 1994). The algorithm was applied to several secretory pathway proteins, and the sequences identified were used to generate peptides for use in peptide stimulated ATPase assays. The BiP score was found to be a good identifier of sequences that could bind BiP and stimulate its ATPase activity in vitro (M. J. Gething, J. Buchner). A yeast ER ATP translocator (Sac1p) has recently been identified. Sac1 deletion mutants are defective in both translocation of proteins into the ER and in their subsequent movement to the Golgi suggesting that ATP is required at two different points. In these mutants, an increased association of proteins with Kar2p was observed, suggesting that one requirement for ATP involves Kar2p release from nascent proteins (P. Mayinger). In support of this, Kar2p mutants that are defective in translocation were shown to be ATPase mutants (J. Brodsky).

Calnexin, a lectin-like ER chaperone was shown to specifically bind monoglucosylated proteins (D. Williams, A. Helenius, W.-J. Ou). Glycoproteins retained in the ER continuously undergo a glucosylation/deglucosylation reaction via the actions of UDP-glucosyltransferase and a glucosidase, allowing them to alternatively bind and release calnexin. Calnexin was shown to promote proper folding of ER proteins in *Drosophila* cells transfected with class I heavy chains and β_2-microglobulin alone or with calnexin. Calnexin markedly increased the yield of properly assembled soluble protein (D. Williams). The question of whether calnexin solely interacts with sugars or also recognizes protein determinants remains controversial.

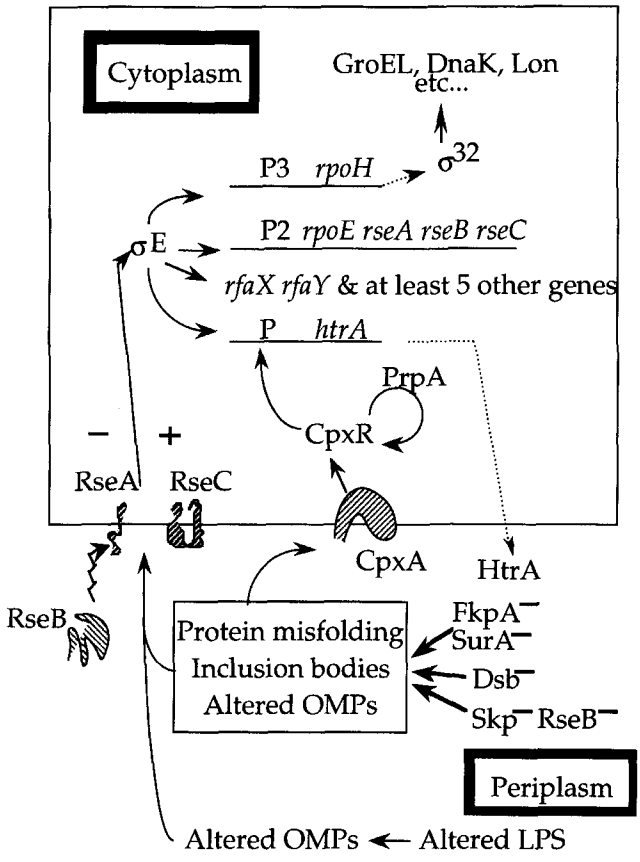

Fig. 2 Signaling pathways induced by protein misfolding in the periplasmic space and the outer membrane of E. coli. Diagram provided by S. Raina and D. Missiakas.

Data on RNase B mutants revealed that calnexin recognized the proteins as long as they were monoglucosylated and was independent of their folding status (A. Helenius). Using thyroglobulin as a substrate, Ou et al demonstrated that protein: protein recognition may be more important for maintaining the interaction of this protein with calnexin. This raises the possibility that calnexin has the capability to interact with different structures, and that the substrate itself may define the predominant interaction observed.

Mutant proteins that are unable to attain a native conformation are often retained in the ER complexed with one or more ER chaperones and eventually degraded by a poorly understood mechanism. A surprising finding reported by J. Brodsky, is that at least some ER degradation may actually take place in the cytosol. This required re-translocation of ER proteins back into the cytosol, was ATP dependent, and utilized the cytosolic proteasome. Calnexin was also required, although the role of calnexin in this ER degradation is not currently understood (A. McCracken, J. Brodsky).

ER chaperones are transcriptionally upregulated in response to the accumulation of unfolded proteins in the ER. The most proximal protein in the yeast signaling response is a transmembrane protein (Ire1p/Ern1p) with homology to cdc2 kinases. This protein was shown to have serine/threonine kinase activity, and similar to plasma membrane receptor kinases, to dimerize and trans-phosphorylate itself (M. J. Gething, P. Walter, R. J. Kaufman). Yeast 2-hybrid studies using the cytosolic domain revealed that Ire1p binds to the yeast transcription factor, Gcn5. Yeast strains deficient in Gcn5 were unable to mount an ER stress response (R. J. Kaufman).

Although ER chaperones are thought to protect a cell from the adverse effects of accumulated unfolded proteins, prolonged exposure to agents that affect protein folding is ultimately lethal. Apoptosis resulting from prolonged tunicamycin treatment was shown to occur via calcium release from the ER, which leads to the activation of ds RNA dependent kinase, PKR, a tumor suppressor (R. J. Kaufman). Overexpression of BiP/Grp94 apparently prevents apoptosis or necrosis by preventing calcium release from the ER in response to oxidative stress (H. Liu).

The compartment of *E. coli* comparable to the eukaryotic ER is the periplasm. Much headway has been made in understanding how the accumulation of misfolded proteins in this compartment is sensed and signaled. Transcription of the *rpoE* gene encoding sigma factor σ^E is responsive to the accumulation of aberrant exported proteins (Fig. 2). The $E\sigma^E$ polymerase transcribes the *htrA* gene which encodes a periplasmic protease HtrA involved in degrading misfolded proteins. How is signaling for increased HtrA production transduced from periplasm to cytoplasm? Raina and Missiakas have identified four additional loci that affect *htrA* transcription. Mutations were mapped to the *cpxR/cpxA* operon and three new genes, *resA*, *prpA* and *prpB*. Evidence of two sensing pathways is accumulating (Fig. 2). In one, upregulation of *htrA* is accomplished by the activation of the two component system CpxR/CpxA where CpxA is a membrane sensor and CpxR is a phosphorylatable regulatory protein. Two phosphoprotein phosphatases, PrpA and PrpB, regulate the cellular level of phosphorylated CpxR. In the second pathway, RseA located in the inner membrane modulates σ^E activity by direct protein–protein interaction. Misfolded proteins are sensed by periplasmic RseB and a membrane protein RseC which bind to RseA to transmit the signal to the cytoplasm, presumably via a conformational change in RseA which releases σ^E. C. Gross obtained evidence that RseB and RseC can independently transduce signals regarding the state of the periplasm to σ^E. In addition a novel peptidyl prolyl isomerase, SurA, was identified which participates in the folding of monomers. Accumulation of unfolded monomers in *surA* strains triggered induction of σ^E.

K. Ito described the properties of FtsH, an *E. coli* member of the AAA family. FtsH appears to be a dynamic quality-control machine, in the author's words, which recognizes altered membrane proteins. For example, FtsH degrades SecY in an ATP-dependent reaction when it is not associated with SecE. However, FtsH binds but does not degrade denatured alkaline phosphatase, suggesting a chaperone activity as well.

PROTEOLYSIS

M. Sherman described studies showing that *E. coli*'s trigger factor (TF) binds to GroEL. TF is a co-chaperone in the degradation of a fusion protein involving GroEL and the ClpP protease. Since TF also binds to large ribosomal subunits and has high levels of prolyl isomerase activity (F. Schmid), it is possible that TF is a transfer chaperone between the machine that chaperones nascent polypeptide chains as they emerge from ribosomes and the GroEL/ES complex.

D. Missiakas described the *E. coli hslVhslU* (*clpQ/clpY*) operon, whose overexpression prevents Hsp induction by puromycyl peptides. HslV is a multimeric protease that degrades peptides independent of ATP. It is related to a subunit of the catalytic core of eukaryotic proteasomes. HslU is related to the ClpX protein of *E. coli* which presents polypeptides to the ATP-independent protease ClpP. Chaperone protease pairs such as ClpA , which has ATPase but not protease activity alone, and its protease partner ClpP are emerging as major quality control complexes. H.-C. Huang also described the properties of HslV, the proteasome b subunit homolog, and HslU, related to the ClpX ATPase, purified from *E. coli.* In contrast to the ATP-independent protein degradative activity described above, the peptidase activity of this complex was ATP-dependent, and it was described as having properties intermediate between *E. coli*'s ClpP and the eukaryotic proteasome. S. Wickner presented evidence using the plasmid P1 RepA protein in vitro that the interaction of an inactive or damaged protein with one of these ATP-dependent chaperones can result in either refolding to an active form or unfolding for presentation to a protease. In this system, ClpA takes the place of DnaK, DnaJ and GrpE in activating the DNA-binding activity of RepA protein.

Each heat shock meeting brings new inducers of the response. One of our favorites at this meeting was the neutral cysteine protease inhibitor N-acetyl-leu-leu-norleucinal in human cell lines (M. Zhou). On first glance, one might think that this rather hydrophobic peptide might bind Hsc70 and compete for binding to Hsf, thus allowing activation. But, this induction is apparently inhibited by cycloheximide, calling for a more complex hypothesis, perhaps involving a cysteine protease in the induction pathway.

REGULATION

The roles that protein phosphorylation play in transcriptional activation during heat shock are still unclear. O. Bensaude reported that the phosphorylation of the largest subunit of RNA polymerase II is increased in heat-shocked human cells. Phosphorylation of this subunit is thought to contribute to promoter clearance and entry into elongation. It was suggested that stress activated MAP kinases might contribute to this phosphorylation and contribute to the highly efficient transcription of heat shock genes.

One of the most exciting new finds in regulation is the role of DNA-dependent protein kinase (DNA-PK) in the activation of heat shock genes. The regulatory subunit of DNA-PK is also known as Ku antigen. This kinase has been implicated in repair of DNA double strand breaks, V(D)J recombination and transcriptional regulation. It was recently shown to phosphorylate Hsf1 in vitro (D. Kim). In cells lacking DNA-PK, heat induced Hsp60, Hsp70 and Hsp90 normally but Hsp25 was not induced, indicating a role for DNA-PK in its regulation (P. Burgman). This may explain the uncoupling of Hsp70 and Hsp27 induction that has been observed previously in several different systems. In Rat-1 cells overexpressing Ku antigen, induction of Hsp70 by heat is specifically suppressed (G. Li). Using in vivo genomic footprinting, it was shown that Hsf1 was not bound to its promoter in heat-shocked cells overexpressing Ku. Rat cells constructed to overexpress Ku subunits were more sensitive to thermal stress than wild-type cells (L. Li). The major difference was in decay of thermotolerance which was faster in the mutants. Hsp70 expression but not other major hsps was blocked in cells overexpressing Ku70. It was concluded that Hsp70 induction is not essential in these cells for development of thermotolerance but it contributes substantially to its maintenance.

Another kinase system implicated in regulation of Hsf1 and Hsp70 expression in mammalian cells is MAP kinase (N. Mivechi). Inhibition of MAP kinase phosphatase MKP1 with sodium vanadate resulted in increased extracellular signal-regulated kinase ERK1 activity, which links the kinase cascade to downstream transcription factors, and increased phosphorylation of Hsf1 in unheated cells, delayed Hsf1 activation by heat, and inhibited induction of Hsp70. B. Chu described in vivo and in vitro experiments which suggested that phosphorylation of Hsf1 by MAP kinase inhibits its ability to activate heat shock promoters before the DNA binding step (Chu et al 1996).

An emerging theme in the regulation of the response is enhancement and synergizing of heat shock by other agents. Aspirin given to rats enhances their hyperthermic response to heating and increases Hsp70 expression in several tissues 3- to 4-fold over heat alone (N. Holbrook).

C. Holmberg showed that the protein kinase C activating phorbol ester TPA significantly enhanced the heat shock response in human erythroleukemia K562 cells. L. Vigh introduced a new drug BRLP-42 that enhances the induction by other agents of hsps in cells and animals. This drug was developed by BioRex R&D and, among other things, it was said to stimulate cell surface expression of Hsp70, resulting in an antitumor immune response. Although the molecular structure of BRLP-42 was not revealed, it appears to interact with membrane lipids to affect membrane fluidity and perhaps transmembrane signaling as well.

It is clear from studies of Hsp induction in vivo that there is much more to learn about the layers of regulation of the heat shock response. For example, different glial cell types in the mammalian brain have different profiles of induction of Hsp70 mRNA when rabbits are subjected to fever-like increases in body temperature (I. Brown). During spermatogenesis in *Drosophila*, Hsp23 and Hsp27 are expressed in both stressed and unstressed gonadal tissue (R. Tanguay). Neither of these proteins is further induced by heat, but other Hsps are, and in the apparent absence of Hsf. Hsp70 regulation is very complex in early *Drosophila* embryos (Z. Wang). Pole cells of the primordial germ line produce Hsp70 upon heat shock in division cycle 12 but lose this ability after cycle 13. Translocation of Hsf from cytoplasm to nucleus occurs independent of heating and correlates with acquisition of heat inducibility.

M. Blake reported that Hsp expression could be induced in aorta in vivo by the antihypertensive drug clonidine, an α_2 adreneric receptor agonist. This was unexpected since previously it was reported that drugs causing hypertension induce Hsp70 in aorta. Heat shock mRNA and protein increased but no HSE binding activity indicative of Hsf1 activation was found, suggesting an atypical induction pathway. The cytokine IL1β is produced by activated monocytes and plays key roles in immune and inflammatory responses including fever. Heat shock activates Hsf1 and represses transcription of the ILIβ gene in monocytes. The promoter for this gene contains an HSE and S. Calderwood presented data suggesting that Hsf1 may be a transcriptional repressor of IL1β expression. Wound healing is another in vivo venue where stress proteins are likely to play major roles. M. Oberringer showed that levels of Hsp70 in different wounds correlated directly with wound healing. Hsp70 accumulated mainly in endothelial cells in tissue sections of the wounds. Both bFGF and vEGF can induce Hsp70 in human vascular endothelial cells. As pointed out by F. White (1980) a number of years ago, the vascular endothelium as the cell layer separating blood from tissue, e.g. the blood–brain barrier, is likely to play a major role in vertebrate stress responses.

The heat shock response is altered in aging cells. A. Liu showed that more monomeric Hsf1 is located in the nuclear fraction of older cells in culture and that upon heat shock, these cells contain much less Hsf1 trimer than young cells. It was suggested that these changes contribute to the attenuation of the heat shock response seen in several model systems of aging. Age-related changes in chaperone function may contribute to the pathogenesis of Alzheimer's disease. In vitro, human Hsp27 and *E. coli* Hsp25 inhibited amyloid formation (N. Eberhardt).

HEAT SHOCK TRANSCRIPTION FACTORS

T. Yura compared the amino acid sequences of σ^{32} from a number of Gram-negative bacteria. The most highly conserved region, the RpoH box, has the consensus sequence Q(R/K)(K/R)LFFNLR. This sequence overlaps with region C, which is involved with DnaK/DnaJ/GrpE mediated negative regulation of synthesis and stability of σ^{32}. More information emerged on the negative regulation of mammalian Hsf1 from studies of mammalian cells treated with salicylate (M. Kline). Hsf1 is converted to a DNA bound form that is not transcriptionally activated under these conditions. A subsequent heat shock converts Hsf1 to a hyperphosphorylated, transcriptionally active form. A region containing basic amino acid residues was implicated in negative regulation. In addition, mutation of a site of constitutive serine phosphorylation resulted in constitutive transcriptional activity in the absence of heat shock. The heat-induced puffing of polytene chromosomes in *Drosophila* has a venerable place in the history of the field. N. Winegarden presented evidence that Hsf binding and puffing can be induced by salicylate without transcriptional activation. Hyperphosphorylation of Hsf did not occur in response to salicylate and, in fact, salicylate treatment prevented heat-induced phosphorylation of Hsf and transcription. How is Hsf regulated by phosphorylation? Work by R. Voellmy on human Hsf1 provides some clues. Inhibitors of protein kinase C significantly reduced heat-induced hyperphosphorylation of Hsf1. Protein phosphatase inhibitors enhanced heat-induced expression of Hsps. It was suggested that the activity period of Hsf1 can be fine-tuned by the phosphorylation state of Hsf1.

Mutagenesis and activity studies of yeast Hsf1 reported by H. Nelson indicated that replacement of several conserved residues results in either up- or down-regulation of transcriptional activity. The N-terminal transcriptional activation domains had no detectable structure in vitro suggesting that they adopt their functional conformations in contact with other proteins. A novel model with a wild-west flavor was presented in which the transcriptional activation domain

functions as a polypeptide lasso to contact the transcriptional machinery.

K.-D. Scharf and L. Nover described recent work on the functional domains of tomato Hsf1 and Hsf2. The HR1/2 region is necessary for oligomerization but not for activator function. An adjacent cluster of basic amino acid residues is required for nuclear import. The heat-inducible Hsf2 forms cytoplasmic heat stress granules with other Hsps. Short C-terminal peptide motifs with a central Trp residue are essential for the activator function of Hsf2. In vitro studies of the interaction of the C-terminal transcriptional activation domain of human Hsf1 are underway in B. Gurley's lab. C.-X. Yuan reported that this domain binds the TATA binding protein in vitro.

THIOLS AND THE INDUCTION PATHWAY

A.-P. Arrigo's lab has discovered a change in cellular physiology that correlates with increased levels of several different small hsps including human Hsp27, *Drosophila* Hsp27 and human α,β-crystallin. Elevated levels of glutathione were found in mouse L929 cells expressing these proteins and this may in part explain their protective activity against oxidative stress. It is unclear at present how overexpression of small Hsps stimulates glutathione accumulation. Activation of the cellular stress response has been correlated with an increased GSSG:GSH ratio in cells by as much as 200-fold in the presence of 10 different inducers (J. Zou and R. Voellmy). An unfavorable GSSG:GSH ratio may put cells at risk for proteotoxicity. Reducing agents like DTT block induction of the response by many agents that damage proteins and they also increase survival of severely heat shocked cells. H. Liu showed that the oxidized form of dithiothreitol induced the ER chaperones Grp78 and Grp94, resulting in protection of a kidney epithelial cell line from cell death induced by the alkylating agent iodoacetamide. Previously the authors showed that thiol depletion, and not protein alkylation, is the most important condition for Hsp70 transcriptional activation in iodoacetamide-treated cells (Liu et al 1996).

THERMOTOLERANCE, PROTECTION AND CHEMICAL CHAPERONES

Mitochondria have long been implicated as targets of stressors that induce Hsps. L. Bornman showed that an in vivo heat shock protects the respiratory activity of rat myocardial mitochondria from injury caused by hydrogen peroxide. Hsp70 and heme oxygenase were among the proteins induced. Despite the strong evidence linking induction of Hsps to acquired thermotolerance, every heat shock meeting brings examples of thermotolerance without new Hsp synthesis, reminding us that cells have more than one mechanism for achieving this critically protected state. This year, M. Borrelli showed that mitotic mammalian cells whose chromosomes are condensed and essentially shut down can still acquire thermotolerance after a heat shock (Borrelli et al 1996). A model was presented invoking the redistribution of constitutive members of Hsp families to sites of thermal damage. A. Laszlo concluded that the increased heat resistance of oxidative stress-resistant mammalian fibroblasts which overexpress catalase occurred without elevated expression of the major Hsps.

Both the chemical chaperone trehalose, which can reach intracellular levels of 0.5 mol/l, and Hsp104 make contributions to yeast thermotolerance (B. Futcher). Yeast mutants lacking an enzyme in the trehalose biosynthetic pathway are 500-fold more sensitive to heat shock (Elliot et al 1996). M. Singer showed that trehalose stabilizes proteins against thermal denaturation both in vivo and in vitro. This chemical chaperone also prevented aggregation of proteins which appeared to be stabilized in a partially folded, molten globule-like form. The chemical chaperones glycerol, trimethylamine N-oxide and dimethylsulfoxide inhibited the formation of PrP^{Sc}, the scrapie prion isoform implicated in pathogenesis (J. Tatzelt). These compounds are thought to stabilize the cellular prion protein PrP^{C} in its non-pathogenic conformation (Tatzelt et al 1996).

The role of Hsp27 phosphorylation in thermotolerance has been controversial. E. Hickey presented a study that begins to clarify some of the ambiguities. In an attachment-dependent rodent cell line, Hsp27 mutants unable to be phosphorylated do not provide increased thermal resistance. However, in a tumorogenic variant of this cell line, non-phosphorylatable Hsp27 mutants do confer added thermal resistance. It is suggested that stabilization of F-actin by Hsp27, which depends on phosphorylation, may be important only for increased thermal resistance in attachment-dependent cells. The general chaperone activity in Hsp27 is not dependent on phosphorylation at least in vitro and may be a separate function.

G. Perdrizet coined the term 'stress conditioning' to describe the cross-protection that occurs when heat shock and other agents are used to induce changes in gene expression and cell physiology resulting in protection of tissues and organs against ischemic injury. Ischemia and reperfusion injury are common consequences of surgical therapy. One of the most dramatic effects of stress conditioning was seen in operations with rabbits in which an 88% incidence of paralysis occurred in unconditioned animals following spinal cord ischemia induced by 20 min of aortic occlusion. In contrast, none of the stress conditioned animals became paralyzed. Surgeons frequently need to make skin flaps to cover surgical wounds. Some breast cancer patients are denied

post-mastectomy breast reconstruction because of their high risk of tissue necrosis in these flaps. Using a rat model, R. Udelsman showed that prior heat induction of hsps resulted in increased survival of similar musculocutaneous flaps. Trials with breast cancer patients are underway.

HSPS OUTSIDE OF CELLS

More reports of Hsps working on the outside of cells surfaced at this year's meeting. Several of these have substantial medical implications. A 38 kDa protein antigenically related to Hsp90 was detected in the media of the aquatic fungus, *Achlya* (J. Silver). Evidence favoring a selective release mechanism over cell lysis was presented. Treatment of leukemic K562 cells with an anticancer alkyl-lysophospholipid resulted in increased amounts of Hsp70 on the cell surface (C. Botzler). Increased sensitivity of treated cells to NK cell killing was correlated with elevated levels of Hsp70 on the cell surface. R. Burd reported that treatment of Scid and nude mice with a fever-like whole body hyperthermia resulted in reduction in the mass of implanted human breast carcinomas. The tumors produced large amounts of Hsps including their expression on the surface. This may have attracted NK cells which underwent a 500-fold increase in the tumors. Bacterial chaperonins can activate T cells and there is now evidence that they can also stimulate human joint fibroblasts to secrete cytokines such as IL-1, possibly contributing to inflammation in arthritis and other diseases (A. Coates).

A fascinating out-of-cell experience was described by J. Hill. Early pregnancy factor (EPF) is the same as eukaryotic Cpn10. EPF acts as cytokine since it is capable of stimulating embryonic growth. Both eukaryotic and prokaryotic Cpn10 are molecular chaperones, but only the eukaryotic version has the activities of EPF. EPF binds to a subset of CD4+ lymphocytes and it can suppress lymphocyte proliferation and delayed-type hypersensitivity reactions (K. Summers). EPF was previously reported to be present in maternal serum during the early stages of gestation and is involved in the regulation of embryonic cell proliferation.

Activity dependent neurotrophic factor (ADNF) is related to the Cpn60 family. ADNF, a 14 kDa neuroprotective protein, enhances neuronal cell survival. D. Brenneman emphasized the remarkable activity of a 14 amino acid peptide at femtomolar concentrations (about 10–15 molecules per cell) related to ADNF and Cpn60. This peptide is part of ADNF released by astroglial cells in response to a vasoactive intestinal peptide. This peptide blocked neuronal cell death from a variety of stressors. Cpn60 antisera triggered neuronal cell death in cultures and this could be prevented by ADNF. These observations may open new strategies for treating neurodegenerative diseases. How about immune responses to Hsp60 in autoimmune diseases? G. Birnbaum presented data from experimental autoimmune encephalomyelitis indicating that immune responses to hsp have a role in acute and inflammatory diseases of the central nervous system.

The literature on the release and surface of expression of stress proteins was reviewed recently (Multhoff and Hightower 1996). Cell biologists want to know the molecular details of these externalizations (Smalheiser 1996). To help unravel the mechanisms behind these unconventional release pathways, yeast has recently entered the arena in a paper showing that mutants blocked in the ERñGolgi pathway still release the expressed mammalian protein galectin-1 which is released from mammalian cells by a non-ERñGolgi route. The authors consider this to be evidence of a new export pathway distinct from the classical secretory pathway in yeast (Cleves et al 1996).

HSPS AND THE NATURAL ENVIRONMENT

The role of the heat shock response in the adaptation of animals to naturally changing environments and to those damaged by human pollution is an underrepresented area of the field. M. Feder and colleagues compared two *Drosophila* sister strains created by site-specific homologous recombination. The control strain carried remnants of the transgene construct and the other carried 12 extra copies of the *hsp70* gene. Third instar larvae and pupae of the extra-copy strain produced more Hsp70 and had higher levels of thermotolerance under ecologically-relevant heating conditions (Feder et al 1996). Is there a cost to the organism of expressing Hsp70? Using these same strains, R. Krebs showed that the first instar larvae carrying the extra copies had reduced growth rates and increased mortality following heat shocks. Apparently first and third instar larvae behave very differently. There does appear to be a trade-off since larva to adult survival following heating was higher in larvae with higher amounts of Hsp70, but less for the same lines held at 25°C.

J. Chapple measured Hsp70 levels and thermotolerance in marine mussels undergoing seasonal changes in their natural environment. High, naturally induced levels of Hsp70 correlated positively with thermotolerance. A positive correlation between levels of Hsp70 and thermotolerance in a natural population of desert fish has also been demonstrated (Norris et al 1995). During studies of threatened populations of salmonid fishes in northwest USA, L. Weber found that the threshold induction temperature of Hsp70 accumulation correlated well with growth inhibition in fish. Preliminary field studies on a salmon population in the Yakima River showed a correlation between induction of stress proteins and reduced population viability. Weber concluded that the threshold

induction temperature for the heat shock response is a useful physiological endpoint for stress management of salmonid populations.

CONCLUDING REMARKS

It has become impossible to summarize adequately all of the interesting aspects of stress responses and molecular chaperones presented at the Cold Spring Harbor meeting. The variety and information density are just too great to be covered in a single mini-review. However, an effort was made to include material from poster presentations as well as the oral sessions. Necessarily, the topics selected were a combination of those emphasized at the conference and those that tickled our fancy. If your favorite topic was not covered, we can only hope that colleagues with different interests and perspectives will contribute additional reviews on this data- and idea-rich conference.

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