Inhibitors and chemical probes for molecular chaperone networks

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The molecular chaperones are central mediators of protein homeostasis. In that role, they engage in widespread protein–protein interactions (PPIs) with each other and with their “client” proteins. Together, these PPIs form the backbone of a network that ensures proper vigilance over the processes of protein folding, trafficking, quality control, and degradation. The core chaperones, such as the heat shock proteins Hsp60, Hsp70, and Hsp90, are widely expressed in most tissues, yet there is growing evidence that the PPIs among them may be re-wired in disease conditions. This possibility suggests that these PPIs, and perhaps not the individual chaperones themselves, could be compelling drug targets. Indeed, recent efforts have yielded small molecules that inhibit (or promote) a subset of inter-chaperone PPIs. These chemical probes are being used to study chaperone networks in a range of models, and the successes with these approaches have inspired a community-wide objective to produce inhibitors for a broader set of targets. In this Review, we discuss progress toward that goal and point out some of the challenges ahead.

Molecular chaperones help ensure protein homeostasis (i.e., proteostasis), playing essential roles in the folding, trafficking, sequestration, and turnover of proteins (1). There are ~150 genes for molecular chaperones in the human genome, including the heat shock proteins Hsp110, Hsp90, Hsp70, Hsp60, Hsp27, etc. and the associated proteins such as co-chaperones, TCP-1 ring complex (TRiC),2 protein-disulfide isomerases (PDIs), peptidyl-prolyl cis-trans isomerases (PPLases), calnexin/calreticulin, and more (2, 3). Together, the coordinated activity of these factors serves to balance proteostasis and protect cells from protein misfolding and/or aggregation. Other articles in this Review Series cover the structure and function of the individual chaperone families in more detail. Here, we focus on the roles played by chemical probes in understanding their activity (4, 5). For example, our knowledge of Hsp90 biology has benefitted from the availability of chemical inhibitors, which can be applied to cells or organisms to ask how Hsp90 might be involved in a process. In this Review, we briefly introduce how chemical probes are developed and then outline how these ideas are being applied to chaperones.

What is a chemical probe?

One simple definition of a chemical probe is as follows: a small molecule that, at a given concentration, selectively inhibits the function of a biological target (6). It is essential that a chemical probe be selective for the intended target. Otherwise, it is difficult to ascribe its activity in cells or organisms to the function of the intended protein (7). Accordingly, the community of chemists and chemical biologists has developed an intuitive, experimental workflow that can be used to understand whether a molecule might be sufficiently selective to be considered a chemical probe. In 2010, Frye (6) published an influential commentary that coalesced many of these emerging ideas, and this concept has been expanded and extended by others (8, 9). From a pragmatic perspective, a good chemical probe is typically evaluated through a combination of chemical, biochemical, and genetic experiments (Table 1). Often, this process starts with discovery of an active molecule in a high-throughput screen. Then, a medicinal chemistry campaign is used to create analogs that reveal the relationship between the compound’s chemical structure and its activity in vitro (e.g., binding and/or functional assays) and in cells (e.g., cell growth, gene expression, or another phenotype). This correlation is typically referred to as a structure–activity relationship (SAR). An important (and sometimes overlooked) product of an SAR campaign is the selection of a negative control molecule, which is structurally similar to the active molecules but does not bind to the target. Finally, this process is often coupled with determination of the solubility, metabolism, permeability, and lifetime of key analogs. Together, these studies provide a chemical and pharmacological basis for understanding how much active compound is present and whether it would be expected to bind to the intended target under those conditions.

From this starting point, the putative probe and its controls are then evaluated in a series of cell-based experiments that are
intended to establish confidence that, at a given concentration, it will primarily bind to the intended target and not others. A classic method to assess selectivity is to immobilize the compound on a bead and determine whether it will preferentially "pull down" the intended target from cell lysates (see Table 1). Often, this experiment is supported by a combination of other assays, including cellular thermal shift assays (10), drug-resistance screens (11), and/or testing of the compound in cells in which the putative target has been knocked down, knocked out, or removed by CRISPRi (12). Together, the results of these experiments are used to assess whether a molecule is selective enough to be considered a chemical probe. Because so many different experiments are needed to understand selectivity, the evaluation process often takes years and involves multiple independent laboratories (typically in both academics and industry). Thus, many groups continue using a probe during its evaluation period, often confirming any results with independent methods. It is also important to note that the criteria needed to define a chemical probe are different from those needed to define a drug/therapeutic, i.e. selectivity is relatively more important for probes, whereas safety is of high value for drugs (13, 14).

Where does one learn whether an existing compound is considered a good chemical probe? On-line resources that collate this information, such as Chemical Probes (www.chemicalprobes.org) and Probe Miner (https://probeminer.icr.ac.uk/#/), are places to start. In addition, reference tools are available for rooting out the worst, most promiscuous molecules, such as pan-assay interference molecules (15) and protein aggregators (16). PubChem (https://pubchem.ncbi.nlm.nih.gov/) provides another guide, as it can be used to determine other assays in which a molecule has been found to be active (17). Finally, Open Science Probes (https://www.sgc.ffm.uni-frankfurt.de/) describes a collection of industry-derived tool molecules that have already undergone extensive validation (18). Together, these resources make it easier for the casual user to become quickly informed about a molecule's suitability for his/her experiment, including using it at the proper concentrations.

### Categories of chemical probes

In the case of targets in the chaperone network, it is worth considering two major classes of chemical probes: (i) those that inhibit the enzyme activity of a chaperone, and (ii) those that inhibit protein–protein interactions (PPIs) at the connection between two chaperones. These designations are somewhat arbitrary, but the methods for finding and improving them can be quite different, so a brief review of their characteristics is warranted.

#### Inhibitors of enzyme activity

The simplest case of a chemical probe is a molecule that binds at an enzyme's active site. The structure of these inhibitors is often based on the enzyme's substrate or product; thus, in a cellular context, it must bind tight enough to compete for the natural ligand (e.g. ATP). In contrast, allosteric inhibitors bind to a distal pocket (i.e. away from the active site) and only indirectly disrupt enzyme function, so they might not be competitive binders. The major target enzymes in the chaperone network include the ATPases, including TriC, Hsp70, Hsp90, and Hsp60. These chaperones use ATP hydrolysis to power conformational motions that are coupled to their function (albeit not directly (19, 20)). Thus, compounds that inhibit nucleotide binding in these proteins would be expected to block chaperone activity.

#### Inhibitors of PPIs

Not all of the chaperones have enzymatic activity; for example, small heat shock proteins (sHSPs), Spy, trigger factor, clus-

### Table 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Key molecules were examined for metabolic stability, reactivity, solubility, pharmacokinetics and other pharmacological properties</th>
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<tr>
<td>Biochemical</td>
<td>Immobilized compound will &quot;pull down&quot; the target; this interaction is competitive with free compound but not with related controls</td>
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<tr>
<td>Genetic</td>
<td>Compound affects known biomarkers</td>
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tively small BSA values (<2,000 to 4,000 Å²) (32–34). Moreover, the most “druggable” PPIs also tend to be those with tight affinity (K_d < 500 nM), likely because those contacts involve a closely spaced combination of hydrophobic and polar residues that facilitates tight inhibitor binding. Thus, not all PPIs are considered equally “druggable.” If PPI targets are placed into four quadrants based on their BSA and affinity values, then those with weak affinity and large BSA values are usually the most difficult. Conversely, targets with small BSA values and/or tight affinity tend to be more tractable.

**Targets in the chaperone network: nodes and edges**

The chaperones and co-chaperones are physically linked to each other through a series of protein–protein interactions, existing as a PPI network (35, 36). In this parlance and borrowing from the systems biology lexicon, we term the major chaperones (i.e. Hsp70, Hsp90, Hsp60, and TRiC) as “nodes.” In turn, these nodes are connected by a series of “edges” that represent the PPIs. As will be detailed below, we find these designations useful when considering chemical probes of the chaperone network; specifically, enzyme inhibitors target the ATP-utilizing enzymes of the nodes, whereas PPI inhibitors target the edges.

However, one caution in this nomenclature is that the edges should not be considered equivalent. Indeed, the physical connections between chaperones come in a great variety of shapes and sizes. For example, a short peptide sequence from the C terminus of Hsp70, EEVD, binds to the tetratricopeptide repeat (TPR) domain that is present in a family of co-chaperones (Fig. 1), such as CHIP and HOP (37, 38). The EEVD–TPR interaction is of relatively tight affinity (K_d ~ 0.5 μM), and it involves a small surface area (BSA ~ 1,100 Å²) (Fig. 2) (39, 40). By comparison, the interaction between Hsp60 and Hsp10 is weaker (K_d ~ 7 μM; Figs. 1 and 2A) and involves a 5-fold bigger contact surface area (BSA ~ 5,500 Å²) (41). More globally, we have shown a subset of chaperone PPI structures in Fig. 1 and collated the BSA values from available PDB-deposited structures of chaperone complexes and matched these to measured K_d values in Fig. 2A. Together, this information, although certainly not inclusive, drives home the point that inter-chaperone contacts (“edges”) have quite distinct topologies. For example, the measured K_d values range nearly 6 orders-of-magnitude (from 0.04 to >100 μM), whereas the BSA values can be compact (~700 Å² for the Hsp27 system) or large (>20,000 Å² for the Hsp90–Cdc37 contact).

Based on this analysis, some of the chaperone PPIs are expected to be relatively more difficult to inhibit. For example, PPIs with weak affinity (>500 nM) and large BSA values (>4,000 Å²), including Hsp90–p23, Hsp60–Hsp10, and Hsp90–Cdc37, are predicted to be particularly challenging (Fig. 2B). Other contacts, such as the ones between Hsp70–BAG1 and Hsp70-HOP, are predicted to be relatively tractable. Recent examinations of published PPI inhibitors have shown that small molecules (<500 Da) can often be used to inhibit a subset of PPIs, whereas the ones with larger BSA values typically need larger molecules, such as peptides or macrocycles (34). Therefore, it is reasonable to speculate that many different types of chemical scaffolds may be needed to inhibit the full suite of chaperone PPIs. In the following sections, we discuss a few examples of PPI systems that have been successfully targeted, with a focus on Hsp70, Hsp90, Hsp60, and sHSPs. In this discussion, we also comment on the current status of each molecule’s ongoing evaluation as a chemical probe, according to the criteria in Table 1.

**Inhibitors of the Hsp70 sub-network**

Hsp70 is called the “triage” chaperone (42) because it plays keys roles in both protein folding and turnover (43, 44). Hsp70s are composed of a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD) (45). ATP binds in the NBD, and the misfolded clients interact with the SBD. This chaperone is assisted by co-chaperones, including the J-domain containing proteins (JDPs) and nucleotide exchange factors (NEFs). Accordingly, there are at least two conceptual ways of targeting Hsp70: block its ATPase activity or change its PPIs targeting Hsp70: block its ATPase activity or change its PPIs targeting Hsp70: block its ATPase activity or change its PPIs. Molecules targeting the ATP-binding cleft include VER-155008 and apoptozole (Fig. 3) (47, 48), which have been recently reviewed (49). VER-155008 competes with nucleotide for binding, as shown by crystallography (47), and this molecule has been shown to have the anti-proliferative activity expected of an Hsp70 inhibitor in HCT116 cells (50). Similarly, immobilized apoptozole will pull down Hsp70 from
A549 (adenocarcinoma) cells (48), and the molecule induces apoptosis in that system. However, Hsp70s, as compared with kinases or Hsp90s, have an unusually tight affinity for ATP ($K_d \sim 100–500 \text{ nM}$), such that competition for cellular ATP (1–10 mM) creates a significant challenge. Covalent controls (71) are expected to increase our understanding of how these compounds might work. Still, although the potency and pharmacokinetics of this chemical series remain un-optimized (i.e. $EC_{50} \sim \text{micromolar}$), there is reason to be optimistic. For example, immobilized dihydropyrimidines pull down Hsp70 from cell lysates (56), and treatment with analogs, such as MAL3-101 and MAL1-27, has been shown to induce known Hsp70 biomarkers (58). Additional evidence for target engagement comes from studies in which yeast treated with an agonist, SW02, was partially protected from genetic deletions of a JDP (56). Moreover, treatment with MAL1-27 (also called 115-7c) protects against polyglutamin (polyQ) aggregation (56). Finally, acquired resistance to MAL3-101 in rhabdocarcinoma cells was mapped to an hsp70 gene (58). Together, these results provide support for selectivity in cells. The next steps for these molecules include expanded medicinal chemistry efforts to increase their potency and identification of additional negative controls. Given the importance of JDPs to Hsp70 biology (25, 54), this chemical series seems worth careful exploration.

The other major category of Hsp70 PPIs is the one with the NEFs, including the BAG family of proteins that bind to the NBD through a conserved BAG domain (61). The NEFs are important mediators of Hsp70 function because they control the release of clients from the complex (62, 63). Thus, blocking NEF binding to Hsp70 would be expected to increase the dwell time of clients in the chaperone complex, favoring their degradation in some cases (63). A series of rhodacyanine-benzothiazoles (Fig. 3; Table 2) have been identified that inhibit this PPI. These molecules were first described by Wadhwa et al. (64) in phenotypic anticancer screens and only later were they found to bind to cytoplasmic and mitochondrial Hsp70 family members in pulldowns. NMR studies showed that the compounds of this series bind in a deep, allosteric pocket on Hsp70 (63, 65). Binding to this site favors the ADP-bound form of Hsp70 and disrupts binding to BAG proteins through a conformational change. As expected from the natural role of the NEFs, treatment of cells with these compounds induces degradation of particularly sensitive “client” proteins, such as FoxM1 (66), Akt (67), RIP1 (68), and inhibitor of apoptosis proteins (69). Medicinal chemistry campaigns (>400 analogs) produced more potent molecules ($EC_{50} \sim 30 \text{ nM}$) and inactive controls (JG-258) and allowed initial correlation between in vitro activity and cellular functions (70, 71). Target engagement in cells and animals has also been explored using genetic approaches; for example, overexpression of a point mutant of BAG3 (R480A) that cannot bind to Hsp70s gives a similar phenotype to compound treatment in breast cancer cells (66). In addition, whole-genome CRISPRi studies revealed that knockdown of Hsp70 family members gives rise to compound sensitivity (71). Most recently, JG-231 and other analogs have been characterized in vitro for liver microsome stability and in mice for maximal-tolerated dose and pharmacokinetics (71). This pharmacological information enables use of the compounds in some animal and tissue models. For example, they were used to identify a role for Hsp70–BAG in breast cancer initiation (66), tau homeostasis (72), Dengue viral replication (73), and castration-resistant

### Table 1. Characterization of chaperone inhibitors

<table>
<thead>
<tr>
<th>Chaperone</th>
<th>PDB ID</th>
<th>Organism</th>
<th>Binding partner</th>
<th>BSA (approximate $A_2^1$)</th>
<th>Affinity ($K_d$, $\mu M$)</th>
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<tbody>
<tr>
<td>Hsp70</td>
<td>5NRO</td>
<td>E. coli</td>
<td>J protein</td>
<td>DnaJ</td>
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<td>NEF</td>
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<td>Bag2</td>
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<td>3A8Y</td>
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<td>HspBP1</td>
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<td></td>
<td>Chip</td>
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<td></td>
<td>4NMJH</td>
<td>Human (Hsp27)</td>
<td>α-crystallin domain-IPV</td>
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<td>5.0 - 10</td>
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</table>

*ADP state

**Figure 2.** PPIs between chaperones and their binding partners. A. table of BSA and affinity values for PPIs between chaperones. B. categorization of PPIs based on BSA and affinity values. Based on retrospective analyses of PPI inhibitors, certain quadrants are comparatively easier (green), challenging (gray), or difficult (red) to inhibit with drug-like small molecules.
Figure 3. Selected chemical probes for molecular chaperones. See text for citations and details.
Inhibitors of the Hsp90 sub-network

Hsp90 is a dimeric chaperone composed of three domains: an N-terminal ATPase domain, a middle region, and a C-terminal dimerization motif. In addition, this protein binds to a number of co-chaperones, including Aha1, p23, and Cdc37 (see Fig. 1). The best-known Hsp90 inhibitors are enzyme inhibitors that bind in the N-terminal domain, such as geldanamycin and its analogs (e.g. 17-AAG) (81). Some of these molecules are clinical candidates (82, 83), and they have been extensively explored for selectivity, including screening against a panel of ATP-binding proteins (84), so they are generally considered to be good chemical probes (85). Indeed, these compounds have been crucial in expanding our knowledge of Hsp90 function, including being used to identify its clients. Molecules of this type have been extensively reviewed (81), so they will not be discussed further.

Alternative ways of inhibiting Hsp90 have also been explored. For example, the natural products novobiocin/ coumermycin (86, 87) and sansalvamide A (88) served as inspiration for the development of inhibitors directed against the C-terminal domain (Fig. 3; Table 2). For example, Blagg and co-workers (89–91) and others (92, 93) have synthesized analogs of novobiocin, complete with negative controls (i.e. inactive molecules), and information about the binding site. Treatment with these compounds induces degradation of Hsp90 clients in multiple cancer cell types. In addition, they may disrupt Hsp90 dimerization (94) and co-chaperone interactions (95, 96), suggesting that they are bona fide Hsp90 PPI inhibitors. Similarly, McAlpine and co-workers (88) have produced biotinylated analogs of sansalvamide A and shown that they pull down Hsp90 from cells and disrupt binding to some client pro-

### Table 2: Subset of reported chaperone inhibitors along with a summary of ongoing studies that have evaluated their suitability as chemical probes

<table>
<thead>
<tr>
<th>Target</th>
<th>Chemical series</th>
<th>Chemical probe</th>
<th>Biological activity</th>
<th>Biological activity</th>
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<td>Hsp70</td>
<td>Dihydroxypyrrolinines</td>
<td>MAL-102 (52, 55, 56)</td>
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In this way, molecules from these two series are progressing toward becoming chemical probes. Interestingly, treatment with certain analogs of novobiocin and sansalvamide A does not induce a stress response in cells, which is considered a hallmark biomarker of the canonical, competitive Hsp90 inhibitors (85, 97). This finding highlights the importance of using multiple assays for assessing selectivity (see Table 1), as molecules with different MoAs might not always share the same biomarkers.

In addition to these chemical series, a number of other reports have introduced leads toward potential Hsp90 PPI inhibitors (98–102). Although these chemical series, such as celasterol, are relatively early in their analysis as chemical probes, further work may expand the suite of available chemical series for the Hsp90 sub-network.

**Inhibitors of the Hsp60 sub-network**

Hsp60—Hsp10 and its prokaryotic ortholog, GroEL—GroES, play important roles in protein folding (103). Hsp60 is thought to be located in the mitochondria of eukaryotes, where it helps stabilize client proteins. In addition to their ATPase activity, these systems involve multiple types of PPIs, including interactions between Hsp60 protomers and those between Hsp60 and the regulatory component (i.e. Hsp10; see Fig. 1). This system also likely interacts with the Hsp70 sub-network through direct PPIs. Most of the Hsp60 inhibitors that have been identified thus far originated in phenotypic screens, and only later did the pulldown studies suggest Hsp60 as a potential target. It is not yet clear whether these compounds are enzyme inhibitors (i.e. targeting “nodes”) or whether they disrupt PPIs (i.e. act on “edges”).

The chemical series described as Hsp60 inhibitors thus far are structurally diverse (Fig. 3; Table 2) and include 2-pyrenothiazole-pyrimidine-2,4-diamines, such as KHS101 (104), gold porphyrins (105), pyrazolo-pyridazines (106), phenoxyacetanilides (107), and the natural products suvanine (108), epolactaene (109), and myrtucommulone (110). Although more work remains to verify the selectivity of these molecules in cells, the striking lack of similarity in these chemical structures is suggestive of different binding sites or MoAs. However, none of these putative Hsp60 inhibitors has yet been subject to extensive medicinal chemistry or the full spectrum of analyses that are needed to give great confidence in their use as probes (see Table 1). Overall, given the central role of Hsp60–Hsp10 in mitochondrial protein quality control (111), it seems worth a greater investment in chemical probe discovery for this system. For example, KHS101 has been shown to disrupt energy metabolism in glioblastoma (104), suggesting a cancer-specific role for Hsp60 in mitochondrial function. These efforts might also benefit from screens focused on finding inhibitors of the folding activity of the prokaryotic GroEL–GroES system using in vitro assays (112).

**Inhibitors of the sHSPs**

The sHSPs are chaperones that lack enzymatic function; rather, they seem to operate by binding directly to each other and to their client proteins and co-chaperones, such as BAG3 (26). Thus, the only way to inhibit these systems is to target their PPIs. The sHSPs engage in a number of distinct interactions, such as the one between conserved α-crystallin domains (ACDs) that are known to stabilize sHsp dimers (see Fig. 1). Another, nonoverlapping interaction is the one between the ACD and the IXI motif that is found in the C terminus of some sHSPs (113). Finally, the N-terminal domain of some sHSPs also seems to make interactions within larger oligomers (114). Thus, sHSPs are a rich source of PPIs, which could become targets for chemical probes. However, the structural complexity of the system has hindered development of such molecules. Aptamers directed at Hsp27 (HSPB1) (115), diterpenes that seem to bind to Hsp27 (116), and oxysterols, such as compound 29, that bind to the ACD of α-crystallin (HSPB5) (117) have been identified (Fig. 3; Table 2), but their selectivity in cells has not been extensively explored.

**Other inhibitors**

We have focused this discussion on molecules that target a handful of heat shock proteins (i.e. Hsp70, Hsp90, Hsp60, and sHSPs). However, the proteostasis system includes other chaperones that are not classified as heat shock proteins but could be important targets. For example, Kelly and co-workers (118) have recently described inhibitors of PDIs, including information on target validation, MoA, and medicinal chemistry. These molecules activate the unfolded protein response, so they have promise in improving quality control in the endoplasmic reticulum. Recent efforts are also producing new inhibitors of the FK506-binding protein (FKBP) family of PPLases (119, 120), including the first selective inhibitors ofFKBP51 (121). Such molecules might be especially good probes of steroid hormone receptor biology (120). The broader protein quality control field also benefits from the availability of chemical probes that inhibit proteins that are not widely considered to be chaperones, including VCP/p97 (122), the proteasome (123), the Sec61 channel (124), and the integrated stress response (125).

Although we lack the space to adequately describe these molecules or evaluate their validation as chemical probes (see Table 1), they collectively serve to provide a wider chemical toolbox for studying proteostasis.

**Outlook for the future**

Chemical probes are powerful tools for studying and perturbing the chaperone network. Despite the production of probes for a handful of chaperone systems, such as Hsp70 and Hsp90, there is much more work to be done. For example, there are no validated probes for major nodes, such as TRiC. Likewise, hundreds of PPIs (“edges”) lack chemical tools. An optimistic vision for the future is one in which each chaperone node and edge has a well-validated inhibitor. Although this goal is certainly ambitious, there is legitimate reason for hope. New technologies, such as CRISPRi, high content screening, cryo-EM (126), isoelectric-focusing capillary electrophoresis (127), and others, are accelerating the rate of probe discovery and optimization. At the same time, increasingly sophisticated chemical libraries, such as macrocycles (128) and natural product–inspired libraries (31), which tend to be enriched in PPI inhibitors, are being built. It seems likely that these
advances will combine to produce additional chemical probes for a wider range of chaperones and their PPIs.

Acknowledgments—We thank our many colleagues in the proteostasis and chemical biology fields, whose combined wisdom we tried to reflect here. We also apologize to those groups whose work we were not able to include, and we thank Dan Schwarz for helpful discussions.

References


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