In vitro evolution of uracil glycosylase towards DnaKJ and GroEL binding evolves different misfolded states

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Author Contributions: The study was designed by OM, PG and GS; samples preparations were made by OM. Experiments were performed by OM. Data were analyzed and the manuscript was written by OM, PG, GS.

Competing Interest Statement: none.

Classification: Protein evolution.

Keywords: Protein-protein interaction, protein-evolution, promiscuous binding, chaperones

Abstract

Natural evolution is driven by random mutations that improve fitness. In vitro evolution mimics this process, however, on a short time-scale and is driven by specific outcomes, such as binding to a high-affinity bait. Here, we used directed in vitro evolution to determine how DnaKJ and GroEL change the sequence and structure space of the *E. coli* protein Uracil glycosylase (eUNG). Using yeast surface display, we generated a random library of eUNG, and selected it for binding to chaperones GroEL or DnaK+DnaJ+ATP (DnaKJ). We found that these chaperones select and enrich for mutations causing eUNG to misfold. Evolution in the presence of either chaperones thus resulted in the accumulation of mutations in buried and conserved positions, with a tendency to increase positive charge. However, GroEL selected for more exposed and less structured proteins that were highly sensitive to protease cleavage, while DnaKJ selected for partially structured misfolded species with a tendency to refold, making them less sensitive to proteases. In a more general context, our results show that ATP-fueled chaperones can purge promiscuous misfolded protein mutants from the system, and thereby avoiding their potential detrimental effects, such as forming wrong interactions with native proteins or form aggregates, compromising cellular function.

Introduction

HSP70 (DnaK) and Hsp60 (GroEL) are ancient, highly conserved classes of molecular chaperones (1) that act as polypeptide unfolding enzymes (2). Both can use the energy of ATP-hydrolysis to forcefully unfold stably misfolded structures in aggregated polypeptides and thereby assist misfolding-prone proteins to reach and transiently stay in the native state, even under stressful conditions inauspicious to the native state (3). As such, these chaperones can buffer less stable genotypic variations (2, 4–8). In the case of Hsp70-Hsp40-Hsp110 (DnaK-DnaJ-GrpE in *E coli*), chaperone-assisted unfolding-refolding occurs via DnaJ-binding motives, enriched with bulky hydrophobic amino acids on the surface of misfolded structures in aggregated polypeptides (9) and DnaK-binding motives, generally consisting of five consecutive hydrophobic residues, flanked by positive charges in un-structured polypeptide segments (10). In the case of Hsp60 (GroEL-GroES in *E coli*), chaperone-assisted unfolding-refolding occurs via cooperative binding of exposed hydrophobic surfaces on aggregation-prone misfolded polypeptides, to a ring of hydrophobic

motives displayed by the apical domains of the GroEL heptameric oligomers (11, 12). With the help of such chaperones, proteins can tolerate more destabilizing mutations, alleviating the evolvability penalty from loss of free energy. Yet, increasing the mutation load of a protein also increases its tendency to develop non-specific promiscuous interactions with other proteins in the very crowded cellular milieu, which is counter-productive. Because of their sticky nature, solvent-exposed hydrophobic residues are particularly problematic, and are thus constrained by purifying selection to maintain low hydrophobicity at a protein surface and reduce the likelihood of generating non-specific promiscuous sticky patches (13, 14).

DnaK-DnaJ and GroEL preferentially interact with the misfolded states of proteins, with a strong preference for hydrophobic patches that are abnormally exposed to water. Following ATP-dependent interaction with client misfolded proteins and their consequent active unfolding by the chaperones, the affinity of Hsp70 for the unfolded state was found to be significantly tighter than the affinity for the native state (15, 16). A key aspect of the unfolding-refolding mechanism is that ATP hydrolysis adjusts the structure, and by this the relative affinity of the chaperone for the client towards weaker affinity (16, 17) This reversibility is important because binding of first DnaJ, then of DnaK, or of GroEL oligomers, can inhibit aggregation which, following forceful unfolding and release of the unfolded polypeptide, can at the same time favor the productive refolding to the native state. Acknowledging this multistep ATP-dependent binding-unfolding-release mechanism of HSP60 and HSP70 chaperone activity, we aimed to determine the sequence and structural consequences of in vitro evolving a protein towards chaperone binding. For the *in vitro* evolution, we used the *E.coli* protein Uracil glycosylase (eUNG). This is a 229 amino acid protein, excises uracil residues from the DNA as a result of mis-incorporation of dUMP residues (18). Folded eUNG binds with nanomolar affinity to the UNG inhibitor Ugi. Therefore, Ugi-binding can serve as a proxy for the structural integrity (native foldedness) of eUNG (assuming the incorporated mutations did not directly compromise binding). *In vitro* evolution was done by generating a library of randomly mutated Uracil glycosylase (eUNG-RL) proteins displayed on yeast surface, with FACS selection for strong binding either to purified $GroEL_{14}$ oligomers or to $DnaK+DnaJ_2+ATP$. Binding of Ugi to the selected yeast-displayed variants was used to distinguish between mutant eUNGs that retained their native conformation and mutant eUNGs that lost their Ugi-binding ability, and likely turned into a misfolded species, prone to engage in promiscuous interactions with other native or misfolded proteins and aggregate (assuming that

only folded eUNG will bind Ugi, see Figure 1). Both DnaKJ and GroEL were found to select for misfolded eUNG that did not bind Ugi. Still, differences were observed between GroEL- and DnaKJ-selected mutant proteins, with the former driving less compact states. Overall, the modulation of a protein evolutionary landscape orchestrated by these chaperones resulted, with evolutionary cycles, in the increasing accumulation of promiscuous mutants with non-native structures, suggesting that the DnaKJ and GroEL chaperones can salvage the proteome from the mildly deleterious destabilization effects by some useful new mutations, while purging mutants with dramatic aggregation-prone effects that can affect cellular functions.

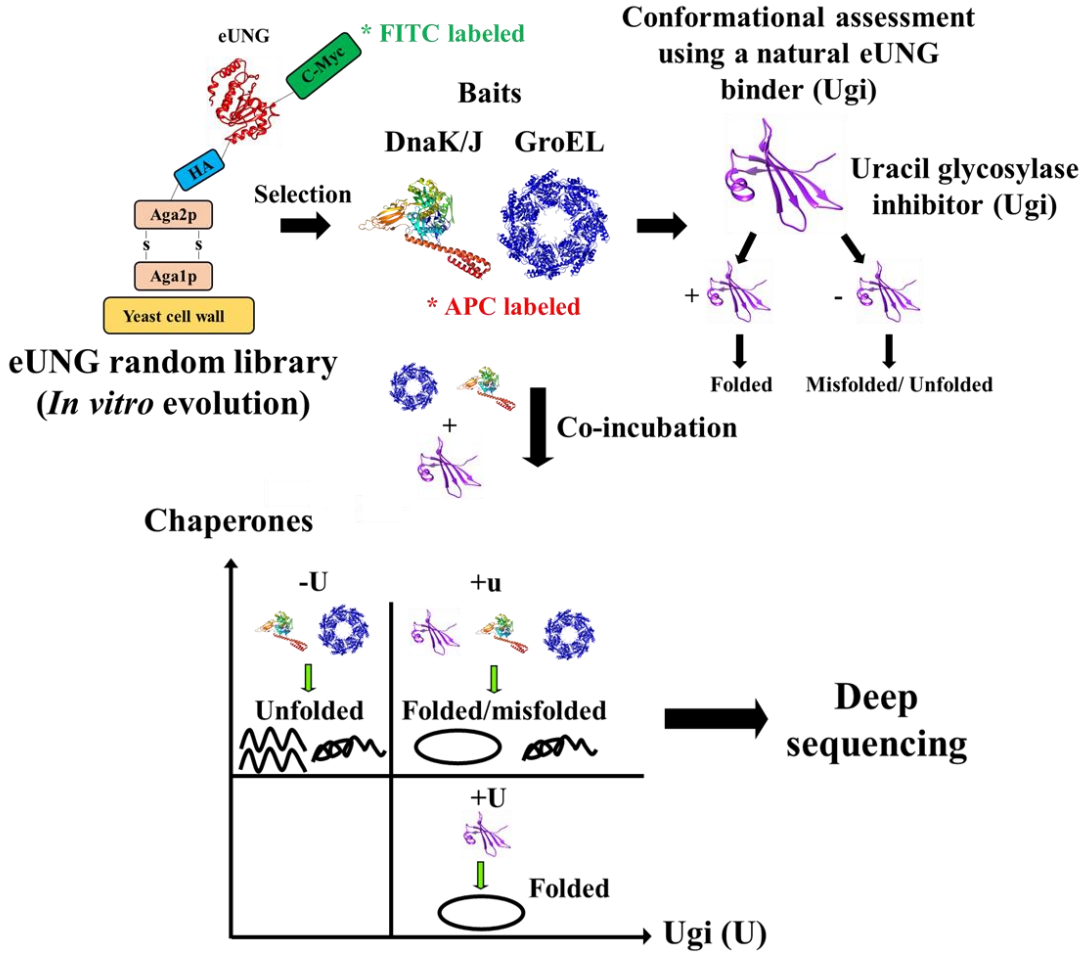

Figure 1. Experimental setup**.** A yeast surface display library of randomly mutated eUNG was prepared and selected against purified chaperones: DnaK+DnaJ+ATP (KJ) or GroEL-ATP. Folded and misfolded/folded variants were separated by their ability to bind fluorescently labeled uracil glycosylase inhibitor (Ugi). Selected clones binding Ugi are assumed to be folded, while those not binding Ugi are assumed to be misfolded or unfolded. Enriched populations were co-incubated with both chaperones and Ugi, to distinguish between folded and misfolded populations. We expect to see one populations, out of possible three, that binds only the chaperones, but not Ugi. eUNG variants from enriched populations were sequenced to study the relation between evolution of mutations and the baits used to enrich them.

Results

Evolving eUNG for chaperone binding

Chaperones have been shown to buffer evolution by increasing the allowed mutation load for marginally stable proteins, preventing their misfolding and aggregation (6, 19). Here, we examined the outcome of *in vitro* evolution of randomly mutated eUNG using chaperones as baits. First, we validated that externally supplied chaperones can specifically recognize and bind a protein displayed on the yeast surface when it is misfolded and not in its native conformation. Heat pre-misfolded fire-fly luciferase is known to bind DnaKJ and GroEL (20). Luciferase was inserted into the pCTcon2 yeast display vector C-terminal to Aga2p, where it is exposed to the surface, and incubated with pure $GroEL_{14}$ oligomers or DnaK+DnaJ+ATP. These were incubated on ice, or were subjected to mild heat treatment (42ºC) that is known to cause luciferase to irreversibly misfold in several minutes (without affecting the viability of yeast). We found that, as expected, the heat-denatured luciferase on the yeast-surface bound DnaKJ, and to a lesser extent GroEL, whereas native luciferase on ice did not bind the chaperones (Figure 2). This experiment confirms that one can use yeast surface display to select for binding of misfolded proteins by chaperons.

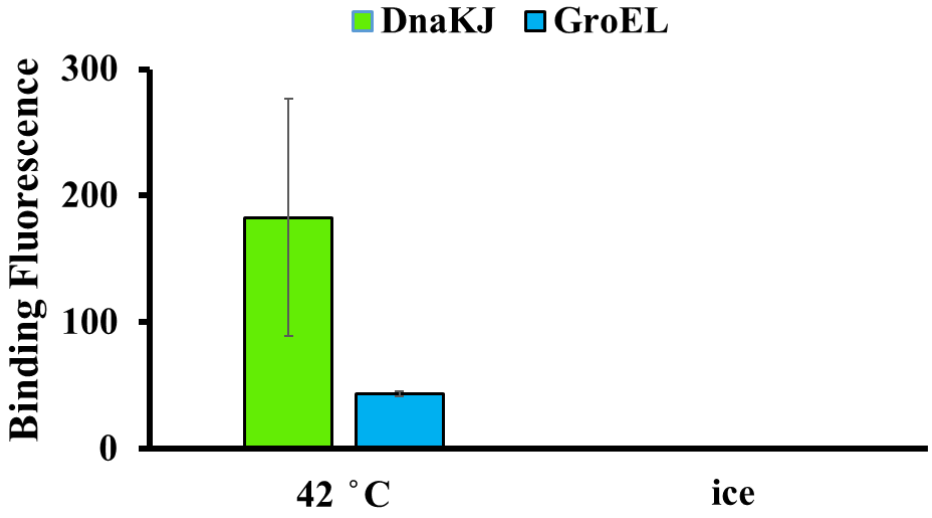

Figure 2. Chaperones bind mis-folded luciferase. Binding of labeled GroEL and DnaKJ to luciferase expressed on yeast surface. For denaturation, luciferase was incubated at 42˚C for 5 min with purified GroEL and DnaKJ protein. Binding fluorescence was analyzed by FACS. Controls were incubated on ice. Both chaperones were labeled with APC. GroEL conc. = 1uM, DnaK/J = 2/0.4uM. Y-axis - APC binding fluorescence (640nm) of normalized values.

Evolving eUNG for chaperone binding

In vitro evolution of a randomly mutated protein is a powerful tool to study how the bait is dictating the fate of the evolved protein. Most commonly, the bait consists of another protein, where enhanced binding between the two is the outcome. Here, we evolved eUNG-RL against purified DnaK+DnaJ+ATP (DnaKJ) or GroEL proteins. The random eUNG library was constructed by error-prone PCR, incorporating on average ~2-4 nucleotide substitutions per clone. The unsorted eUNG-RL underwent deep mutational scanning to determine the genetic complexity of the starting library. Using Enrich2 (see materials and methods) and Matlab (R2017a) the number of mutation-reads per position was extracted. Output reads were used to calculate the log-ratio of eUNG-RL (Table S1). The log-ratio is the common log of the total reads per position divided by the total number of reads including the number of WT reads normalized by the same calculation for the

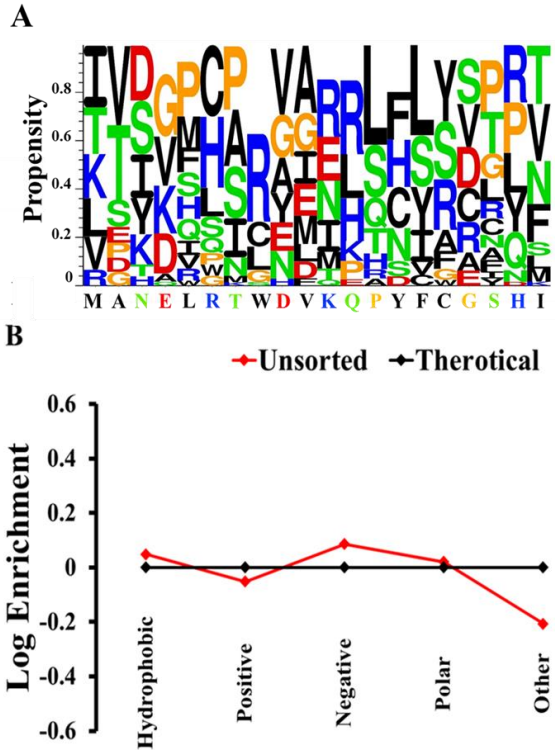

Figure 3. Natural evolution differs from *in-vitro* evolution in sequence space. **A.** Sequence logos display for the probability of WT amino acids to turn to any of 20 other amino acids in the unsorted library. Each WT amino acid and its corresponding possible mutation in the unsorted library was identified and its frequency was calculated as reads of possible mutations by the total number of reads for a possible mutations using Eq.4 where R_M denotes mutation reads over eUNG amino acid sequence. **B.** Hydrophobic, positive, negative, polar and other amino acids enrichment in the eUNG-RL (red) relative to the theoretical calculated values (black) assuming single-nucleotide mutations of the WT eUNG sequence. Analysis was done using MATLAB V.2017a

unsorted library using Eq. 1 (see materials and methods). Log ratio > 0 indicates a positive selection and log ratio < 0 a negative selection for a given position. Figure 3A shows the logo of eUNG-RL, and Figure 3B shows the enrichment of residue types of eUNG-RL relative to the theoretical calculated values, taking into account the propensity of amino acid changes upon single nucleotide mutations. This has been done as the frequencies of mutations requiring 2 nucleotide

changes were very low, as expected. Probing the subset of amino acids reachable by single-nucleotide mutations is in line with natural evolution.

Having established that pure GroEL and DnaKJ (+ATP) bind preferentially to a yeast surface-exposed misfolded luciferase, but not to a natively-folded one, and having established a random library of eUNG-RL, we next used DnaKJ and GroEL as bait against eUNG-RL. Each round of selection was evaluated by *in vitro* co-incubation of the yeast cells with the chaperones and with Ugi. The progress of selection against DnaKJ and GroEL was found to be accompanied by a loss of binding to Ugi, indicating that the chaperones selected mostly for misfolded eUNGs (Figure 4A). Strikingly, the binding pattern for Ugi versus DnaKJ appeared to be mutually exclusive. Furthermore, tighter binding towards DnaKJ evolved more rapidly (after fewer evolution cycles) than binding towards GroEL (as judged by the increase in the APC signal). This suggests that the random library was selected for presumably misfolded eUNG–RL mutants with higher affinity towards DnaKJ, than towards GroEL.

Thermodynamic characterization of selected eUNG clones as function of chaperone binding

To further investigate the degree of native foldedness of eUNG in the different selected populations, twenty single-clones from each population were isolated and sequenced. The mutational profile from each variant was used to calculate the change in free energy of folding of the individual mutations ($\Delta\Delta G$ [kcalmol^{-1}]) (21), from which a $\Sigma\Delta\Delta G$ was estimated for each variant (Figure 4B). For comparison, we also selected eUNG-RL against *E.coli* cell extract, and selected for clones that did bind Ugi (called WT CE+U). Clones binding either GroEL or DnaKJ had negative $\Sigma\Delta\Delta G$ values (destabilized), with no significant difference between the GroEL or DnaKJ selected populations (Figure 4B). This is in strong contrast to the WT CE+U population, for which no change in calculated $\Sigma\Delta\Delta G$ was found.

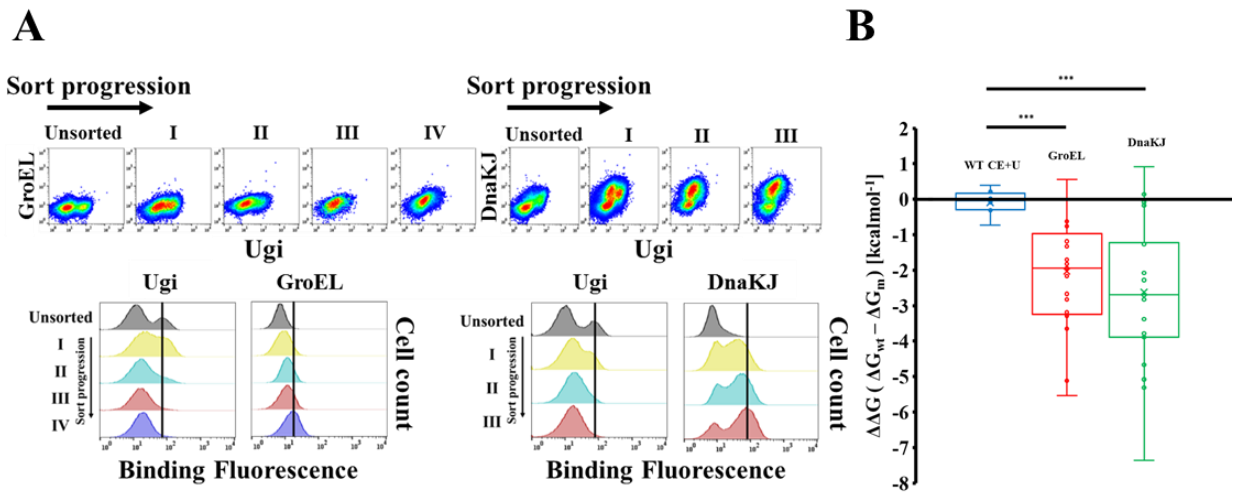

Figure 4. eUNG-RL selection against GroEL or DnaKJ results in loss of Ugi binding as a function of loss of free energy. **A.** eUNG-RL was selected against either GroEL (left) or DnaKJ (right), monitoring chaperon and Ugi binding after each round of selection. Progression of sort is indicated in roman numerals. Top, Scatter plots and bottom histograms of GroEL [1uM] and Ugi [5uM] as bait, binding yeast displayed eUNG-RL at each round of sort. After four rounds of selection, Ugi binding is not observed. Top right, scatter plots and histogram fit (bottom) of DnaKJ [2 or 0.4uM] and Ugi [5uM] with eUNG library at each round of sort. The gradual decrease in Ugi binding is visible. Black bar indicate maximum binding intensity for each substrate (chaperones or Ugi). Scatter plots: x axis – Ugi binding (FITC) and y axis – DnaKJ/GroEL binding (APC). Histograms: x axis – FITC/APC channel (530/640nM respectively) and y axis – cell count. DnaKJ was labeled by APC and Ugi was labeled by FITC. **B.** Free energy calculations for different sorted populations of eUNG. Box plot of the ΣΔΔG eUNG variants sorted against WT cell extract and chaperones as a function of +/- Ugi binding. WT CE+U stands for eUNG-RL selected against E.coli cell extract, with the clones selected binding Ugi and cell extract. ΔΔG was calculated using SDM and represents the difference in free energy between the eUNG wt and the mutant. Values represent the ΣΔΔG of mutation for a single variant.

Analyzing the mutational landscape of chaperone-sorted populations by deep sequencing. Mutations in the core of proteins or in conserved positions will more frequently translate in loss of folding free energy (22, 23). The degree in which a protein is destabilized changes the equilibrium between locally folded, transiently unfolded, stably unfolded (which is rare for globular proteins because of the tendency of exposed hydrophobic residues to avoid water), and stably misfolded conformations. Here, we examined the relation between the position and conservation of mutations in the different eUNG-sorted populations and their tendency to bind Ugi. Each sorted population and the unsorted eUNG-RL underwent deep mutational scanning and evaluated as explained above, for the unsorted eUNG-RL. Output reads were used to calculate the log-ratio of each population (Table S1). Both GroEL and DnaKJ-selected population's log-ratios showed reduced genetic complexity relative to the unsorted library (eUNG-RL). For mutations

that were significantly higher than the mean of log-ratio > 0 (frequent mutations), the mean accessible surface area (ASA) and ConSurf conservation score (CS) were calculated (Table S2) (24, 25). The respective ASA and CS values of the different selected eUNG populations are shown in Figure 5A. As expected for a folded population, the mutations in eUNG sorted against WT CE+U were mostly surface-exposed and had a low conservation score. Conversely, mutations in eUNG sorted against either GroEL or DnaKJ were characterized by low ASA (buried) and high CS. Buried and conserved mutations increase the susceptibility of a protein to become non-native, explaining the loss of Ugi-binding in these populations. The results shown in Figure 5A are in line with those shown in Figure 4, with both suggesting that sorting against chaperons results in mutations that result in the protein being non-natively folded.

Susceptibility to protease cleavage of the sorted populations

Trypsin and Proteinase K are serine proteases that cleave after Arginine/Lysine or hydrophobic amino acids, respectively (26, 27). Cleavage is dependent on the recognized site to be exposed, with buried sites being mostly shielded. Therefore, loss of native structure renders a protein more sensitive to digestion by a limiting concentration of a protease. Accordingly, chaperone-binding enriched populations should be more sensitive to protease digestion in accordance with their respective ASA, CS and their loss of Ugi binding. This hypothesis was examined by incubating enriched yeast populations with limiting concentrations of trypsin or proteinase K, for 5 min. The conditions were preset such that no more than 50% of the natively folded WT eUNG was cleaved. Protease digestion was measured by FACS as loss of FITC signal (due to yeast surface displayed eUNG being cleaved) and the percentage of remaining fluorescence was plotted. The WT CE+U binding population was almost as resistant to proteolysis as the WT (Figure 5B). On the other hand, the GroEL-binding population was completely degraded, in line with it being comprised of less compact misfolded proteins. Surprisingly, the DnaKJ selected population was only partially digested, despite displaying the same reduction in $\Sigma\Delta\Delta G$ (Figure 4) and high ASA and CS as the GroEL-selected population. This would suggest that the structural characteristics of the DnaKJ-selected population differs from that of GroEL-selected population.

To get a closer look at the difference between the GroEL- and DnaKJ-sorted populations, we took the last round of sort of both and re-analyzed their binding to DnaKJ or GroEL (Figure 5C). In addition, we also added GrpE and ATP+GroES, respectively to the DnaKJ- and GroEL-

sorted eUNG. The co-chaperone GrpE functions as a nucleotide exchange factor to promote dissociation of ADP from the nucleotide-binding cleft of DnaK, and thereby driving substrate dissociation, which can lead to its refolding. Figure 5C upper panel shows the DnaKJ sorted population to have some residual Ugi binding characteristics, particularly after the addition of GrpE. Conversely, the GroEL-sorted population had no Ugi binding activity, even in the presence of DnaKJ+GrpE (second panel). The reciprocal experiment shown in the third panel shows that GroEL binds the DnaKJ-sorted population, however, in the absence of DnaKJ no Ugi binding was observed. GroEL also bound GroEL sorted population, again without Ugi binding. Interestingly, GroEL bound two distinct DnaKJ-sorted populations (third panel), one stronger than the other, while the GroEL selected population was homogeneous. This suggests that a more versatile eUNG was selected by DnaKJ, in line with the limited proteolysis observed. This also suggests that sorting against GroEL results in mutations that are less compact and therefore with more unfolded segments than sorting against DnaKJ. This is in line with our initial observation that heat-denatured luciferase binds DnaKJ better than GroEL (Figure 2).

Previous reports have shown that both DnaKJE and GroELS recognize misfolded proteins and use energy from ATP hydrolysis to unfold and provide them with a new chances to reach the native state (28–30), even under out-of-equilibrium conditions unfavorable the native state (31, 32) Yet, whereas GroEL can only unfold misfolded polypeptides which are smaller than 55 kDa and cannot disaggregate already formed proteins aggregates (33), DnaK can solubilize and unfold large polypeptides from already formed stable aggregates (34). Our results further suggest that there is a fundamental difference between the two polypeptides unfoldases: while DnaKJE may target misfolding-prone protein mutants that can still be refolded to the native state and thereby assist them to evolve into new proteins, GroEL may target protein mutants that are more extensively damaged in their structure and contribute to their purging from evolution.

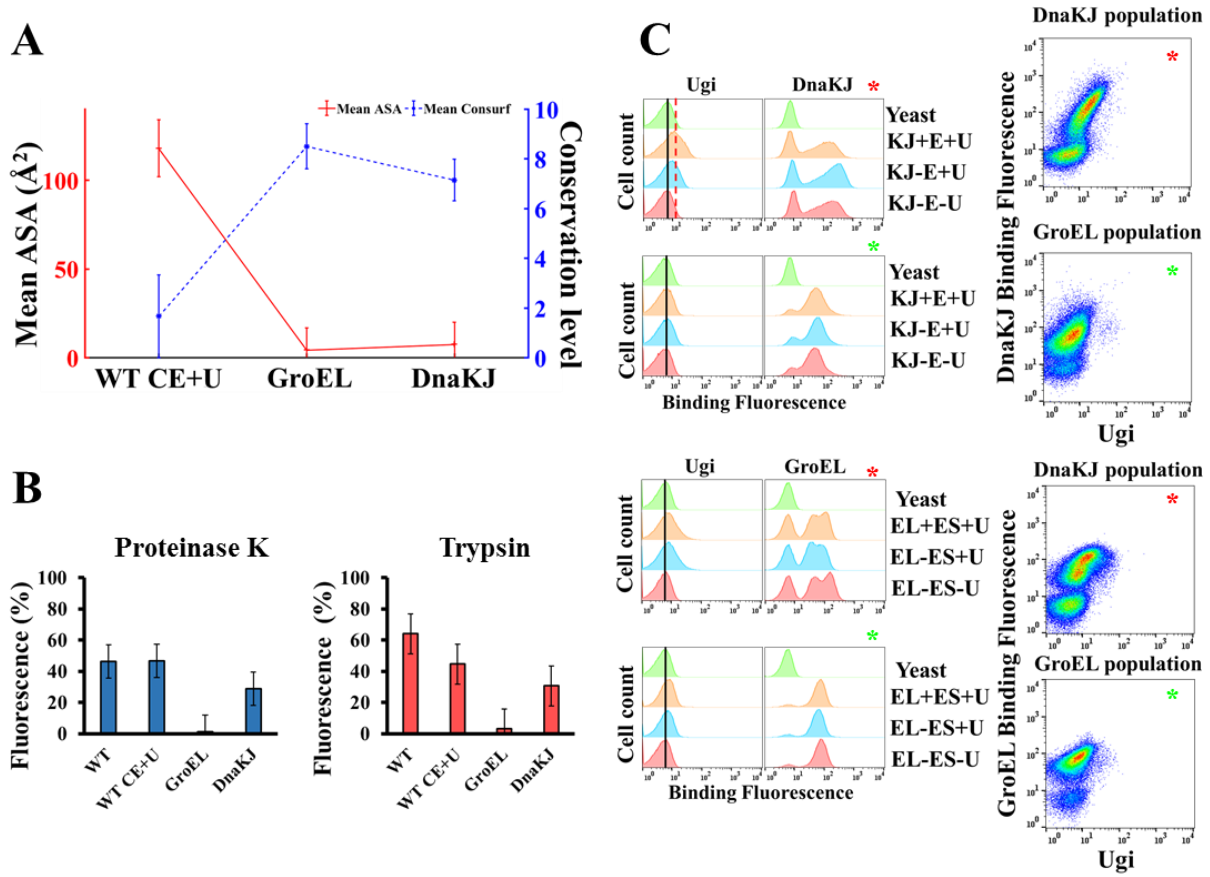

Figure 5. Biophysical properties of chaperone enriched populations. A. Negative correlation between accessible surface area (ASA) and ConSurf conservation score for WT CE, DnaKJ and GroEL selected populations. ASA and conservation scores were calculated using PDBePISA and ConSurf servers, respectively. Each selected population mean log ratio was calculated and positions with values significantly higher (p.value = 0.05 or 0.01) were used to calculate the mean ASA and ConSurf values. ASA is plotted with blue broken line and each population is marked with asterisk. ConSurf is plotted with red solid line and each population marked by a dot. **B.** Proteinase K (left) or Trypsin (right) treatment of enriched co-incubated populations. Each population was labeled with FITC and incubated with a limiting concentration of trypsin or Proteinase K, such that following 5 minutes at 37˚C over 50% of the WT native eUNG resisted the protease treatment. Controls were incubated only with PBSX1. Change in fluorescence was analyzed using FlowJO and plotted as percentage of remaining fluorescence. **C.** Re-analysis of DnaKJ (panels 1, 3) or GroEL (panels 2, 4) selected population for DnaK [2μM]+DnaJ [0.4μM]+GrpE [1μM] +ATP (4mM) (panels 1, 2) or GroEL [1μM] GroES [1μM] +ATP [4mM] binding (panels 3, 4), respectively. Each was monitored also for Ugi [5μM] binding. Left panels show DnaKJ and GroEL selected population after refolding with their controls. KJ+/-E+/-U and EL+/-ES+/-U letters are initials for incubations with DnaKJ (KJ), GrpE (E), GroEL (EL), GroES (ES) and Ugi (U). Increase in Ugi binding affinity is depicted by the broken red line. Non-binding sub-populations are marked with black line. Right panels – scatter plots of DnaKJ and GroEL after refolding with DnaK+DnaJ+GrpE and GroEL+GroES.

Residue-dependent enrichment of selected populations

So far, we discussed the general properties of the different evolved populations (Figures 4 and 5). Next, we analyzed the mutation space of the evolved populations on a per-residue basis (Figure 6). The sequence space of the eUNG protein was divided by two parameters: 1. Log-ratio of the frequency to obtain a mutation at a given position of a sorted library divided by the frequency of the unsorted library (R0). 2. Frequency of a mutation in a specific amino-acid divided by the number of reads at the given position normalized by the unsorted library (Table S3). The first represents the spatial distribution (SD) of mutations per position in relation to the unselected library (positive or negative). The second, designates as specific spatial distribution – SSD, which describes the frequency of a specific position to be mutated into a specific amino acid (rather than any). Principle component analysis (PCA), which correlates population variance further verifies this conclusion (Figure 6A). PCA transforms a large set of variables into a smaller one, retaining most of the information content. PCA clearly identified the similarity between selections against GroEL and DnaKJ in the two main principle components, which is distance from WT CE+U. In addition, the log-ratio of SD of the different selected populations was clustered in hierarchal manner over the sequence of eUNG (Figure 6A – heat map). In simple words, it shows the per-residue *in vitro* evolution as a function of the specific bait. This analysis shows that the two populations evolved against purified GroEL and DnaKJ proteins cluster together, and are very different from the WT CE+U population in terms of positions positively enriched for mutations.

Specific spatial distribution (SSD) was the second element that was thought to influence promiscuous evolution. It consists of amino acid residues, which were either negatively or positively selected relative to the starting library, which we have shown to represent random amino-acid frequencies in accordance to random single nucleotide mutations (therefore, the values in Figure 2B are around zero). Yet, as 8 codons code for positively charged residues and only 4 for negatively charged residues, the random library contains 13% Arg+Lys and only 6.5% Asp+Glu (Table S4), and thus the random protein is on average positively charged. Counting the amino-acid types in WT eUNG and averaging them over homologous UNG proteins as retrieved by ConSurf, shows frequencies that are richer in negatively charged residues and poorer in positively charge residues than the random library (Figure 6B), giving the protein a -2 net charge. This suggests evolutionary pressure towards a more neutral charge distribution of UNG than randomly expected, when starting from the eUNG sequence (35). Next, we calculated the amino-

acid frequencies of the different selected populations and grouped them according to positive, negative, polar, hydrophobic and other amino-acids (Figure 6B). Interestingly, GroEL and DnaKJ selections resulted in further enrichment of positively charged amino-acids and fewer negatively charged amino-acids relative to eUNG-RL (which already has excess positive charge amino-acids), which is in contrast to natural UNG evolution (Figure 6B). WT CE+U resulted in fewer negatively charged residues together with fewer polar residues (Table S4), suggesting that evolutionary pressure towards cell extract binding while maintaining Ugi binding, is very different from that dictated by the fitness of a protein for its biological function (which includes avoiding the binding to other proteins in the cell extract). To obtain a more detailed picture, the log enrichment of the individual amino-acid mutations of each population was clustered and compared (Figure 6C). Strikingly, while both GroEL- and DnaKJ-selected populations displayed an increase in positive charge, their amino acid distributions were different. Whereas both selected populations were enriched with arginines, only the GroEL population was enriched with lysines. In addition, GroEL selection favored prolines, while DnaKJ did not. In addition, most of the variance between populations can be explained by the PC1 (Figure 6D). It revealed that chaperone-binding populations and WT CE-binding populations share similar SSDs. Remarkably, the 'eUNG evolution' was most distinct, confirming that in-vivo evolution is completely different from our in-vitro evolution with purified chaperones.

Using a more structural view on the locations of the most abundant mutations in the SSD, we mapped them on the eUNG structure. The log ratio values of the most abundant mutations of the chaperone-binding populations are mapped near the C-terminus (Figure S1), which, as a consequence, is more positively charged. To better understand the observed increased selection for positively-charged residues, observed for selection against chaperone proteins, we calculated the electrostatic potentials of eUNG and Ugi (Figure S2). Ugi shows a strong negatively charged surface pointing towards eUNG, which displays a positively charged surface, albeit weaker, towards Ugi. Therefore, while the increased positive charge in chaperone-selected eUNG populations should benefit the protein-inhibitor complex binding, its lack suggests that it is more prone for misfolding.

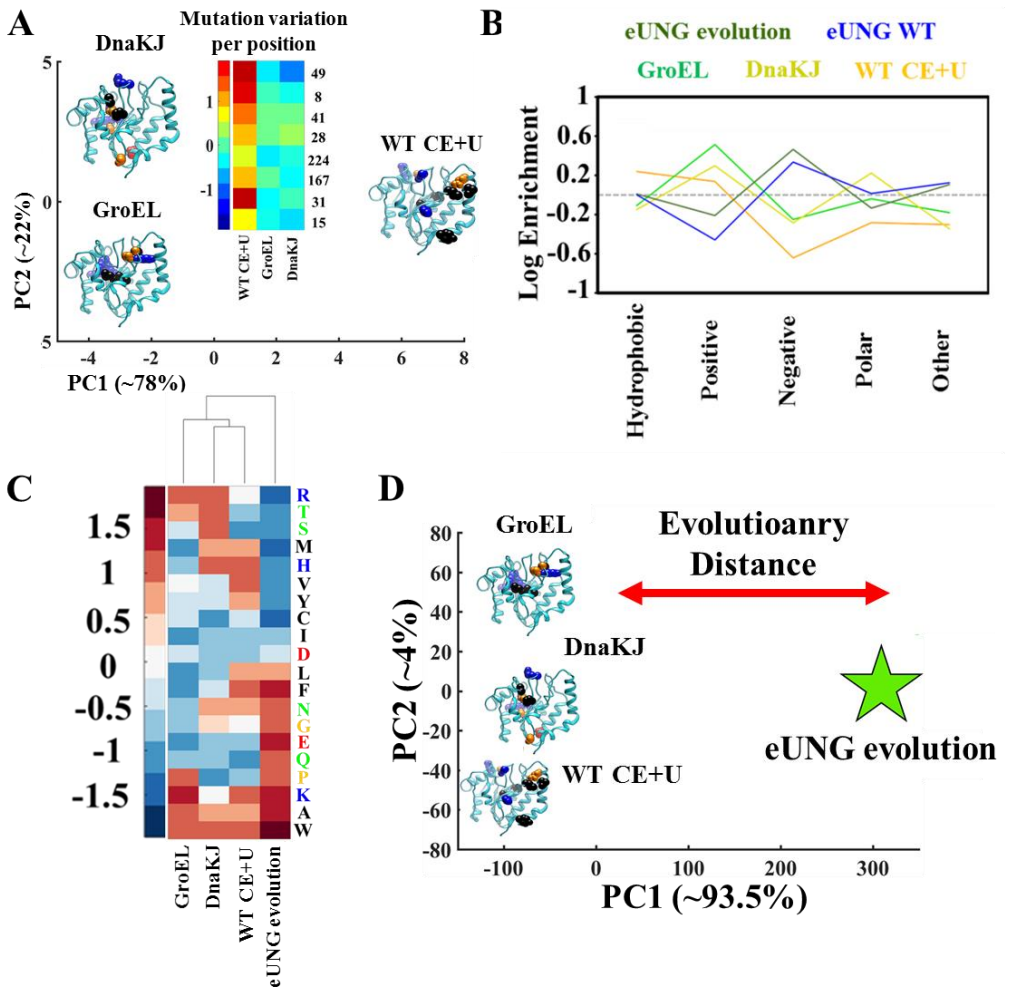

Figure 6. Covariance of enriched chaperones or CE sorted populations. **A.** PCA of the log-ratio values of DnaKJ, GroEL and WT CE+U binding populations. For each population positive log-ratio values were mapped on eUNG (PDB = 1FLZ) structure with their corresponding mutations. Axis percentage reflect the explained variance by a given principle component. PCA was calculated using the log ratio data derived using Eq. 1 (see materials and methods). Insert is the log-ratio clustering of enriched per position mutations in an area of interest of the cluster. Numbers on the right indicate variance position. Data is shown as a heat map. Each population log-ratio was combined to form an n-by-m matrix where n is the row number of the log-ratio in each position of the eUNG sequence and m is the chaperones/CE enriched populations. Red indicates high variance and blue low variance, per position. Values were standardized to have mean of 0 and SD of 1. **B.** Frequency of type of amino acid normalized by the theoretical frequency as calculated from the codon usage (Figure 3B and Table S4). The probability of having hydrophobic, positive, negative, polar or other side chain was calculated for selected populations. **C.** Sequence space of enriched GroEL, DnaKJ and WT CE+U binding populations as well as eUNG natural evolution (from ConSurf). The average frequency of each mutation, along the protein sequence, was normalized by the unsorted library (see Equation 3). The common log of these values was plotted. **D.** Co-variance of enriched population's sequence space. Co-variance was calculated by PCA for the average frequency enrichment relative to the unsorted library (Equation 2 and 3), of each mutation that resulted in WT CE, GroEL and DnaKJ selection. Data was compared to ConSurf frequency matrix (Table S3). X axis resembles evolutionary distance (marked by two-sided arrow) where clustered dots suggest similar SP. Pentagram – ConSurf as evolutionary conservation. Analysis was done using Matlab R2017A).

Discussion

On the role of chaperones in protein evolution

In vitro evolution of eUNG directed for chaperone-binding is very different from its natural variability, as depicted by ConSurf (Figure 6D). Natural evolution has a functional purpose, with selection directed towards keeping fitness and proteostasis (which includes avoiding promiscuity). Evolving eUNG-RL against bacterial cell extract, while keeping structural integrity (as measured by Ugi binding) evolves eUNG yet towards a different sequence space, which favors cell extract binding (including the molecular chaperones therein) and is limited only by foldability not by its fitness. Thermodynamically, our results imply that *in vitro* evolution towards chaperone-binding is accompanied by, and thus possibly because of, a loss of free energy and destabilization. However, as binding to chaperones leads eventually towards sorting the protein for degradation, as is the case of Hsp70 and proteasomal degradation (36), during the process of evolution, ATP-fueled unfoldase chaperones, by virtue of their ability to bind new, excessively unstable mutant proteins, may either "skim out" the excessively destabilizing mutations, or, as shown here with the complete active DnaK+DnaJ+GrpE+ATP system, give some unstable chaperone-binding mutant proteins another chance to refold (Figure 5C, for DnaKJ selected population, addition of KJ+E+U shows Ugi binding). This leads proteins to retain only near-native mutations that can either avoid chaperones or when they are misfolding, to be unfolded and refolded by them, until new stabilizing mutations are selected to evolve new functions (see Fig 7 above the dashed line).

Clear differences were observed between selection against pure $GroEL_{14}$ oligomers (without ATP) and pure DnaK+DnaJ in the presence of ATP. These significant disparities motivated our reexamination of the different responses to partial protease digestion of these populations (Figure 5B). Since trypsin cleaves after lysine or arginine, and prolines leads to backbone breaks and local unfolding, their enrichment in the GroEL-selected sequence space suggests more disordered eUNG conformations, and thus higher protease sensitivity, compared to DnaKJ selected populations. Furthermore, the increase in positive charge and polarity in the GroEL- and DnaKJ-selected populations, resembles properties observed in intrinsically disordered proteins (IDPs) (37). Particularly, the ratio between lysine and arginine was shown to influence the conformational equilibria of IDPs. While an increase in arginine over lysine in the DnaKJ-selected population promotes compactness in IDPs, higher lysine to arginine ratio in the GroEL-selected population promotes weaker phase-separation and results in reduced compactness of the

misfolded species (38, 39). This implies that the equilibria between unfolded, misfolded and native eUNG conformations, greatly differed in the GroEL-, as compared to the DnaKJ-selected population, enhancing susceptibility to protease digestion for the first. Increase in arginine residues might also lead to solubilization of the misfolded state, as arginine has been shown to enhance the solubility of aggregates or unstructured proteins (40). In addition, positive charges surrounding a hydrophobic core have been shown to characterize DnaK-binding motifs (10, 41). As eUNG selection against DnaKJ resulted primarily in an increase in positive charges, it is plausible that this contributes to the observed increase in DnaK binding.

The protease degradation of selected populations (Figure 5B) might mimic *in vivo* evolution, because it may resemble what could have been the natural repartition of the work between ATP-fueled unfolding chaperones and proteases gated by ATP-fueled unfolding chaperones in evolution (31). In our assay, we differ from the conditions in the cell, as the proteins, chaperones and proteases are about tenfold less crowded than in cells. However, it is plausible that our *in vitro* chaperone selection is not so different than in the cell where the chaperones work hand in hand with proteases, and the non-promiscuous new mutants that are slightly less stable, may indeed be actively stabilized into native species, as previously been shown (6, 42). Those that are excessively less stable than the native WT, which cannot be stabilized to the native state by ATP-fueled unfolding/refolding chaperones may instead be hijacked by the AAA+ proteases that are in the same cellular compartment, and will be degraded and eliminated from evolution.

GroEL and DnaKJ chaperones recognize exposed motives at the surface of misfolded proteins that are mostly hydrophobic, which in the case of DnaK are also flanked by positive charges. Noticeably, these can be naturally enriched in mutants through a natural bias in evolution: the codon usage bias dictates that random mutagenesis should more frequently generate spontaneously sticky mutations, mainly hydrophobic and also positive charges that can interact with the negative charges that are often on the surface of native soluble proteins (43) (Figure 6A and B and Table S4). A protein can explore a larger mutational space and even include promiscuity-enhancing mutations due to the chaperones acting as enzymes that can iteratively unfold misfolded but not native protein conformations (30). Whereas the codon usage bias increases the likelihood of promiscuity-enhancing mutations, chaperones have evolved to precisely detect this bias and apply a selection pressure against it (10, 20). Our results show that the chaperones will bind only to those mutants, as they indeed select against destabilizing

mutations. This allows for the subsequent new stabilizing mutations, rendering the protein more native-like, i.e. that is not further recognized by chaperones (6, 7). The result is a feedback loop in which the component that permits in evolution the enhancement of the mutation load, also inhibits the possible enriched promiscuous products of this enhancement, by its destabilization. Inhibiting promiscuity suits well the chaperones, which can be 5-10% of a cell's protein mass (44) and are therefore very expensive, but have value as major organizers of the cellular protein-protein interaction network (45). If chaperones were lacking this feature, then protein stickiness should have been the null hypothesis (14, 46–49). Any time scale for a solution to emerge would have to be found in a chaperone-dependent manner. Thus, along the tree of life, an ever increasing chaperone-addiction was necessary for the formation of the most complex proteomes, such as metazoan, plant and fungi proteomes (4). It also explains why an organism complexity is not reflected by the number of distinct proteins it produces, but rather by the number of new folds (50), the extend of fold combinations and of domain repeats resulting in increasingly long beta-fold enriched proteins, as well as of chaperone-indifferent IDPs, of which some that form toxic amyloids are also chaperone-resistant, along evolution (4). Noticeably, our results suggest that the IDPs that did survive chaperone selection in evolution would have to lack typical chaperone-binding motives, such as clusters of hydrophobic residues, flanked by positive charges, as identified before and here (10, 33, 51, 52). Furthermore, it might explain why core-chaperones have not evolved proportionally with the increased complexity of proteomes. Rather co-chaperones, such as the J-domain proteins, of which DnaJ in a member, which specifically target the Hsp70 unfoldase machineries to bind misfolded protein substrates with high-affinity and use energy of ATP to unfold them. This, while avoiding binding to the natively unfolded and the compact native proteins, which are in general the low-affinity products of the chaperone-mediated unfolding reaction (4). In addition, our results suggest that the Hsp60 and Hsp70 chaperones, alongside being ATP-fueled unfoldases, may also assist the proper assembly of protein oligomers. Chaperon assisted assembly was first suggested by John Ellis and colleagues, who showed that the chloroplast HSP60 (CPN60) was key to the proper assembly of RubisCO L_8S_8 dodecamers (53). It seems that the term chaperones, initially chosen by John Ellis to describe features of this class of proteins, was not inaccurate. As such, chaperones are required for specific protein multimerization, thus it is plausible that they inhibit promiscuous interactions, and allow the formation only of rare specific interactions, in particular native oligomers and thereby limiting

their evolvability (54). In conclusion, we suggest that the evolutionary landscape of protein promiscuity is modulated by molecular chaperones, performing as the molecular "police" of cells (55) to shield promiscuous, counter-productive protein-protein interactions, carried out primarily by misfolding and aggregating proteins, which are cytotoxic and cause degenerative diseases in metazoans. This passively drives specificity, as only binding interfaces, which are not recognized by chaperones are positively selected for, in the course of protein evolution (Figure 7).

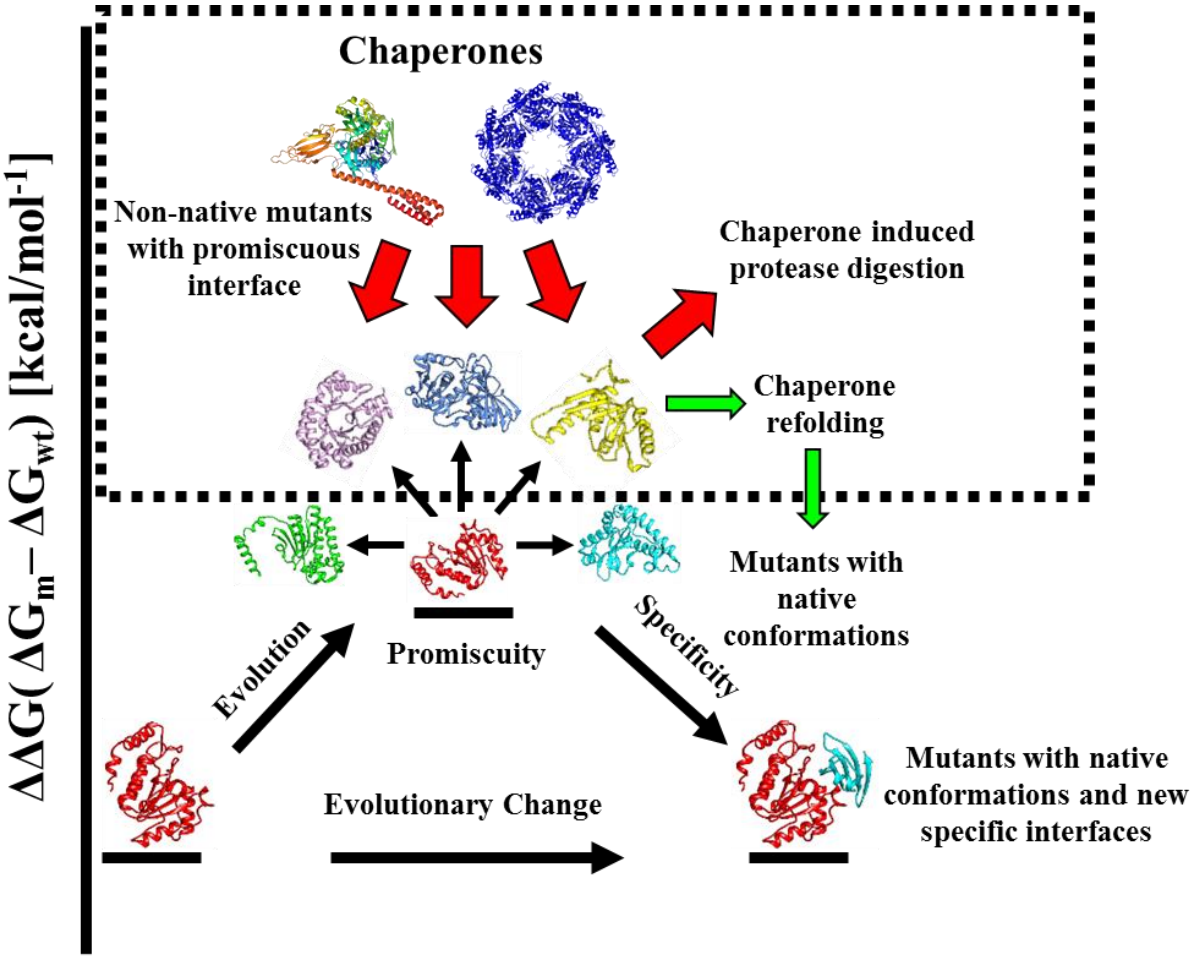

Figure 7. Chaperones modulate the evolutionary landscape of protein-protein interactions. Mutations in proteins can result in the formation of promiscuous interactions with non-target proteins. Chaperones promote evolution by buffering mutations, expanding the sequence space (SP) in which a protein can evolve. However, expanding the number of mutations a protein endures increases the likelihood it will mis-interact via misfolded species. While chaperones can maintain a folded state of a marginally destabilized conformations (green arrow), chaperones mostly selects against unstable promiscuous SPs (red arrows), promoting their degradation, until a near native solution is achieved, which is no longer recognized by chaperones.

Materials and Methods

Yeast library formation

A library of randomly mutated *E.coli* Uracil glycosylase (eUNG) was created on EBY100 yeast by following the procedure described by Benatuil (56). DNA mutagenesis was performed with mutazyme (GeneMorph II random mutagenesis kit catalog # 200550) resulting in an average of ~4 mutations per gene. Amplification of eUNG gene was done with Taq DNA-polymerase as described by Chao (57). Library size was estimated to be ~10^7 by plating serial dilutions on YPD selection plates lacking Tryptophan. Library was further confirmed for correct gene insertion and average mutation by sequencing 20 single clones.

Co-incubation experiments

After final round of selection against chaperones or WT cell extract, enriched populations were collected and incubated both with chaperones or WT cell extract and Ugi. Chaperones and WT cell extracts were labeled with APC (Biotium # 92108) while Ugi was labeled with FITC (Biotium # 92103) as described by the manufacturer. Co-incubated populations were analyzed by Bio-Rad FACS sorter and according to population distribution were sorted out into new vials with SDCCA selection media (57).

Uracil glycosylase inhibitor production

Urcail glycosylase inhibitor (Ugi) gene was ordered from TWIST and later cloned into the pet28 vector.(58) The vector was modified to have a longer linker on the N' terminus for better protease cleavage. Ugi was expressed in BL21(DE3) bacteria, grown at 37˚C until $O.D_{600}$ = 0.6-0.8 and then incubated with 0.1 mM IPTG at 16˚C overnight. Bacteria were pelleted and sonicated and suspended in SUMO protease buffer (50mM Tris-HCL pH 8, 200mM NaCl 10% glycerol 1mM DTT and 1:200 sumo protease both of which were added fresh). Ugi was purified from Ni beads binding 6xHis tag as described in (58) and further purified using ion exchange chromatography. After cleavage Ugi was dialyzed into labeling buffer (0.1M $NaHCO_3$ pH = ~8.3).

Cell extracts production

W3110 *E.coli* WT cell extract was prepared by first inoculating at 1:1000 ratio into 2YT followed by incubation at 37˚C until $O.D_{600}$ of 0.6-0.8. Cells were then harvested by centrifugation at 4˚C

and resuspended in lysis buffer (Tris HCl pH = 8, 200 mM NaCl, 10 mM Imidazole and 10% glycerol). The bacteria were sonicated and cytosolic fraction was collected by 17000 rpm 4˚C for 30 minutes. Supernatant was filtered using 0.4 μM filters and was dialyzed in labeling buffer (0.1M $NaHCO_3$ pH = ~8.3) in double distilled water and in 3.5 MWCO dialysis membrane.

Yeast library sorting

The library was sorted 5 times against 5 mg/ml cell extract labeled with CF640R succinimidyl ester (APC) through amine-coupling. APC labeled cell extract was filtered from excess dye before each sort. Protein expression levels on the yeast surface was detected by labeling the c-myc tag found at the C' terminal of expressed eUNG with FITC coloring. The selection continued until notable binding enrichment was detected by FACS. The selected libraries were then plated and 20 clones were sequenced and evaluated for binding cell extracts or chaperones.

Deep Sequencing

Deep sequencing was done on all the populations. Plasmids encoding the selected populations were extracted using Lyticase (Sigma # L2524). The eUNG gene (636bp) was amplified as 4 fragments of 250 bp each. Amplified plasmid flanks, which are not part of the eUNG gene were discarded in the analysis. Deep sequencing was done using Illumina MiSeq V2 (500 cycles) with paired-ends, 250bp read length and 12M reads. Deep sequencing reads were extracted using Enrich2 (59) and were further processed using Matlab R2017a. Sequence logos images were done using Seq2Logo (60).

Matlab analysis of deep sequencing data

Deep sequencing raw data was extract using Enrich2. The reads were organized in excel files containing reads per mutation along eUNG sequence. Reads were used to write scripts (Extended data) which calculates three parameters; Log-Ratio – SD (Eq. 1), amino acid frequency per position and from it the SSD (Eq. 2 and 3) and the initial amino acid variance, denoted by $V_{AA,}$ of the unsorted library (Eq. 4):

Equation1: $SD = Log10(\frac{\Sigma R_P / \Sigma R_{\Sigma P+ wt}}{\Sigma R_{P-U} / \Sigma R_{\Sigma P - U+ wt}})$

Equation2: $f_{M/P} = \frac{R_{M/P}}{\Sigma R_P}$

Equation3: $SSD = 100*(\bar{x}(\frac{f_{M/\Sigma P}}{f_{M/\Sigma P-U}}) - 100)$

Equation4: $\frac{R_M}{\Sigma R_M}$

ΣR_P – total reads per position, $\Sigma R_{\Sigma P + WT}$ – total reads over all positions including WT sequences, ΣR_{P-U} – unsorted library total reads per position, $\Sigma R_{\Sigma P-U + WT}$ – unsorted library total reads over all positions including WT sequences, $f_{M/P}$ – mutation frequency per position, $R_{M/P}$ – reads of a mutation per position, $\bar{x}$ – mean, $f_{M/\Sigma P}$ – frequency of a mutation over all positions. $f_{M/\Sigma P - U}$ - unsorted library frequency of a mutation over all positions, V_{AA} – initial amino acid variance (the frequency of a possible mutation for an amino acid) displayed as a fraction of: R_M – reads of a specific mutation X of amino acid Y and ΣR_M – sum of all reads of possible mutations for an amino acid Y.

Funding Sources. This research was supported by the Israel Science Foundation grant No. 1268/18 (GS) and Grant 31003A_175453 from the Swiss National Fund (PG)

Acknowledgement. We thank Satyam Tiwari and Tatiana Fomekong T. Mbefo from the University of Lausanne (UNIL), for providing us with purified GroEL, DnaK and DnaJ proteins and Pierre Gevevaux from the University of Toulouse III for giving us the delta DnaJK-DnaJ *E. coli* stain.

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