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Lean forward: Genetic analysis of temperature-sensitive mutants unfolds the secrets of oligomeric protein complex assembly

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Abstract

Multisubunit protein complexes are essential for cellular function. Genetic analysis of essential processes requires special tools, among which temperature-sensitive (Ts) mutants have historically been crucial. Many researchers assume that the effect of temperature on such mutants is to drive their proteolytic destruction. In fact, degradation-mediated elimination of mutant proteins likely explains only a fraction of the phenotypes associated with Ts mutants. Here I discuss insights gained from analysis of Ts mutants in oligomeric proteins, with particular focus on the study of septins, GTP-binding subunits of cytoskeletal filaments whose structures and functions are the subject of current investigation in my and many other labs. I argue that the kinds of unbiased forward genetic approaches that generate Ts mutants provide information that is largely inaccessible to modern reverse genetic methodologies, and will continue to drive our understanding of higher-order assembly by septins and other oligomeric proteins.

Keywords

forward genetics; molecular biology; molecular genetics; oligomeric proteins; random mutagenesis; septins

Introduction

The impetus for this essay is partly my lab's recent studies on temperature-sensitive (Ts)-mutant septins. Also, however, it reflects my awareness that certain important concepts about Ts mutants are unknown to many researchers, but well understood by others who nonetheless cannot readily cite a thorough discussion of those concepts extant in the literature. Based on my own searches, I direct the reader to a thoughtful examination by David Goldenberg of genetic studies of protein folding and stability [1], in which Ts mutants play a major role, and to a research paper by Gordon and King [2] that spells out many of the important concepts and cites a number of other key papers.

Here I wish to consider what we as a field have learned from Ts mutants of oligomeric proteins, using septin filament assembly as an example. In so doing, I hope to point out compelling topics of future septin research, and also to make the broader argument that unbiased forward genetic screens like those done decades ago should still have a place in the modern molecular biology toolkit.

First, a very short historical introduction to Ts mutants: Early genetic model systems – filamentous fungi [3], bacteria [3] and the phages that prey upon bacteria [4] – were the first hosts for Ts mutant screens, before this approach was applied to other models like fruit flies [5], budding yeast [6], fission yeast [7], nematodes [8] and green algae [9]. The primary advantages of Ts mutants were the abilities to study essential genes and to identify temporal aspects of gene function [10]. For the sake of space, I will not discuss cold-sensitive (Cs) mutants, which are equally as powerful as Ts mutants, and can be combined with Ts mutants in very informative ways [11, 12].

Next, a succinct summary of the relevant properties of septin proteins: Septins are GTP-binding proteins found throughout non-plant eukaryotes as heteromeric complexes that are capable of polymerizing into filaments with a variety of cellular functions. Septin structure and function have been extensively reviewed [13–15]. About septin function I will say only two things: (1) filament formation is required for cytokinesis in budding yeast [16], and (2) septins have been implicated in a list of cellular processes that is so long as to be essentially uninformative about what septins actually do at the molecular level.

Septin-encoding genes were first found in yeast Ts screens as the *cdc3*, *cdc10*, *cdc11*, *cdc12* complementation groups in which colony growth ceased at the restrictive temperature due to cytokinesis failure [17]. Cs mutants of *CDC11* were also found in multiple forward genetic screens [11, 18], and my lab recently identified a Cs mutation in *Cdc12* [19]. Homology-based approaches identified a fifth septin expressed in mitotically dividing cells, *Shs1/Sep7* [20, 21], and two expressed only during the gametogenesis-like process of sporulation, *Spr3* and *Spr28* [22–24]. Distinct septin proteins co-assemble in strict stoichiometric proportions into nonpolar, rod-shaped heteromeric complexes (Fig. 1A) that polymerize end-on-end into filaments. There are up to four subunit positions, represented twice, within each septin complex (or “protofilament”) (Fig. 1A) that may be occupied by alternative “isoforms” – distinct polypeptides encoded by the same gene, or by distinct but related genes – in different cell types, or even in the same cytoplasm. For example, in a subset of mitotic yeast hetero-octamers *Shs1* occupies the same “terminal” positions as does *Cdc11* (Fig. 1A) [25]. Septin complexes are like elaborate tubulin heterodimers: both are the stable building blocks of cytoskeletal polymers and are composed of GTP-binding polypeptides often represented by multiple alternative isoforms in a given cell. An important distinction is that the septin GTP-binding pockets face each other across the so-called “G” dimer interface (Fig. 1), whereas alpha tubulin's pocket is buried by a non-pocket interface of beta tubulin.

Finally, a summary of the kinds of conceptual insights that can be made from the study of Ts mutants, using septins as an example: In mitotic yeast cells, septin filaments comprise membrane-associated rings at or near the site of cytokinesis. Ts septin mutants have primarily been used to ask whether these rings are involved in a particular cellular process or required for the normal localization of non-septin factors. Typical experiments along these lines either attempt to identify synthetic growth defects in strains carrying Ts septin alleles and mutations in other genes phenotypes, or look in Ts septin-mutant cells shifted to the restrictive temperature for defects in a given cellular process or localization of a given factor. From a structure/function perspective, Ts phenotypes in septin mutants have mainly been considered to be general evidence of non-lethal septin dysfunction, without much

regard for why elevated temperatures should elicit a loss-of-function phenotype. By sequencing a large collection of Ts and Cs septin mutants isolated in unbiased screens, and mapping the mutations onto atomic-level structures of septin complexes, my lab recently found compelling evidence that Ts and Cs mutations target the G dimer interface in ways that trap the mutant proteins in non-native conformations incompatible with the assembly of functional septin filaments [19]. We also used forward genetics to isolate a new septin mutant that suppresses the Ts phenotype of one of the original *cdc* mutations in a distinct septin subunit, and with this discovery demonstrated that GTP binding promotes, but is not required for, the acquisition of oligomerization-competent conformations at high temperatures [19]. As will be discussed below, the general concept of conformational compatibility applies broadly to the study of Ts mutants in subunits of other oligomeric assemblies.

Diverse Ts mutants have common molecular properties

Early on, a distinction was made between so-called “TL” (thermolabile) and “TSS” (temperature-sensitive synthesis) mutants: TL mutants displayed phenotypes immediately upon temperature upshift, whereas TSS mutants only displayed phenotypes if new synthesis/folding/assembly occurred at high temperature; pre-formed proteins/assemblies were functional upon upshift [26]. Perhaps the easiest mechanism to envision for TL mutants is proteolysis immediately following temperature-induced partial denaturation of the mutant protein. (I note that partial denaturation causes many proteins to aggregate, which often delays or prevents proteolysis. However, whether proteolyzed immediately or sequestered in insoluble aggregates, the misfolded protein is excluded from the normal higher-order structures and rendered non-functional, so I will not dwell on this distinction.) On the other hand, if the folding or assembly state recognized by the proteolysis machinery is transiently sampled by the mutant protein only during its *de novo* folding or prior to higher-order assembly, assemblies constructed prior to temperature upshift will resist destruction, and the mutant will be TSS. This argument predicts that one factor differentiating TL from TSS mutants is the extent of the protein's denaturation at the restrictive temperature: severe unfolding will trigger proteolysis and result in a loss of function at any point along the protein's folding/assembly path. In support of this prediction, most mutations in phage lambda repressor display a direct relationship between thermodynamic stability and intracellular half-life [27].

However, this does not mean that all Ts mutant proteins are at some point subject to high-temperature-induced proteolysis. Unaware of the historical TL/TSS distinction, my lab examined yeast septin ring stability among a panel of Ts septin mutants and found that they fell into two categories corresponding to TL and TSS, which we called “stability” and “assembly” mutants, respectively [19]. No Ts point-mutant septin protein displays any evidence of degradation ([28] and our unpublished data). Yeast septins in particular appear to have exceedingly long half-lives [29], and although a subset are ubiquitinated, this does not appear to trigger proteasomal destruction [30]. Instead, we attributed all of the Ts effects to changes in the conformations of oligomerization interfaces in the mutant septins, leading in some cases to TL phenotypes [19].

Hydrophobic interactions between amino acids in a polypeptide are weakened by high temperatures [31]. Accordingly, thermal denaturation of wild-type proteins is primarily driven by temperature-induced dissolution of hydrophobic interactions [32]. It follows that reducing the number of hydrophobic interactions that maintain a protein's conformation will make it more susceptible to thermal denaturation. A method for designing Ts mutant proteins based solely on predictions from primary sequence [33, 34] involves identifying residues likely to be buried in the hydrophobic core of a protein of interest (Cys, Ile, Leu, Met, Phe, Trp or Val) and replacing them with neutral (Gly) or charged/polar residues (Asp, Glu, Thr). Often several such substitutions must be made in a single protein to produce a strong Ts mutant, but the method was able to generate Ts mutants of a protein (Gal4) for which standard unbiased genetic approaches failed [33].

The substitutions we recently identified among unbiased Ts yeast septin mutants do not conform to this paradigm: 9 of the 11 unique mutations replace Gly with Arg, Asp, or Glu, while the other two are Ser->Phe and Asp->Asn [19]. Structural studies of unbiased Ts mutants of phage T4 lysozyme found that most substitutions primarily affect residues that are buried from solvent, regardless of their hydrophobicity, and are immobile/rigid, as indicated by low crystallographic temperature values ("B-factors") [35]. As can be seen in Fig. 1B, the septin residues targeted in unbiased Ts mutants are indeed relatively immobile, at least in the context of a human SEPT2 homodimer with non-hydrolyzable GTP analogs bound in the pockets. Interestingly, most would be also buried from solvent in the context of a G dimer, but in the monomeric form these residues would be very much exposed. In later sections I also note the specific substitutions identified in other Ts mutants generated by unbiased methods, to allow the reader to compare them to the designer Ts mutation strategy.

A more high-throughput method of designer Ts engineering involves creating a "temperature degron", such as fusing to a protein's N terminus a TL derivative of dihydrofolate reductase (DHFR) that targets the fusion for proteolysis upon temperature upshift [36]. One would predict that such *td* alleles would be TL, assuming that the proteolysis machinery can access the tagged protein at any folding/assembly state, and indeed that was the case for a septin *td* mutant (*cdc12-td*) [19]. A downside of such strategies is that the rather large DHFR appendage may interfere with the function of the tagged protein even at the permissive temperature. Defects in septin function observed in *cdc12-td* cells at all temperatures (unpublished results) demonstrate that this caveat applies to this and likely other *td* fusions.

Oligomeric proteins face inherent challenges during de novo folding

Relatively subtle conformational changes can significantly affect oligomerization interfaces without inducing proteolysis. For monomeric proteins, changes of the same extent might have little effect on activity. Therefore, more drastic temperature-induced misfolding may be required to compromise monomeric protein function. Consistent with this logic, TSS mutants are enriched among the subunits of oligomeric complexes. Moreover, from the perspective of de novofolding, many oligomeric proteins face an inherent challenge: the burial of hydrophobic residues is not only a characteristic of the core of a folded protein, it is also a feature of oligomerization interfaces. In principle, individual subunits continuously

expose hydrophobic surfaces until oligomerization occurs. Likely to avoid this situation, hydrogen bonding and electrostatic interactions are more widely found at oligomerization interfaces than is hydrophobic packing [37]. Additionally, hydrophobic residues can be buried intramolecularly in transient intermediate folding states, before subsequent intermolecular burial. For such proteins, folding and oligomerization are not easily distinguishable. Tubulins are a telling example: native alpha and beta tubulin can only be found in the context of an alpha-beta heterodimer, suggesting that each subunit provides folding information that the other requires [38].

Native septin oligomerization interfaces are not particularly dependent on hydrophobic packing. Considering as hydrophobic Cys, Ile, Leu, Met, Phe, Trp and Val, only 7 hydrophobic residues are among the 24 residues buried by the human SEPT7 G homodimer interface [39], and only 13 of the 39 residues buried by the “NC” homodimer interface of human SEPT2 are hydrophobic [40]. Interestingly, however, the stability of a non-native SEPT2 G homodimer assembled in vitro requires burial of a Phe residue via interaction with the same Phe from the other protomer. In the native SEPT2–SEPT6 G heterodimer, on the other hand, SEPT2 Phe156 binds SEPT6 Lys185 [40], likely through cation-pi interaction. Small, polar residues like Ser and Thr replace Phe156 in other septins. Non-native septin oligomers may thus be more dependent than native ones on hydrophobic packing, which brings us to the phenomenon of non-native septin oligomerization in vivo.

Like many essential genes, point-mutant alleles of the septin-encoding genes *CDC10* and *CDC11* were first identified in the *cdc* Ts screen [17]. However, deletion alleles of *CDC10* or *CDC11* also make cells Ts [16, 41]. These two integral components of essential assemblies are thus somehow non-essential. The satisfying solution to this paradox is that when Cdc10 or Cdc11 is missing, the subunit with which each protein normally forms a G heterodimer (Cdc3 or Cdc12, respectively) now forms a non-native G homodimer, preserving the ability to form filaments [16]. Why are these structures Ts? The *cdc10* Ts phenotype can be suppressed by a mutation in the G interface of Cdc3 that replaces a Gly residue with a hydrophobic Val in a position predicted to be located very close to that occupied by SEPT2 Phe156 [16]. Burial of this Val likely stabilizes the Cdc3(G261V) homodimer at high temperatures. Hence, exposing additional hydrophobic residues on the surface of a monomer can actually improve folding/assembly by a mutant protein at high temperature.

From an evolutionary perspective, if one way to bury exposed hydrophobic residues is via oligomerization, then oligomerization may be an evolutionary strategy to buffer against insolubility caused by mutational drift. Evidence in support of this idea comes from fitness studies of mutants of *E. coli* DHFR [42]. A class of mutants (e.g. Ile->Ala) remained active at high temperatures by forming soluble oligomers (as opposed to insoluble aggregates) of the otherwise monomeric DHFR. DHFR in at least one thermophilic organism is a native homodimer [43], highlighting the utility of oligomerization as a means to prevent protein misfolding at high temperatures, and of exposure of hydrophobic residues as an evolutionary strategy to drive oligomerization.

Dominant Ts mutations define properties of oligomeric assemblies

Dominant Ts (DTS) mutants are rare, but those that have been characterized provide meaningful insights into both the mechanistic basis of TSS mutants, and specific details of higher-order assembly by the mutant proteins. Table 1 summarizes selected examples of DTS mutants that were isolated by unbiased forward genetic screens in a variety of model organisms. Considering the crucial role of shape in the functions of the affected oligomers, I hypothesize that DTS mutations act by subtly altering the conformations of the mutant subunits at the restrictive temperatures, tweaking higher-order structure in ways are incompatible with activity. Remarkably, in the case of proteasome mutants in both *Drosophila* and yeast, Ts phenotypes can be suppressed by mutations in distinct proteasome subunits (Table 1). Thus, when certain mutant subunits co-assemble, the overall assembly regains – if not the native form – at least the structural features that matter for activity. See below for a description of recessive Ts mutations in septin subunits that individually compromise septin oligomerization but together restore higher-order assembly.

We can view these instances of reciprocal suppression by mutations in distinct subunits of heteromeric assemblies as examples of conformational selection or induced fit [44], whereby the two mutant proteins help each other adopt the oligomerization-competent form. Some evidence for conformational selection in the case of septin mutants may be found in the observation that overexpression of the wild-type G dimer partners also suppresses the Ts phenotype of single septin mutants [45], perhaps by populating otherwise rare quasi-native conformations that are compatible for dimerization with the quasi-native conformation the mutant protein tends to adopt. The idea of quasi-native folding intermediates of distinct subunits influencing each other's folding may in fact be central to the study of Ts mutants in oligomeric proteins: rather than destabilizing the native state, some mutations likely bias the spectrum of conformations sampled during earlier steps along the folding path (Fig. 2A,B).

Allosteric regulation targets oligomerization interfaces

If Ts phenotypes reflect mutant proteins “stuck” in non-native conformations, then one would predict that Ts mutations often target points of normal allosteric regulation. Indeed, residues affected in Ts septin mutants cluster around the GTP binding pocket (Fig. 1B), and GTP hydrolysis by septins is thought to have two major allosteric effects on oligomerization interfaces (see Box 1). First, the septin “switch” I and II regions named after the homologous sequences in other small GTPases undergo large movements upon GTP hydrolysis. Residues in the switch II region make key contacts across the septin G interface [40, 53]. Hence, nucleotide phosphostatus influences G dimerization. Moreover, septins bound to the “wrong” nucleotide are unable to G dimerize, apparently due to direct interference by switch II residues. Wild-type human SEPT7 is unable to homodimerize when bound to a non-hydrolyzable GTP analog, but directed mutagenesis of a conserved switch II Asp residue to Ala allows homodimerization [54].

Remarkably, my lab identified a mutation in the same Asp residue in an unbiased forward genetic screen with yeast septins [19]. I isolated a spontaneous suppressor of a Ts allele of the septin *Cdc10*, *cdc10(D182N)*. Although the *cdc10(D182N)* allele was obtained in a random mutagenesis screen [17], others had engineered the identical substitution in different

GTP-binding proteins and found that their binding specificities switched to xanthosine di/triphosphate [55, 56], nucleotides that are not normally present in cells. (Strikingly, the same mutation (*SEPT12(D197N)*) also arose spontaneously in humans, and causes male infertility [57]). Thus, Cdc10(D182N) should be nucleotide-free in vivo, although I cannot exclude the possibility that cellular XMP or unphosphorylated xanthosine occupies this mutant's pocket. I hypothesized that the suppressor mutation altered Cdc3, the septin with which Cdc10 forms a G dimer, to make it compatible for dimerization with Cdc10(empty). I therefore sequenced the *CDC3* gene in the non-Ts, suppressed mutant strain. The Asp210 residue in Cdc3 I found substituted to Gly [19] corresponds precisely to the Asp residue in human SEPT7 whose substitution to Ala allows homodimerization in the GTP-bound state [54]. Thus, Asp210 in Cdc3(GTP) (see Box 2) must interfere with heterodimerization with Cdc10(empty). These experiments provide yet another example of the power of forward genetic approaches to pinpoint critical subunit-subunit interfaces and inform us about what is important for higher-order assembly: in the case of septins, compatibility of G interfaces, not the presence of bound nucleotide. From a practical perspective, it may be possible to introduce both the Asp mutations described above into a single septin protein and allow for the first time the purification of a soluble, nucleotide-free septin G homodimer. A high-resolution X-ray crystal structure of such a protein would be a powerful way to dissect the contributions of nucleotide binding from those of G dimerization in septin folding.

The second major effect of GTP hydrolysis by septins is allosteric coupling of the conformations of the G and NC interfaces. Specifically, GTP hydrolysis appears to induce a “strand slippage” event in which the septin beta3 strand shifts by two residues in register with respect to the beta2 and beta1 strands [58] (Box 1). The consequences of the strand slippage event for higher-order septin assembly are somewhat controversial. Attempts to crystallize human SEPT2 bound to a non-hydrolyzable GTP analog failed unless a critical component of the NC interface – the alpha0 helix – was deleted [53]. These results were interpreted to mean that in a G homodimer in which both protomers carry GTP, the NC interfaces are in conformations that are incompatible with homodimerization, preventing filaments (and therefore crystals) from forming. However, others propose that a polybasic motif implicated in membrane association – which overlaps with the alpha0 helix (Box 1) – is the functionally relevant target of hydrolysis-mediated conformational changes, effectively priming fully assembled complexes for membrane association [58]. This conclusion was based primarily on the ability to obtain crystals/filaments of a fragment of a schistosome septin, *SmSEPT10*, bound to both GTP and GDP [58]. However, the alpha0 helix was also truncated from the crystallized *SmSEPT10* [58], and native *SmSEPT10* lacks a polybasic motif [59]. Hence, these results are certainly open to alternative interpretations.

My lab recently implicated a distinct septin region in allosteric control of higher-order assembly when we identified the substitution Arg363Lys in a *Cs cdc12* mutant yeast strain [19]. We discovered that the region surrounding Cdc12 Arg363 – immediately preceding the hydrophobic heptad repeats – is highly conserved among Cdc12 homologs in other species and is also homologous to the CTE of the GTPase Ran. In Ran, this region allosterically controls the conformation of the switch regions in a way that mimics the effects of GTP hydrolysis [60]. We speculated that the Arg->Lys substitution in the CTE of Cdc12

indirectly influences the conformation of the Cdc12 G interface [19]. This allosteric effect in the mutant might itself respond to decreases in temperature, or might constitutively alter the Cdc12 G interface in a way that influences the NC interface of Cdc11 in a temperature-dependent manner.

How could such a conservative amino acid substitution have such a profound effect? What does Arg do that Lys cannot? I note that especially highly conserved is an RXR motif (where the first Arg corresponds to position 363 in Cdc12) that is known to be recognized by certain arginine methyltransferases [61]. Moreover, in a recent proteomic study Cdc12 was identified as containing methylarginine [62] (see Box 1). It may not be a coincidence that a septin-interacting protein, Hsl7, has an arginine methyltransferase activity in vitro whose in vivo function (if any) remains unknown [63].

Nucleotide-mediated conformational changes stabilize septin oligomers

Careful studies of higher-order assembly using mammalian septins carrying G- or NC-interface mutations clearly point to a requirement for prior G dimerization in order for most NC dimers to form [69]. Our results with Ts and Cs phenotypes in yeast septin mutants [19] are also most easily reconciled with a model in which GTP binding directs septin polypeptides towards functional folding states and away from non-functional conformations. By coupling NC dimerization to stable G dimerization, GTP hydrolysis then “locks” septin subunits together into stable, polymerization-competent hetero-oligomers. By predicting that the stability of NC interfaces will be especially affected by G interface mutations in the septins that form NC homodimers (yeast Cdc10 and Cdc11/Shs1; Fig. 1A), this model explains the TL phenotypes we observed in *cdc10* and *cdc11 shs1* cells [19].

Here I wish to note that ring instability in vivo does not necessarily reflect instability of septin hetero-octamers. In the case of mutants of Cdc10, I propose that at high temperatures filaments fall apart at the Cdc10–Cdc10 NC homodimer interface into “inside-out” hetero-octamers with a central Cdc11 homodimer [16]. On the other hand, I expect that normal, Cdc11-ended hetero-octamers persist in an unpolymerized state in *cdc11 shs1* or *cdc12-6* (see next section) cells in which a pre-assembled ring disappears following temperature upshift [19, 28, 70], predictions that should be tested by appropriate experiments.

Another relevant point on this subject regards an apparent example of “instability” of “wild-type” septin hetero-octamers during normal cycles of yeast septin ring assembly and disassembly: in vivo experiments exploiting covalent pulse labeling with an affinity tag provided evidence of exchange of Cdc12 monomers within pre-existing complexes [29], and suggested that the wild-type hetero-octamer is actually not very well locked together. These results made some sense with the exchange of Cdc12 and Shs1 for the “new” subunits Spr3 and Spr28 during the yeast sporulation process [29], but they do not agree with experiments in mammalian cells that demonstrate a clear lack of subunit exchange in septin complexes [71]. I believe the critical distinction between these experimental approaches is that – for unknown reasons – the SNAP-tagged Cdc12 fusion used in the yeast experiments made cells Ts [29]. Perhaps even at temperatures permissive for growth, hetero-octamers containing Cdc12-SNAP are “fragile” and subject to loss of constituent subunits. The apparent exchange reflects subsequent subunit replacement from a pool of “new” subunits generated

by dissolution of fragile hetero-octamers synthesized after the pulse of label. I propose that native mitotic yeast septin hetero-octamers are in fact inviolate, and that the constituent subunits remain “locked” together throughout the long lives of the polypeptides (or upon sporulation do they part).

All available evidence thus points to a structural role for nucleotide binding and hydrolysis in septin folding and higher-order assembly. Because most Ts yeast septin mutants are TSS [19, 28], however, all the nucleotide-promoted conformational changes that are important in higher-order assembly can still occur without nucleotide binding. This allosteric role of nucleotide in helping septins achieve an “active” conformation is reminiscent of the decades-old idea of “allosteric preconditioning”, by which ligand binding helps overcome free energy barriers during “maturation” of an oligomeric enzyme, but ligand is not a structural requirement of the mature, active form [72] (Fig. 2C).

Hydrophobic interactions in coiled coils are inherently Ts

Burial of hydrophobic residues may not be the driving force behind septin oligomerization interfaces, but hydrophobic residues are a specific feature of another important class of septin-septin interactions away from the nucleotide-binding pocket. Many septins have C-terminal extensions (CTEs) that include at their ends hydrophobic heptad repeats predicted to form coiled coils. These domains are dispensable for septin-septin interactions via the G and NC interfaces [40], although truncations that eliminate the repeats and additional portions of the CTE block polymerization by purified hetero-octamers in vitro [68] and have severe effects in vivo [73]. Lateral interactions that pair filaments together in vitro likely involve hetero-tetrameric coiled coils formed by the CTEs [68]. The pairing and polymerization defects caused by CTE truncations have been taken as evidence for some coupling of lateral associations with longitudinal interactions, but the details of this relationship remain unclear.

Given these properties, it remains a puzzle why the most potent yeast septin Ts mutant allele, *cdc12-6*, encodes a version of Cdc12 truncated by only 16 residues, eliminating only about half of the hydrophobic heptad repeats. Septin rings in *cdc12-6* cells are TL [28, 70]. Temperature affects coiled coil stability, to the extent that coiled-coil-based temperature sensors first found in nature have been recently adapted to synthesize artificial GFP-based thermosensors [74]. It is therefore easy to imagine why the loss of hydrophobic heptad repeats might make a mutant sensitive to high temperature, but we still know too little about what coiled coils do for septins to understand why destabilizing them would make a pre-formed septin ring fall apart.

Folding chaperones modify the characteristics of Ts mutants

Considering the requirement for certain proteins in survival at the temperatures typically used as restrictive temperatures for Ts mutants (33 – 42°C), some fraction of Ts mutants will represent null alleles of genes encoding such proteins. For example, heat shock factors help cells tolerate the thermal instability of essential wild-type proteins and/or the lethal consequences of accumulation of heat-denatured aggregates. Many such heat shock factors also play important roles at normal physiological temperatures by acting as molecular

chaperones. In fact, chaperones are often important (and frequently overlooked) players in the aforementioned folding challenge faced by oligomeric proteins. By recognizing and associating with exposed hydrophobic or highly charged residues, chaperones can protect nascent polypeptides from aggregation or inappropriate intermolecular interactions until the *de novo* folding process is near completion (Fig. 2D).

Looking back again to Ts mutants in phage, we find more insight gleaned from unbiased genetic investigations of chaperone involvement in oligomeric protein folding/assembly. The distal-half tail fiber of phage T4 is made from three proteins, one of which (gp37) trimerizes in a process that normally requires a dedicated chaperone (P38) [75]. However, a mutation in gp37 suppresses the requirement for P38, but only at low temperatures [76]. Sequencing revealed that the mutant gp37 had a duplication of a sequence in its C terminus [77]. Recognizing that the duplicated sequence included hydrophobic heptad repeats, Qu et al. introduced artificial coiled-coil-forming sequences into wild-type gp37 and found that this was sufficient to bypass the need for the P38 chaperone [78]. Moreover, multiple coiled-coil-forming insertions allowed phage function at extremely high temperatures [78]. By promoting early homomeric gp37 associations even at temperatures that destabilized wild-type gp37 assembly/folding intermediates, the extra coiled coils were sufficient to direct the proper assembly of the tail fiber without assistance from P38.

Thinking in reverse, it is apparent that the evolution of chaperones permits a degree of flexibility in genetic drift. Mutations that might otherwise send nascent proteins down dead-end, non-native folding paths – leading to Ts or other organismal phenotypes – can be “buffered” by factors that disfavor off-pathway folding states (Fig. 2D). Additional mutations can subsequently accumulate until a “new”, functional folding trajectory appears [79]. The Hsp90 class of chaperones in particular has been widely considered to represent such an evolutionary buffer [80].

Based on what I have presented above, it should be obvious that engagement by a chaperone protein is a common consequence of temperature-induced misfolding of a mutant protein. What is perhaps less obvious is that a mutant protein might misfold to an extent that triggers chaperone recognition – or delays chaperone release (Fig. 2E) – without necessarily adopting a non-functional conformation. In such a situation, the chaperone inhibits the function of the mutant protein and a loss of chaperone activity would restore mutant protein function to at least some extent. This idea was cleverly exploited in the search for a nuclear protein quality control pathway in budding yeast by identifying a gene of unknown function, *SAN1*, loss-of-function mutations in which were reported to suppress the Ts phenotypes caused by a variety of unrelated mutant nuclear proteins [81]. San1 is now known to recognize exposed hydrophobic residues in “misfolded” nuclear proteins and target them for destruction [82]. If we recall that oligomerization often involves burial of hydrophobic residues exposed on monomeric proteins, we can immediately grasp how chaperones can also engage wild-type proteins whose oligomerization partners are missing. I predict that future research will reveal many additional examples of chaperones acting to eliminate (via degradation or sequestration) proteins that would otherwise function normally. Of course, “normal” function may be restricted to particular genetic backgrounds or environmental

conditions, bringing us back to the concept of chaperones buffering against phenotypic variation.

Conclusion

It can be said that forward genetic approaches attempt to understand a biological system by finding ways to break it, using unbiased mutagenesis to generate screenable phenotypes. We now (think we) know so much about many biological processes that reverse genetic approaches – in which we predict how a specific manipulation should modify the system, and engineer a mutant to test our prediction – have become more popular. I believe the study of septin structure/function illustrates how sophisticated forward genetic approaches – dominant Ts mutants, suppressor screens, etc. – provide insights that we lack the knowledge to obtain by reverse genetics, such as how to re-assemble a broken system in a different way that still works. These approaches have lost none of the incisiveness and elegance that made them so powerful when they were developed many decades ago, and should supplement – not be replaced by – advanced methodologies of the modern era.

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Abbreviations

Cs	cold-sensitive
TL	thermolabile
Ts	temperature-sensitive
TSS	temperature-sensitive synthesis

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Box 1 Allosteric drives septin oligomerization

As illustrated using the structure of a SEPT2–SEPT6–SEPT7 human septin heterotrimer (PDB 2QAG [40]), there are three proposed modes of allosteric connections (double-headed arrows) affecting septin oligomerization interfaces. Mutations causing temperature-sensitive phenotypes likely target one or more of these connections. (1) The “switch” regions comprise loops whose positions change upon GTP hydrolysis. Red spheres indicate a conserved Asp residue in the switch II region making differential contacts across the septin G dimerization interface depending on whether GTP or GDP is bound in the G dimer partner's pocket. Mutating this Asp restores G dimer compatibility to septins stuck in the “wrong” nucleotide state [19, 54]. (2) A “strand slippage” event between two beta strands has been proposed to couple GTP hydrolysis with the conformation of the NC interface [58]. For the schistosome septin (“SmSEPT10”) whose crystal structures were used to reach this conclusion, strand slippage is facilitated by a Lys-Leu-Lys-Leu repeat (blue spheres and sequences) within the beta3 and a Phe-X-Phe (yellow spheres and sequences) sequence in the beta1 strand [58]. Notably, however, the Lys-Leu-Lys-Leu sequences (blue) are poorly conserved in human SEPT7 (“HsSEPT7”), which hydrolyzes GTP in the context of native hetero-oligomers [40]. Conversely, both repeat types are perfectly conserved in human SEPT6 (“HsSEPT6”), which does not hydrolyze GTP [40]. Similar discrepancies are observed for budding yeast septins (“ScCdcX”); Cdc10 and Cdc12 hydrolyze GTP, whereas Cdc3 does not. Strand slippage alters the orientation of the alpha0 helix, an important component of the NC interface that also overlaps with a “polybasic motif” (gray spheres) thought to mediate associations with negatively-charged phospholipids in the plasma membrane. (3) In the budding yeast septin Cdc12, a region between the G domain and the putative coiled-coil-forming domain shares homology to a domain in the small GTPase Ran that allosterically controls the configurations of the switch regions [19]. An Arg-to-Lys substitution in Cdc12 causes cold-sensitive growth defects, perhaps by preventing Arg methylation and “locking” the mutant Cdc12•GDP in a Cdc12•GTP-like conformation [19].

Box 2. Which septin subunits hydrolyze GTP?

Septins are members of the Ras family of small GTPases, and purified native septin complexes are able to hydrolyze added GTP to GDP at low rates that appear to be limited by very slow nucleotide exchange [64–66]. In the context of protofilaments, only a subset of the subunits are bound to GDP [40, 41, 64–66]. The remaining subunits apparently protect GTP from hydrolysis and should therefore be considered “anti-GTPases”. Early attempts to engineer mutations in yeast septins that disrupt GTP binding or hydrolysis were based on homology with Ras and generated misleading results both in vitro and in vivo [47, 67], which in retrospect can be attributed to the fact that septin-GTP interactions are not identical to Ras-GTP interactions.

Structural studies have implicated in septin GTP hydrolysis a Thr residue that coordinates a water molecule interacting with the gamma phosphate of GTP [53]. This residue is missing in certain septins, including those in whose pockets GTP appears in X-ray crystal structures [40]. The absence of the “catalytic Thr” is now widely considered diagnostic of septins that are GTP-bound within higher-order structures. Among the mitotic yeast septins, Cdc3, Cdc11, and Shs1 lack the catalytic Thr. However, I suspect that Cdc11 (and its close relatives Shs1 and the sporulation-specific septin Spr28) hydrolyze GTP via a distinct mechanism, and are in fact largely GDP-bound in vivo. First, the GDP:GTP ratio in septin hetero-octamers purified from wild-type yeast cells (>2:1) [41, 65, 66] is clearly incompatible with hydrolysis by only two of the four polypeptide species in the complex. Second, in the human septin complex whose high-resolution crystal structure has been solved [40], the polypeptide that remains GTP-bound (SEPT6) occupies the position corresponding to Cdc3 in the yeast complex, pointing to this yeast septin as the one that fails to hydrolyze nucleotide. Third, experiments with human septins and non-hydrolyzable GTP analogs suggest that GTP-bound septins do not efficiently form NC homodimers, yet Cdc11 NC homodimerization mediates filament assembly in vitro [68] and in *shs1* cells in vivo [16]. Fourth, in vitro experiments using septin complexes isolated from yeast demonstrate that Shs1 rapidly exchanges its bound nucleotide for exogenous radiolabeled GTP [66], consistent with efficient hydrolysis despite lack of the “catalytic Thr”. Cdc11 displayed the least exchange [28, 66], likely reflecting efficient hydrolysis but inefficient exchange due to especially tight binding to GDP. Thus, I postulate that alternative mechanisms of GTP hydrolysis by septins exist that do not require the “catalytic Thr”.

X-ray crystallography is the only method currently able to identify the phosphostatus of the nucleotide bound by each polypeptide in a heteromeric complex. It will be important to develop new techniques that can address this question more efficiently. Toward this goal, it may be possible to exploit the predicted ability of Asp->Asn mutations in the septin G4 motif (e.g. Cdc10(D182N)) to direct binding of XTP, and/or Gly->Glu mutations in the position equivalent to Cdc12 Gly247 to direct binding of CTP. If yeast nucleotide metabolism can be remodeled to allow XTP/XDP accumulation in vivo, Asp->Asn G4 mutant septins within a hetero-octamer should be the only sources of xanthosine polyphosphates, and Gly->Glu mutants should be the only sources of cytosine

polyphosphates, upon biochemical purification of the hetero-octamers and extraction of bound nucleotides.

Figure 1.

The residues mutated in unbiased Ts septin mutants are relatively immobile but are buried only in the context of a G dimer. **A:** A cartoon illustrating the organization of mitotic yeast septin subunits within a septin hetero-octamer (open shapes). The “G” and “NC” interfaces are noted below. Hetero-octamers polymerize into filaments by associating end-to-end; the “terminal” subunit of another hetero-octamer is represented by a filled shape.

B: The residues corresponding to those found in Ts mutant yeast septins are rendered as spheres in one protomer within a G homodimer of the human septin SEPT2 bound to the non-hydrolyzable GTP analog GppNHp (PDB 3FTQ). The other protomer is in gray. Rainbow colors indicate the average crystallographic temperature value, or B factor, of the atoms within each residue, on a spectrum of dark blue (least mobile) to red (most mobile), as indicated. GppNHp is shown as purple sticks. Adapted from [19].

Figure 2.

Models for effects of Ts mutations, ligand binding, and chaperone association on the free energy landscape of protein folding (or “maturation”). **A,B,D,E:** Idealized free energy landscapes for the folding of a nascent polypeptide with a single major low-energy folding intermediate, illustrating “entrapment” of a Ts-mutant protein at the restrictive temperature by (B) a non-native folding intermediate, or (E) prolonged association with a chaperone protein. Dashed lines indicate parts of the energy landscape rendered inaccessible by the condition in question. **C:** The historical concept of allosteric “preconditioning”, by which ligand binding facilitates protein folding. The dashed line indicates the effect of preconditioning. Adapted by permission from Macmillan Publishers Ltd: Nature [65], copyright 1971.

Table 1

Dominant temperature-sensitive mutants highlight structural attributes of oligomeric assemblies

Oligomeric assembly (organism)	Mutant allele(s) (protein affected)	Amino acid substitution(s), (change in properties)	Putative molecular explanation
Filamentous septin ring (budding yeast)	<i>cdc12-1</i> [46] (filament subunit)	G->E (small, neutral to bulky, charged) [19, 47]	Remodels GTP binding pocket to allow CTP binding and quasi-native folding, but incompatible with filament assembly at high and low temps [19]
Collagen IV fibers (Drosophila and nematode)	<i>DTS-L3</i> (fly alpha1 subunit [48]) <i>emb-9</i> (worm alpha1 subunit) [49]	<i>DTS-L3</i> : G->D, G->A, K->N, A->T <i>emb-9</i> : G->D or G->E (small, neutral to bulky, charged)	Affect G-X-Y repeats, strain packing within triple-helical structure of collagen fiber that is inherently Ts
Barrel-shaped proteasome (Drosophila and yeast)	<i>DTS5</i> , <i>DTS7</i> (fly core beta subunits) [50] <i>crl3-2</i> (recessive ; yeast regulatory cap subunit) [51]	<i>DTS</i> : T->I or G->R [50] <i>crl3-2</i> : G->V (various)	Unknown; <i>DTS5</i> and <i>DTS7</i> both suppressed by core alpha subunit C->Y mutation [52]; <i>crl3-2</i> suppressed by core alpha Y->C [51].