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Why Molecular Chaperones Buffer Mutational Damage: A Case Study With a Yeast Hsp40/70 System

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ABSTRACT

The malfunctioning of molecular chaperones may result in uncovering genetic variation. The molecular basis of this phenomenon remains largely unknown. Chaperones rescue proteins unfolded by environmental stresses and therefore they might also help to stabilize mutated proteins and thus mask damages. To test this hypothesis, we carried out a genomewide mutagenesis followed by a screen for mutations that were synthetically harmful when the RAC–Ssb1/2 cytosolic chaperones were inactive. Mutants with such a phenotype were found and mapped to single nucleotide substitutions. However, neither the genes identified nor the nature of genetic lesions implied that folding of the mutated proteins was being supported by the chaperones. In a second screen, we identified temperature-sensitive (ts) mutants, a phenotype indicative of structural instability of proteins. We tested these for an association with sensitivity to loss of chaperone activity but found no such correlation as might have been expected if the chaperones assisted the folding of mutant proteins. Thus, molecular chaperones can mask the negative effects of mutations but the mechanism of such buffering need not be direct. A plausible role of chaperones is to stabilize genetic networks, thus making them more tolerant to malfunctioning of their constituents.

THE remarkable constancy of phenotypes in face of genetic alteration was recognized decades ago (Shmalhausen 1949; Waddington 1957; Lewontin 1974). Recent technical developments have revealed that the degree of genetic variation hidden under common phenotypes is greater than expected. Targeted mutagenesis of entire genomes has demonstrated that often as many as half of the genes can be individually inactivated without causing visible alteration of a phenotype (Goffeau *et al.* 1996; Oliver 1996; Gerdes *et al.* 2003; Kobayashi *et al.* 2003). Studies with microorganisms demonstrated that strains carrying dozens of harmful mutations may still have relatively high fitness (Cooper and Lenski 2000; Funchain *et al.* 2000; Sliwa *et al.* 2004). This genetic robustness has attracted renewed interest and a number of general explanations for its occurrence have been proposed (Kirschner and Gerhart 1998; Hartman *et al.* 2001; Siegal and Bergman 2002; Hermisson and Wagner 2004; Wagner 2005). Among the most appealing hypotheses are those assuming that the ability to resist genetic perturbations results from the ability to cope with environmental challenges. The genetic robustness would be simply congruent with the environmental one or derived from it in a course of evolution (Wagner *et al.* 1997; Miklejohn and Hartl 2002). Molecular chaperones seem to be an especially plausible example of such adaptation. These molecules, also known as heat-shock proteins (hsp's), are able to disaggregate and refold proteins destabilized by a variety of environmental stresses (Estruch 2000; Gasch *et al.* 2000). The realization that they may also uphold the functionality of genetically damaged proteins came from studies of endosymbiotic bacteria. These organisms experience elevated rates of mutation accumulation due to the operation of genetic drift. They also have a high cellular level of GroEL chaperone. This was interpreted as an adaptation to a high incidence of mutational destabilization of proteins (Moran 1996). Subsequent experiments confirmed that bacteria carrying multiple mutations grow better when the chaperones GroEL or DnaK are overexpressed (Fares *et al.* 2002; Maisnier-Patin *et al.* 2005). But, although it was shown that the mutations were present in the bacterial genomes and that the levels of chaperones were enhanced, there is no evidence that the mutations caused a massive misfolding and that this led to upregulation of the chaperones' expression (Moran 1996; Maisnier-Patin *et al.* 2005). Moreover, it is known that overexpression of the chaperones facilitates folding of many native proteins, not only the mutated ones (Genevaux *et al.* 2004;

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Vorderwulbecke *et al.* 2004). In eukaryotes, a decreased activity of the Hsp90 chaperone results in unmasking of heritable phenotypic variation. This indicates that the chaperone's activity dampens expression of genetic variation (Rutherford and Lindquist 1998; Queitsch *et al.* 2002; Rutherford 2003). Again, interpretation of these findings is not straightforward because of the complex role of Hsp90 in the cellular metabolism. Hsp90 maintains activity of key signaling factors, including steroid hormone receptors and protein kinases (Zhao *et al.* 2005). Hsp90 is also needed for the development of a correct structure of chromatin and therefore it affects epigenetic inheritance (Sollars *et al.* 2003). Thus, the scarcity of Hsp90 may have a general epistatic effect and not a direct effect due to insufficient aid in the folding of mutated proteins. As a result, although the extent of phenotypic buffering by Hsp90 is impressive, its molecular underpinnings remain unknown (Sangster and Queitsch 2005).

This work is based on the assumption that virtually all chaperones should be able to participate in buffering genetic variation because they are all likely to support folding of mutationally damaged proteins (Sangster *et al.* 2004). Although several chaperones are essential to a cell's viability under nonstressful conditions, the position of the Hsp70 family of chaperones is special because they are found at the beginning and at the crossroads of all major pathways of protein folding (Young *et al.* 2004). They are also engaged in numerous specialized processes such as protein translocation, remodeling, and degradation (Lund 2001). Therefore alteration of their activity is likely to be highly pleiotropic, as is the case for GroEL and Hsp90. A special feature of *Saccharomyces cerevisiae* is that it has two Hsp70's dedicated to aiding the folding of newly synthesized proteins. These are Ssb1 and Ssb2, the chaperones responsible for the bulk of Hsp70 binding to ribosome-bound nascent chains (Pfund *et al.* 1998; Craig *et al.* 2003; Yam *et al.* 2005). The peptide-binding activity of Ssb is dependent on the ribosome-associated complex (RAC) of an Hsp40 protein, Zuo1, and of another Hsp70 protein, Ssz1 (Yan *et al.* 1998; Michimoto *et al.* 2000; Gautschi *et al.* 2001; Huang *et al.* 2005). Ssb, Zuo1, and Ssz1 form a functional triad. Deletion of a single constituent results in inactivation of the whole system and a decrease in the rate of growth (Gautschi *et al.* 2002; Hundley *et al.* 2002). Although the RAC complex appears to be specialized, components of the complex also participate in other activities. For example, Ssb is involved in post-translational transportation of some proteins (Siegers *et al.* 2003) while Zuo1 and Ssz1 may perform some regulatory functions outside the ribosomal context (Eisenman and Craig 2004). Nevertheless, in yeast the cotranslationally acting Ssb's appear functionally distinct from multipurpose Hsp70's, such as Ssa1–4 (Craig *et al.* 2003). This is in contrast to mammalian systems where a multipurpose Hsp70 is also recruited to the cotranslational folding by a ribosome-tethered Zuo1 homolog (Hundley *et al.* 2005). For these reasons, we selected the Ssb–RAC system as especially attractive for the study of the phenotypic buffering that results from the activity of chaperones during folding of nascent proteins.

We used a classic genetic approach. We chemically mutagenized a yeast strain and screened for mutants whose lethal (slow-growth) phenotype was apparent only in the absence of active Ssb–RAC. Tests of complementation with a genomic library and subsequent gene cloning allowed us to identify a sample of single-locus mutations that interacted genetically with the Ssb–RAC. However, there was no clear indication that the role of the chaperones was to stabilize the mutant proteins. Subsequently, we looked for thermosensitive mutants to identify proteins of reduced spatial stability (Varadarajan *et al.* 1996; Hampsey 1997). We then tested whether the thermally unstable proteins were also sensitive to the absence of Ssb–RAC but found no evidence that the chaperones can stabilize such proteins.

MATERIALS AND METHODS

Strains and media: A haploid clone acquired from the Yeast Deletion Project collection, BY4742 *zuo1*Δ *ura3 leu2 his3 lys2*, was used in all experiments. For the purpose of genetic analysis, the *kan* gene used to construct *zuo1*Δ was replaced with the *hph* gene that conferred resistance to hygromycin B (Goldstein and McCusker 1999). This strain was transformed with a pRS416 *URA3* plasmid (ATCC 87521) containing the *ZUO1* gene. This plasmid and those used in complementation tests contained a centromere and therefore were present as one or a few copies per yeast cell. Standard synthetic complete (SC) and omission media (*e.g.*, SC–uracil) were used when appropriate. Loss of the p*ZUO1 URA3* plasmid was selected for on complete synthetic medium with 0.1% 5-fluoroorotic acid (5-FOA) (Boeke *et al.* 1984). All experiments were carried out at 25°, except when stated otherwise.

Mutagenesis and screening: A sample of 1 ml of overnight culture of cells grown on SC–uracil was treated with 30 μl of ethyl methanesulfonate (EMS) and incubated for 30 min at 30°. Cells were washed, diluted eightfold in fresh medium, and allowed to grow for 24 hr. The resulting culture was plated for single colonies on solid medium and allowed to grow for 5–6 days. To screen for effects dependent on the absence of p*ZUO1 URA3*, the colonies were replica plated onto 5-FOA plates and allowed to grow for 2–4 days. In a screen for thermosensitivity, the colonies were replica plated onto SC–uracil plates and incubated at 36° for 1–2 days. Clones showing signs of slow growth were retested by streaking the master colonies to single cells on the two compared media (*e.g.*, SC–uracil and 5-FOA).

Complementation: To identify the gene mutant in a given clone, each mutant strain was transformed with a library of random fragments of the yeast genome inserted into pBS32 *LEU2* (ATCC 77163). Transformations were performed using a standard procedure (http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html). The transformed cultures were spread on SC–ura–leu plates and incubated for 2–3 days. The subsequent characterizations were different for the two classes of mutants. In the case of the mutants whose phenotype was thought to be dependent on the absence of Zuo1p, the transformants were replica plated onto 5-FOA

plates to force the loss of the p*ZUO1 URA3* plasmid. These were then screened for good growth, which indicated that the cloned gene complemented the mutant and relieved the requirement for Zuo1p. The library plasmids were isolated from these transformants and used for retransformation of the original mutants. Strong growth upon acquisition of the plasmid marked genuine complementation. The genomic fragments present in these plasmids were identified by sequencing their ends in reactions primed from the adjacent sequences of pBS32. Single genes from the complementing clones were amplified by PCR and subcloned into a pRS413 *HIS3* plasmid (ATCC 87518).

In the screen for thermosensitive mutants, the plates with transformants were replica plated on SC–ura–leu plates and incubated at 36°. The library plasmids were isolated from colonies that were able to grow at this temperature; the identity of chromosomal fragments was determined as described above.

Allele replacement: To compare the phenotypes of the mutant alleles to those of gene deletions, the open reading frame of the nonessential p*ZUO1*-dependent alleles was replaced with the *kan*MX4 cassette by a one-step replacement technique (Wach *et al.* 1994). Acquisition of resistance to geneticin and positive results of PCR amplifications from the expected constructs were used to confirm each replacement.

In other experiments, the thermosensitive alleles were replaced by wild-type ones. The screen for a clone with a successful replacement was done by incubation of a post-transformation culture at 36°. The sequence of DNA in a locus in question was determined to confirm that the replacement actually happened and that the observed phenotypic change was not caused by a compensatory mutation in another locus.

In all cases of DNA sequence analysis at least two independent PCR products were sequenced. The sequences obtained from wild-type genes matched those published in the Saccharomyces Genome Database (http://www.yeastgenome.org). The mutations reported here are the reproducible sequence deviations from the wild-type alleles.

RESULTS

Phenotypes dependent on *zuo1*Δ: The goal of this experiment was to find strains with impaired growth due to a synthetic effect of inactivation of the Ssb–RAC complex and acquisition of a random mutation. We used EMS to mutagenize a haploid strain of yeast in which the gene coding for the Zuo1 protein was deleted from the chromosome (*zuo1*Δ) but expressed from a plasmid, p*ZUO1 URA3*. We then compared growth of ~20,000 colonies on media selecting for either presence or absence of the plasmid. We found 93 mutant strains that grew well on the former but poorly on the latter medium.

We hypothesized that the growth defects observed on the *zuo1*Δ background were caused by point mutations and that the mutated loci could be complemented by their wild-type alleles. To test this assumption, we transformed every mutant, initially harboring p*ZUO1*, with a library of single-copy plasmids carrying random fragments of the yeast genome. We expected that complementing plasmids would allow sustained ability to grow after losing p*ZUO1*. Successful implementation of this approach required that three conditions were met for every mutant: the efficiency of transformation was high, the rate of spontaneous reversion to fast growth was low, and the difference in the growth rate between the background and the candidate colonies of complemented mutants was easy to detect. These requirements were fulfilled for 37 mutant strains. The library plasmids from these transformants were isolated and used to retransform the original mutant strains and to retest the ability to complement the growth defect. Plasmids passing this test were found for 32 mutant strains. In all cases, at least one library plasmid contained *ZUO1*; for 8 mutants, we also found unique complementing chromosomal regions. We carried out two further tests on the latter. First, we found which individual genes from the fragments provided complementation. We also sequenced the same genes from the chromosomal loci of the mutant strains. For two strains, we did not find a mutation in the genes that were responsible for complementation, *RRP1* and *TRK1*. These genes were identified as the only ones shared by all library fragments complementing a given mutant. For the remaining six mutants, we identified a single gene in which the results of both tests agreed; that is, the cloned wild-type allele complemented the growth defect and the chromosomal allele contained a mutation. All eight cases of complementation are described in Table 1. The mutations were transitions at GC sites, as expected for the EMS mutagenesis (Sega 1984). No substitutions other than those reported in Table 1 were found. Thus, the occurrence of three substitutions in a single clone of *sac1* was striking but was confirmed by repeated cloning and sequencing.

Figure 1 illustrates the phenotype of mutants in which either *ZUO1* or a complementing gene were sufficient to improve growth. Strains carrying mutations in nonessential genes were compared with strains derived directly from them by deletion of the mutated locus. In the cases of *ARC18*, *VPS2*, and possibly *SPE3* and *SAC1*, the negative growth effect of the substitution was stronger than that of the deletion compared with strains missing *ZUO1*. We also asked whether the strains differ in the maximum growth rate measured in liquid cultures. The results generally corresponded to those obtained in the dot test although not all mutant strains were suitable for assays in liquid cultures (see supplemental Figure 1S at http://genetics.org/supplemental/).

To summarize this experiment, we did find yeast mutants whose growth was greatly impaired in the absence of p*ZUO1*. However, most of these were complemented only by library clones containing *ZUO1*. This implies that these were not simple single-gene loss-of-function mutations that could be complemented by supplying a wild-type allele. A caveat to this is that strains harboring multiple loss-of-function mutations would also be complemented only by *ZUO1* as one clone could not complement several mutations. However, it would require that such mutations were present only in the 24

TABLE 1

Complementation of mutants with a *zuo1*Δ-dependent slow-growth phenotype

Gene	Gene product	Cellular localization	Length (aa)	Base mutation	Amino acid mutation
		Complementation of characterized mutations			
RPB2	RNA polymerase II core subunit	Nucleus	1224	1583 C → T	528 Pro → Leu
SCH9	cAMP-dependent protein kinase	Cytoplasm	824	1378 G → A	460 Glu → Lys
SPE3	Spermidine synthase	Cytoplasm, nucleus	293	475 C → T	159 Gln → STOP
ARC18	Arp2/3 complex subunit	Actin patches	537	494 G → A	494 Trp → STOP
VPS27	Vacuolar protein sorting	Endosome	622	509 G → A	170 Trp → STOP
SAC1	Phosphoinositide phosphatase	ER, Golgi	623	3 G → A	1 START → Ile
				79 G → A	27 Ala → Thr
				638 G → A	213 Gly → Asp
		Complementation of uncharacterized mutations			
RRP15	Constituent of pre-60S ribosomal particles	Nucleus	250		
TRK1	Potassium transporter	Plasma membrane	1235		

strains in which only *ZUO1* provided complementation, at least two per strain, while none of them occurred in the 8 strains with mapped loci. Having the proportion of the class with zero events, $p_0 = 8/32 = 0.25$, we can use Poisson distribution to calculate the frequency of the class with a single event ($p_1 = 0.347$, which gives the expected number of such mutants, 11.1, in contrast to the zero observed) and the frequency of the class with multiple mutations ($p_m = 0.403$; expected 12.9, observed 24). The ratios of expected/observed are clearly inconsistent with the hypothesis that our results were confounded by strains with multiple mutations by *ZUO1* ($\chi^2 = 20.632$, d.f. $= 1$, $P \ll 0.001$).

Thermosensitivity and dependence on *zuo1*Δ: Our second approach was to design a screen in which we identified conditionally structurally unstable proteins and then demonstrated their phenotypic dependence on the chaperones at the permissive conditions. To implement this idea, we tested whether thermal sensitivity of mutated proteins, a known marker of spatial instability, was associated with sensitivity to the absence of functional Ssb–RAC at the permissive temperature. We reasoned that a link between the ts- and *zuo1*Δ-dependent phenotypes would be the most straightforward method for interpreting essential genes. A mutationally caused instability of such proteins might be enhanced by either heat or absence of the chaperones. Either would result in an insufficient level of an essential protein and, consequently, in a growth defect.

We mutagenized the *zuo1*Δ strain harboring *pZUO1 URA3* as above. Survivors were screened for temperature-sensitive (ts) growth after replica plating and incubation at 36°. A total of 125 strains whose ts phenotype was reproducible were identified. These mutants were streaked to single cells on 5-FOA medium at 25° to force the loss of *pZUO1 URA3*. In 54 mutant strains, we could unequivocally demonstrate that there were no signs of poorer growth after losing the p*ZUO1* plasmid. The remaining strains were further analyzed to test the association between sensitivity to thermal stress and absence of chaperones. We transformed the mutants with the genomic library and selected for restoration of growth at 36°. Of the 47 mutant strains that transformed with high efficiency, complementing plasmids were found for 38 mutant strains. These strains, complemented for the ts effect, were then tested for dependence on Zuo1p at 25°. For 22 of them, we decided that the apparent association between ts and dependence on *zuo1*Δ could not be confirmed because, while the complementing plasmid clearly cured the ts phenotype, it did not alleviate the *zuo1*Δ dependence.

For the remaining 16 mutant strains, the association between ts and the sensitivity to absence of Zuo1p could not be excluded although it was not clearly evident. The observed small differences in growth could have been caused by differences in relative plasmid stabilities or in nutritional properties of the media used. To exclude such nuances and map the complementation effects to single genes, we first determined the stretch of DNA that was shared by clones complementing a given mutant strain. We found 18 essential genes, distributed among 14 chromosomal fragments. We analyzed the DNA sequence of all corresponding genes in the appropriate mutants. For 6 mutants, we found substitutions of a single base in a single essential gene. To confirm that these were the critical changes, we amplified the corresponding wild-type alleles, transformed the mutants, and selected for the ability to grow at 36°. We found revertants of temperature sensitivity in all six strains. Restoration of the original DNA sequence was confirmed in every case.

Figure 2 presents the six strains whose ts effect was reverted by restoring the original nucleotide at the point of mutation. However, reversion of the ts phenotype was not associated with loss of the sensitivity to the absence of *ZUO1*. Thus, association between the two phenotypes was not confirmed although these strains were regarded as the most likely to provide examples of such pleiotropy. Only in the case of *rrp1* did we find

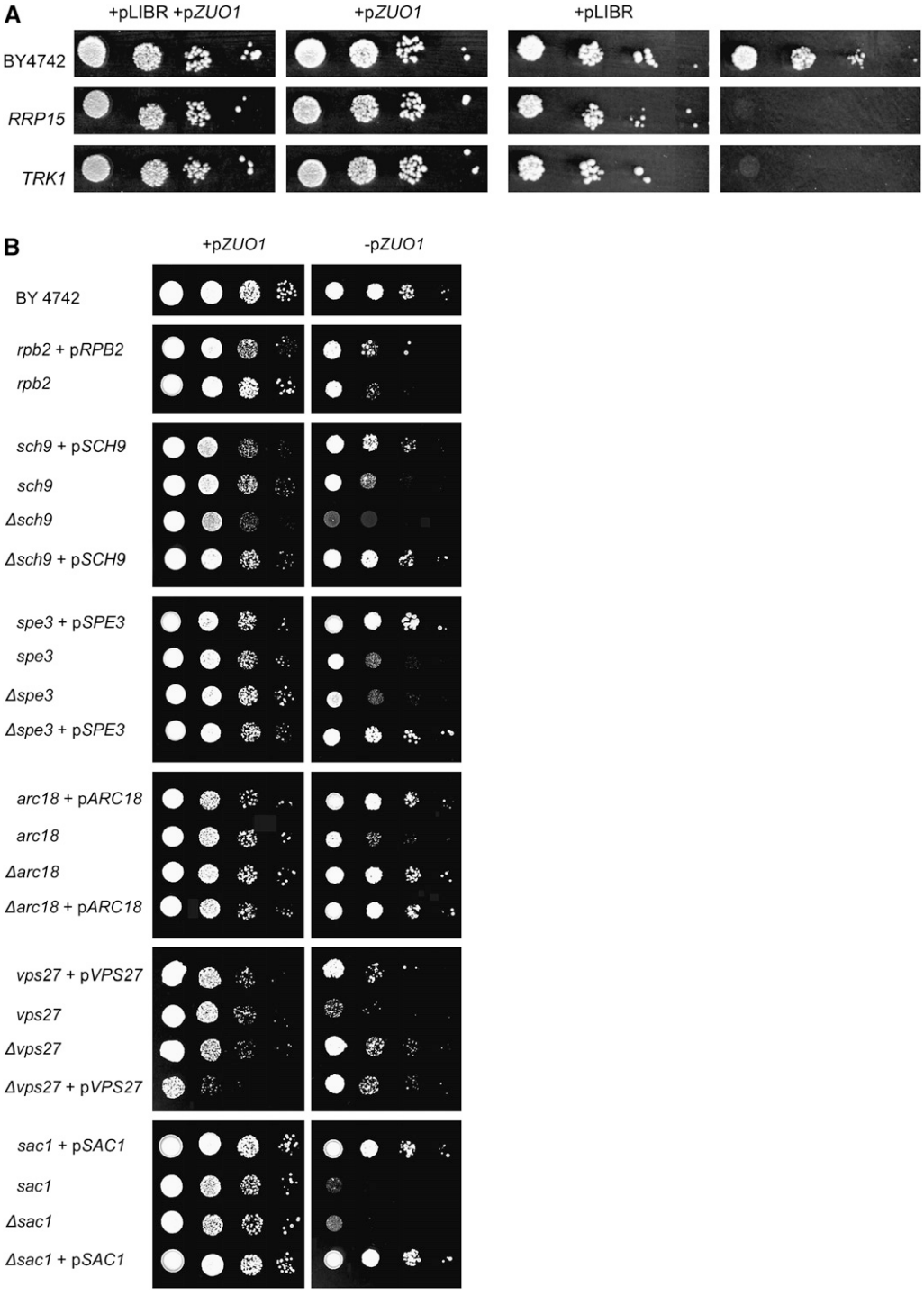

FIGURE 1.—Mutants dependent on the activity of cotranslational chaperones. The mutants were derived from BY4742 deleted for *ZUO1*; this strain serves as a control. (A) The two mutants in which mutated genes were not identified. (Left) The presence of p*ZUO1* was sufficient to support growth of mutants whether library plasmids, pLIBR, were present or not. (Right) In the absence of p*ZUO1*, the library plasmids containing *RRP15* or *TRK1* were needed to support growth. (The chromosomal copies of these genes were found nonmutated; see text). Comparing the left and right side shows that the growth defect appears only when *ZUO1* is absent. (B) The six mutants in which the mutated locus was identified. (Left) In the presence of p*ZUO1*, mutants grew well whether or not wild-type alleles of the mutated genes were supplied on plasmids. For the nonessential genes (all except for *RPB2*), the comparisons were extended on strains in which the mutated genes were deleted. (Right) In the absence of p*ZUO1*, the plasmids carrying cloned wild-type alleles were needed to support growth of mutants. The overall conclusion is again that growth defects appear or are enlarged in mutants when *ZUO1* is absent. This observation was not always true for the deletants; see text for comments.

the phenotypic difference between the mutant and wild type in the background. However, the molecular basis of this genetic interaction could be very special. It is probably not coincidental that *rrp1* and *nop4* (as well as *rrp15*, identified in the first screen) are mutations in genes required for maturation of the 60S ribosomal unit (SUN and WOOLFORD 1994; HORSEY *et al.* 2004; DE MARCHIS *et al.* 2005), the complex to which Zuo1p is anchored. It indicates that Zuo1p participates in biogenesis of the ribosome either passively as its future constituent or actively as a working enzyme. This hypothesis is appealing in that Zuo1p can bind both nucleic acids and ribosomal proteins (ZHANG *et al.* 1992; YAN *et al.* 1998). We therefore suggest that the interaction between *zuo1*Δ and *rrp1*, the only one producing the searched co-occurrence of ts and *zuo1*Δ depencence, is not rooted in the need of active Zuo1p for cotranslational folding of the mutant Rrp1p. Thus, co-occurrence of ts and dependence on *ZUO1* was confirmed for none of the original 125 mutations.

Most of our ts mutations remained unidentified. We therefore decided to include in our tests a set of

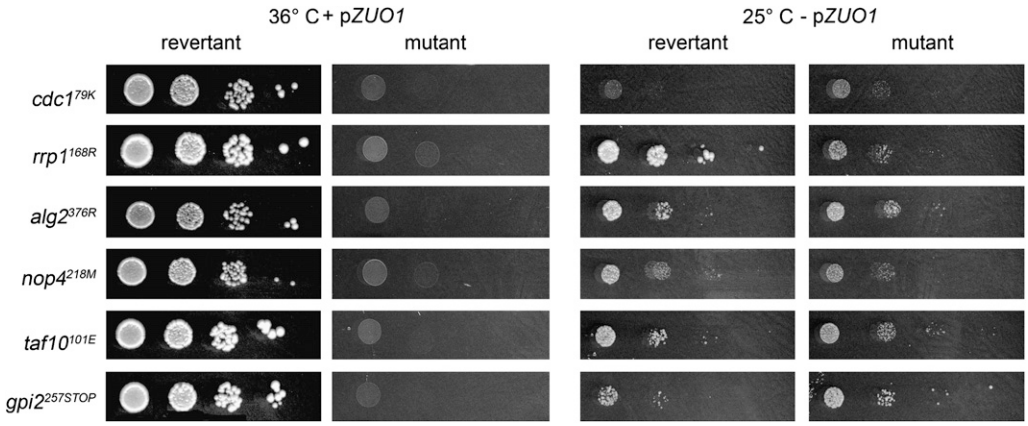

FIGURE 2.—Test for correlation between the ts- and *zuo1*Δ-dependent growth effects. (Left) At high temperature and in the presence of p*ZUO1*, reversion of the named mutation was necessary and sufficient to uphold growth of the ts mutants. (Right) At lower temperature and in the absence of p*ZUO1*, the reversion of the ts mutation did not result in a better growth. It shows that thermosensitivity does not correlate with sensitivity to the absence of the chaperones.

well-characterized ts mutations that were all in the same S288C-derived strain (DAVIERWALA *et al.* 2005). These were *arp2-14*, *cdc12-1*, *cdc42-1*, *exo84-102*, *nip7-1*, *nop1-3*, *prt1-1*, *rfc5-1*, *sec1-1*, *sec7-1*, *sec15-1*, and *sec18-1*. An additional three ts mutations, *stu1-5*, *stu2-10*, and *myo2-14*, were obtained from T. Huffaker (BREW and HUFFAKER 2002). We confirmed the thermosensitivity of these strains and then deleted *ZUO1* in each of them to test for a link between the ts phenotype and sensitivity to the loss of chaperone function. The results were negative for all mutants (see supplemental Figure 2S at http://genetics.org/supplemental/), confirming that proteins that are unstable at elevated temperatures are not dependent on the RAC–Ssb complex at normal temperatures.

DISCUSSION

Our study provides further evidence that malfunctioning of molecular chaperones is likely to uncover cryptic mutational variation. Thus, chaperones may be counted among the factors promoting genetic robustness of an organism (DE VISSER *et al.* 2003). However, we suggest that it is necessary to reconsider what is the basis of this phenomenon. In particular, we did not find support for the hypothesis that molecular chaperones directly interact with mutationally destabilized polypeptides and facilitate their refolding. Consider first the mutants that grew slowly due to a synthetic effect of an EMS-induced mutation and inactivation of the cytosolic chaperone system RAC–Ssb. Most of them remained unidentified because they were complemented only by library clones containing *ZUO1*. The insensitivity to complementation by specific library clones suggests that these were not simple loss-of-function mutations but ones involved in defects of other types (HERSKOWITZ 1987). There were strains (*RRP5* and *TRK1*) in which a *zuo1*Δ-dependent growth defect was compensated by acquisition of additional copies of the wild-type *RRP5* and *TRK1* but were not mutated in these genes. It appears as if doubling the dose of these proteins helped to overcome defects in other aspects of cellular metabolism. In four strains, the *zuo1*Δ-dependent mutations were nonsense mutations in nonessential genes (*arc18*, *vps27*, *spe3*, *sac1*). The chaperones probably did not restore functionality to these truncated proteins; rather, their activity helped in alleviating toxicity of malformed proteins. The conjecture that the proteins were toxic was supported by the observation that complete deletion of the mutant alleles resulted in alleviation of the growth defects. There were two missense mutations located in loci whose deletion would be lethal (*RPB2*) or very harmful (*SCH9*). However, mutations in a core subunit of RNA polymerase II or an important protein kinase are known to be extensively pleiotropic (ARCHAMBAULT and FRIESEN 1993; JORGENSEN *et al.* 2002; WILSON and ROACH 2002). Therefore, it is possible that the absence of chaperones did not decrease the stability of the altered proteins but rather widened the spectrum of their negative pleiotropic interactions. Having these negative or ambiguous results, we performed an entirely new screen that was designed to search specifically for mutations that lead to destabilization of protein structure (ts phenotype) and whose negative effects would be alleviated by the activity of molecular chaperones (presence of Zuo1p). Such an association was not found. In addition, no link was found between temperature sensitivity and dependence on Zuo1p in a collection of ts mutants.

Molecular chaperones are best known for their effectiveness in preventing the massive misfolding and aggregation of proteins in the face of strong environmental stress. Therefore, it appears especially plausible to invoke their direct assistance in reactivation of mutationally impaired proteins. However, there is an alternative explanation of the buffering role of the chaperones. They uphold functioning of a multitude of other proteins and therefore an insufficient level of their activity will result in improper behavior of their client proteins even if the latter are not mutated.

Impaired folding of the chaperones' clients, in turn, would change the cellular environment and sensitize it to malfunctioning of the proteins that happened to be mutated (Sangster *et al.* 2004). In this way, the chaperones would buffer mutations without any physical interaction with mutated proteins. At higher levels of cellular organization, the chaperones—with their ability to form weak, dynamic, and temporary interactions with a great number of proteins—appear to be important stabilizing factors of cellular networks (Csermely 2004; Soti *et al.* 2005). Indeed, a study devised to map the genomic network of gene interactions in yeast found that genes coding for molecular chaperones exhibited the highest number of interactions, confirming their role as global stabilizers (Tong *et al.* 2004).

Despite a decade of interest in the role of molecular chaperones in the masking of genetic variation (Moran 1996; Rutherford and Lindquist 1998), there is a dearth of experimental examples that the chaperones act by improving folding of mutationally defective proteins (Rutherford 2003; Sangster *et al.* 2004; Maisnier-Patin *et al.* 2005; Sangster and Queitsch 2005). Neither of our experiments provide support for this hypothesis. Thus, we suggest that chaperones buffer mutations by stabilization of interactions between proteins rather than by direct assistance of their folding. Further experimental tests are needed to evaluate both hypotheses. Advancement in basic research on the molecular chaperones will be also important. It is interesting to note that recent years have witnessed an important change in perception of their cellular role. Once seen mostly as helpers in disassociation and refolding of proteins, molecular chaperones are now known to be involved also in their degradation. The chaperones, together with the ubiquitin pathway, form cellular systems of protein quality control. An older view was that molecular chaperones save destabilized proteins from entering the degradation pathway (Wickner *et al.* 1999). Newer concepts postulate that the chaperones may actually cooperate with the ubiquitination enzymes in detecting destabilized chains (Esser *et al.* 2004; McClellan *et al.* 2005; Bukau *et al.* 2006). The quality control is normally tuned to maintain the balance between folding and degradation of *native* proteins because, even for the wild-type genes, as many as 30% of the ribosomal products do not attain the proper structure and therefore they need to be detected and removed (Schubert *et al.* 2000). From this perspective, the hypothesis that the function of chaperones is to save mutationally destabilized proteins has to be considered especially carefully. There is no indication that molecular chaperones can distinguish proteins destabilized by random events or environmental stress from those destabilized by mutations. If the cellular surveillance against misfolded polypeptides were lowered to save mutated proteins, massive misfolding and aggregation of native proteins could develop and result in an array of cytotoxic effects (Goldberg 2003).

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