Identifying separation-of-function mutations

Dissecting protein function: an efficient protocol for identifying separation-of-function mutations that encode structurally stable proteins

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ABSTRACT Mutations that confer the loss of a single biochemical property (separation-of-function mutations) can often uncover a previously unknown role for a protein in a particular biological process. However, most mutations are identified based on loss-of-function (LOF) phenotypes, which cannot differentiate between separation-of-function alleles vs. mutations that encode unstable/unfolded proteins. An alternative approach is to use over-expression dominant negative (ODN) phenotypes to identify mutant proteins that disrupt function in an otherwise wild type strain when over-expressed. This is based on the assumption that such mutant proteins retain an overall structure which is comparable to that of the wild type protein and are able to compete with the endogenous protein (Herskowitz 1987). To test this, the in vivo phenotypes of mutations in the Est3 telomerase subunit from Saccharomyces cerevisiae were compared with the in vitro secondary structure of these mutant proteins as analyzed by circular dichroism spectroscopy, which demonstrates that ODN is a more sensitive assessment of protein stability than the commonly used method of monitoring protein levels from extracts. Reverse mutagenesis of EST3 which targeted different categories of amino acids also showed that mutating highly conserved charged residues to the oppositely charged amino acid had an increased likelihood of generating a severely defective est3− mutation which nevertheless encoded a structurally stable protein. These results suggest that charge swap mutagenesis directed at a limited subset of highly conserved charged residues, combined with ODN screening to eliminate partially unfolded proteins, may provide a widely applicable and efficient strategy for generating separation-of-function mutations.
INTRODUCTION

A fundamental tool for *in vivo* analysis of biological pathways employs gene inactivation. Prior to the availability of genome-wide resources, mutations in individual genes were commonly recovered from forward mutagenesis screens. Although labor-intensive, such approaches had the benefit of recovering rare novel alleles that conferred the loss of a single biochemical property. More recently, with the completion of the genome sequence of *Saccharomyces cerevisiae*, high throughput methods have been used to generate a collection of mutant strains which inactivate gene products, either through deletion of non-essential genes or conditional depletion of essential genes (Winzeler et al. 1999; Ben-Aroya et al. 2008; Yan *et al.* 2008; Li *et al.* 2011). The availability of these genome-wide reagents has permitted the analysis of genetic networks on a large scale, with the resulting information used to place genes in pathways, identify points of intersection between different pathways and assign gene function to novel ORFs (Tong *et al.* 2001; Dixon *et al.* 2009). However, an inherent caveat of these studies stems from the nature of the genetic reagents. Many proteins have more than one biochemical activity (for example, more than one interaction with other proteins; Venkatesan *et al.* 2009), and thus complete inactivation of gene function is potentially pleiotropic (Costanzo *et al.* 2010); an additional source of pleiotropy is the aneuploidy that has been observed in a subset of yeast deletion strains (Hughes *et al.* 2000). Furthermore, conditional depletion of essential genes through the use of temperature-sensitive alleles or degrons requires a temperature shift (Hartwell *et al.* 1970; Dohmen *et al.* 1994), which can itself confer a phenotype even in the wild type situation (Paschini *et al.* 2012).

A potential solution is to employ missense mutations, referred to as separation-of-function (sof) alleles, which surgically eliminate a single biochemical function while leaving other activities (and presumably the structural integrity of the protein) intact. The advantages of this particular class of mutations in elucidating biological pathways, particularly in essential genes, can be substantial (Zhou
and Elledge 1992; Nugent et al. 1996; Umezu et al. 1998). However, recovery of sof− alleles has been a logistic hurdle even for single genes, as such mutations are most often recovered from genetic screens that employ loss of function phenotype(s), followed by careful analysis to ensure that only one function has been impaired (for examples, see Rudge et al. 2001; Laurençon et al. 2003; Bertuch and Lundblad 2003; Mu et al. 2008). As a consequence, the amount of experimental effort necessary to identify and characterize sof− mutations has been a substantial barrier to recovering such mutations on a large scale. The obstacles stem from two technical hurdles. This first challenge is identifying the rare mutations that alter a specific biochemical property of a protein among the much larger set of hypomorphic mutations that alter non-specific properties (such as protein stability/folding). A second hurdle is the presumption that a large number of mutations must be screened, since very few amino acids are likely to yield a separation-of-function phenotype when mutated. If candidate mutations are generated by reverse genetics, the problem is further confounded by the decision of which amino acid substitution(s) would be most likely to result in sof− defects.

Towards the goal of developing a widely applicable protocol for generating sof− alleles, we examined several approaches that might alleviate the two obstacles described above. Our experimental system was the EST3 gene, which encodes a small (181 amino acid) subunit of yeast telomerase with two distinct categories of sof− alleles and easily monitored in vivo LOF and ODN phenotypes when mutated (Hughes et al. 2000; Lee et al. 2008; Lee et al. 2010). Est3 can also be expressed as a soluble structurally stable protein in E. coli (Lee et al. 2010), thereby providing an opportunity to assess the secondary structure of mutant and wild type proteins by circular dichroism. In this study, examination of the properties of a large panel of mutant Est3 proteins revealed a striking correlation between in vivo ODN phenotypes and in vitro protein stability, resulting in the conclusion that ODN assays can provide a facile means of identifying mutant proteins that are structurally intact. We also used a systematic set
of mutations introduced into ~20% of the amino acids in the Est3 protein to assess the phenotypic consequences of mutating different categories of amino acids to alanine vs. a residue with a high potential to disrupt protein folding. Alanine mutagenesis has been the default option for reverse mutagenesis (Cunningham and Wells 1989; Baase et al. 1992; Wertmann et al. 1992), on the assumption that such a substitution should have a minimal effect on protein structure (Dao-pin et al. 1991; Lim et al. 1992). We show instead that introduction of a residue that is potentially disruptive to protein structure is in fact more useful in distinguishing between mutant proteins that are unfolded (and thus do not have a ODN phenotype) vs. proteins that have a defect in a specific activity (with a strong ODN phenotype).

Based on our analysis of several different categories of mutations, we propose that charge swap mutagenesis directed at highly conserved charged amino acids, combined with an assessment of ODN phenotypes to eliminate unstable proteins, is an effective strategy for identifying sof alleles in proteins.

MATERIALS AND METHODS

Yeast strains and plasmids

All genetic analyses were performed in two isogenic strains, YVL2967 (MATa ura3-52 lys2-801 trp1-Δ1 his3-Δ200 leu2-Δ1) or YVL3057 (MATa est3-Δ ura3-52 lys2-801 trp1-Δ1 his3-Δ200 leu2-Δ1 /p EST3 CEN URA3). Missense mutations in EST3 were introduced into either pVL1024 (2µ LEU2 ADH-EST3) or pVL2537 (CEN LEU2 EST3); a complete list of the yeast plasmids used in this study is shown in Table S1. Standard genetic methods were used to introduce plasmids into yeast, and telomere length was assayed as described previously (Lendvay et al. 1996; Paschini et al. 2010).

Expression and purification of wild type and mutant Est3 proteins
His\textsubscript{10}-SUMO-Est3 proteins cloned in the pET-His-Smt3 vector (Mossessova and Lima 2000) were grown in \textit{E. coli} BL21(DE3) to OD\textsubscript{600} ~1.0, cold-shocked on ice for ~1hr, and 1.0 mM IPTG added to induce protein expression. Cells were allowed to grow post-induction for 24 hrs at 15\textdegree, harvested and resuspended in lysis buffer (buffer A: 100 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{3}PO\textsubscript{4} pH 7.5, 100 mM Na\textsubscript{2}SO\textsubscript{4}, 10\% glycerol, 10 mM imidazole and 3 mM \(\beta\)-ME) with an EDTA-free protease inhibitor cocktail tablet (Roche) and PMSF (phenylmethylsulfonyl fluoride), as recommended by the manufacturer, followed by lysis by sonication and cell debris separation by centrifugation at 25,000xg for 20 minutes. The soluble cell lysate (supernatant) was decanted, and the pelleted cell debris was resuspended with lysis buffer to the same volume as the supernatant. Aliquots of the pellet and supernatant fractions were resolved on a 13\% SDS-PAGE gel, and solubility was assessed by Western blot analysis against the His tag to compare the His\textsubscript{10}-SUMO-Est3 bands from the two fractions (Figure S1).

Soluble Est3 mutant proteins were purified from the clarified cell lysate, which was subjected to Ni\textsuperscript{2+}-affinity chromatography by gravity-flow (GE Healthcare). The His\textsubscript{10}-SUMO-Est3 proteins were subsequently eluted with buffer A containing 300 mM imidazole and concentrated in a 10,000 MWCO concentrator (Millipore) to 2 ml, without buffer exchange, and incubated overnight at 4\textdegree with the Ulp1 SUMO isopeptidase to cleave the His\textsubscript{10}-SUMO tag (Mossessova and Lima 2000). Cleaved proteins were purified via size-exclusion chromatography (Superdex75, GE) on an AKTA FPLC system in buffer B (100 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{3}PO\textsubscript{4} pH 7.5, 100 mM Na\textsubscript{2}SO\textsubscript{4}, 5\% glycerol and 3 mM \(\beta\)-ME). Contaminating ~14 kDa His\textsubscript{10}-SUMO tag was separated from the ~20.6 kDa Est3 protein by a second round of Ni\textsuperscript{2+}-affinity chromatography in buffer B containing 20 mM imidazole. Est3 eluted at >95\% purity, as assessed on Coomassie-stained gels (Figure S1), and was concentrated and buffer-exchanged to 50 mM potassium phosphate buffer (pH 7.4) using a 9000 MWCO protein concentrator (Pierce). Concentrations were obtained using an extinction coefficient of 15930 M\textsuperscript{-1} cm\textsuperscript{-1}. 
Circular dichroism (CD) spectra and melting curves: acquisition and analysis

All CD experiments were carried out in a 0.05 cm cuvette using a Chirascan-plus spectropolarimeter (Applied Photophysics) equipped with a Quantum Mechanics Peltier temperature controller. CD spectra were obtained in 50 mM potassium phosphate buffer (pH 7.4). Buffer baselines were acquired using the flow-through buffer from the protein concentrator for each mutant. CD spectra of wild type and mutant Est3 proteins (~10 μM) were collected from 180 to 300 nm in 1 nm increments, over a temperature range of 5° to 75° with steps of 10° and equilibration times of 3 minutes per step. A subsequent experiment, with same wavelength range and equilibration time, collected data points between 30° to 50° with steps of 2° for better estimation of the melting transition. Data acquisition and initial processing like baseline subtraction and curve smoothing was performed using ProData Viewer (Applied Photophysics), with further processing in Microsoft Excel. The midpoint temperatures of the thermal denaturation curves at 208 and 222 nm were determined from fits to the Boltzmann sigmoid equation using Prism GraphPad software.

RESULTS

An in vivo dominant negative effect on telomere replication correlates with in vitro stability of mutant Est3 proteins

We previously proposed that the overall structure of mutant Est3 proteins should be largely intact for those proteins that are capable of disrupting telomere replication when over-expressed in a wild type strain (i.e. ODN), whereas the inability to confer an ODN phenotype would indicate an improperly folded protein (Lee et al. 2008). To test this assumption directly, we assessed the in vitro properties of
mutations in four amino acids of the Est3 protein, est3-W21A, est3-D86A, est3-R110A and est3-V168E. As shown previously, strains expressing these four alleles exhibit severe loss-of-function (LOF) phenotypes, comparable to that of an est3-Δ null strain (Lee et al. 2008). Two of these mutations (est3-R110A and est3-V168E) also represent distinct categories of sof¯ alleles: V168 is part of a group of residues that provide a binding site on Est3 for interaction with telomerase whereas R110A defines a separate activity that is not required for either association with telomerase or enzyme activity (Lee et al. 2008; Lee et al. 2010). Despite comparable LOF phenotypes, these four mutations show striking differences with regard to their ODN phenotypes. Over-expression of either Est3-R110A or Est3-V168E conferred a substantial telomere length decline in a wild type strain (i.e. in the presence of the wild type Est3 protein expressed from its native genomic locus), whereas over-expression of the Est3-W21A or Est3-D86A proteins had no impact (Lee et al. 2008 and Figure 1A; see also below).

To examine whether this differential ODN response was a reflection of protein stability, recombinant versions of the Est3-W21A, Est3-D86A, Est3-R110A and Est3-V168E proteins were examined relative to the wild type Est3 protein following expression in E. coli. Structure stability was initially assessed based on expression and solubility of His10-SUMO tagged Est3 mutant proteins, by evaluating the partitioning of E. coli-expressed protein into insoluble vs. soluble fractions after cell lysis by Western blot analysis (Figure S1). The wild type His10-SUMO-Est3 protein expressed well and partitioned roughly equally between the insoluble and soluble fractions, whereas expression of His10-SUMO-Est3-D86A was markedly reduced, exhibiting expression levels only 30% of the wild-type and yielding very little, if any, soluble protein. Although expression of His10-SUMO-Est3-W21A was less impaired, subsequent purification revealed that this mutant protein eluted in the void of the size-exclusion column (data not shown), indicating that it formed soluble aggregates. Thus, both of these
Identifying separation-of-function mutations

mutant proteins exhibited a marked loss of structural stability in vitro, providing a potential explanation for their failure to disrupt wild type yeast telomere replication in vivo when over-expressed.

In contrast, the mutant Est3-R110A and Est3-V168E proteins were readily expressed and yielded sufficient soluble protein to allow secondary structure analysis by circular dichroism (CD). Soluble wild-type and mutant His_{10}-SUMO-tagged proteins were purified by Ni^{++} affinity followed by size-exclusion chromatography to remove the cleaved SUMO tag (Figure S1), and secondary structure was evaluated by CD as a function of temperature. Far-UV spectra were collected from 5° to 75° with 10° increments, and a second set of spectra were obtained with 2° increments over a more limited temperature range to more carefully monitor the melting transition. The spectrum of wild-type Est3 suggests a mixed α/β structure, with double minima at 208 nm and 222 nm and a global maximum at 195 nm (Figure 1B). This spectrum remained constant with respect to temperature until 35°, after which increased temperature unfolded the protein, with the thermal transition completed by 55°. Comparison of the spectra of the Est3-R110A and Est3-V168E mutant proteins with wild type Est3 revealed a strikingly similar secondary structure content and thermal stability, as indicated by the spectra shown in Figures 1B and S2 and the melting curves at 208 nm and 222 nm in Figure S3. In fact, the Est3-V168E protein appeared to be slightly more stable than Est3 or Est3-R110A (Figure 1B). The ability of these two mutant proteins to assume an overall structure which was essentially indistinguishable from that of the wild type Est3 protein supports the correlation between structure stability and the ability of these two mutant proteins to exert a dominant negative impact on telomere length when over-expressed.

Mutagenesis of hydrophobic amino acids to alanine can often fail to identify functionally relevant residues
In the example above, changing valine 168 to a charged residue resulted in a mutant protein with a profound effect on Est3 function. However, mutating this same residue to alanine had no effect on telomere replication (Lee et al. 2008 and see below). This result runs counter to the usual convention, in which mutation to alanine is the default choice when assessing the potential contribution of an amino acid to protein function, in order to minimize any possible perturbation of protein structure (Cunningham and Wells 1989; Moreira et al. 2007). However, an alternative premise is that amino acid changes which are likely to be highly detrimental to protein folding would provide a more effective means of distinguishing between residues that are important for stability vs. those that perform a discrete biochemical function. This approach is based on the rationale that if a residue is located in the interior of the protein, mutating it to a charged residue is likely to disrupt the structural integrity of a protein (Dao-pin et al. 1991; Lim et al. 1992); this destabilized mutant protein should confer a loss-of-function phenotype but will be phenotypically silent in the ODN assay. In contrast, mutating a surface residue which is critical for a specific activity (such as a protein interaction) to a non-conservative amino acid substitution is less likely to affect protein structure; as a consequence, such mutations should exhibit defects in both the LOF and ODN assays.

To examine this idea in a systematic manner, we examined the behavior of mutations introduced into hydrophobic amino acids; although such apolar residues are frequently solvent inaccessible, hydrophobic patches on protein surfaces can contribute to protein-protein interfaces (Lijnzaad and Argos 1997). A total of 18 hydrophobic amino acids in Est3 that exhibited a high degree of conservation (Figure S4) were selected for mutagenesis to either alanine or glutamic acid. The resulting panel of mutations was examined for both LOF and ODN phenotypes following transformation into est3-Δ and EST3 strains, respectively (Figures 2, S5, S6 and S7). Figure 2A compares the consequences on telomere length in the LOF assay, which shows that this panel of conserved hydrophobic residues was
remarkably tolerant to alanine mutagenesis, with very modest declines in telomere length observed in a limited subset of the mutant strains. Not unexpectedly, introduction of a glutamic residue in place of these 18 hydrophobic residues had a far more substantial impact on Est3 function, with 12 mutant strains displaying a severe impairment in telomere length maintenance. The differential response to alanine vs. glutamic acid mutagenesis suggested that the more severe phenotypes were due to disruption of protein structure. However, when this collection of hydrophobic → glutamic acid mutations were examined in the ODN assay, only four mutant proteins were capable of conferring an effect on telomere replication in the presence of the wild type Est3 protein (Figures 2B, S6 and S7). Notably, the extent of the ODN defect paralleled the magnitude of the LOF defect for each of these four mutations. For example, est3-V75E and est3-V168E, which both behaved like null mutations when introduced into an est3-∆ strain in the LOF assay (Figure 2A and Lee et al. 2008), had a pronounced impact in the ODN assay (Figure 2B). Similarly, est3-L6E and est3-L18E exhibited comparable effects in both the LOF and ODN assays (Figures 2A, S6 and S7), although the modest phenotypes in both assays indicate that these two residues are less critical for Est3 function.

The fact that the ODN phenotype was comparable to that of the corresponding LOF phenotype in each case argued that the glutamic acid residues introduced at these four sites did not interfere with protein structure. Consistent with this, the CD spectra of the purified Est3-L6E mutant protein, as well as the mid-point of the melting curves at 208 nm and 222 nm, were indicative of an overall structure that was highly similar to that of the wild type Est3 protein (Figures S2, S3 and S8), similar to the results for the Est3-V168E protein (Figure 1). The remaining mutations which conferred extremely short telomeres in the LOF assay were phenotypically silent in the ODN assay (Figures S6 and S7), suggesting that their in vivo defects were due to impaired structural stability. This was confirmed for est3-I22E and est3-V157E, as the comparable mutant proteins expressed very poorly in E. coli and with greatly reduced
solubility (Figure S1), indicating that these hydrophobic residue side-chains are likely internalized and important for structural integrity of the protein. These observations provide additional support for the premise that the ODN assay can provide a rigorous means of distinguishing between mutations that impair structural stability of a protein vs. those that are potential separation-of-function alleles (Figure 2C). The results with this panel of 36 mutations (the in vivo dataset is summarized in Figure S9) also demonstrate that mutagenesis to residues other than alanine may be necessary in order to identify hydrophobic residues that are important for function. In particular, the potential contribution of valine 168 to Est3 function would have been overlooked in a mutagenesis strategy that relied only on alanine substitutions.

One unexpected observation from this analysis was that expression and solubility of the Est3-V157A protein in E. coli was reduced, and the Est3-I22A protein formed soluble aggregates (Figure S1 and data not shown). This was unanticipated, as the est3-I22A and est3-V157A yeast strains were indistinguishable from a wild type strain with regard to telomere function, at least as assessed under laboratory conditions (Figure 2A). Presumably, additional features of the in vivo milieu (such as association with other subunits of the telomerase complex) which are not recapitulated in the E. coli expression system contribute to function (and presumably stability) of these two mutant Est3 proteins in yeast. This indicates that an examination of the properties of wild type and mutant Est3 proteins expressed in E. coli provides a very stringent assessment of protein stability.

Comparing charge-swap and alanine mutagenesis of charged residues in Est3

Although the above analysis supports the ODN assay as a strategy for identifying functionally important residues, hydrophobic residues proved to be an inefficient target, as only two (V75 and V168) among 18 residues qualified as strong sof” alleles. Moreover, neither V75 nor V168 could be distinguished on the
basis of amino acid conservation (Figures S4 and S9), arguing that this criterion would not be helpful in restricting mutagenesis to a more limited subset of hydrophobic amino acids.

Because charged residues are often located on the surface of a protein, mutagenesis of this class of amino acids might provide a more enriched category of candidate sof⁻ alleles. To test this, we conducted a similar systematic analysis of 18 charged residues in Est3, which were mutated to either alanine or to a charged residue (as a charge swap) and analyzed for ODN and LOF phenotypes. Unlike the situation with the hydrophobic residues, amino acid conservation was a strong predictor of whether a charged residue would generate a potential sof⁻ mutation, as shown by the summary in Figure 3. In particular, mutations in four (K68, K71, R110 and D166) of the six invariant or highly conserved residues conferred a strong LOF phenotype which was also accompanied by a pronounced ODN phenotype. In contrast, only one residue (D164) of the 8 moderately conserved amino acids gave rise to a notable telomere replication defect when mutated (although mutations in two additional residues in this category conferred more modest phenotypes).

For the charged residue mutations that gave rise to a moderate to strong phenotype in the LOF assay, we assessed whether each mutation conferred an ODN phenotype of equivalent magnitude by monitoring telomere length under the appropriate genetic conditions. This comparison identified eight mutations (est3-K3E, est3-K68A, est3-K71A, est3-K71E, est3-R110A, est3-R110E, est3-D164A and est3-D166R) with ODN phenotypes that were strikingly similar to their corresponding LOF phenotypes (Figures 1, 4, S10 and data not shown). To further test our hypothesis that these mutations should encode structurally stable proteins, the protein stability of six of these mutant proteins were examined (since est3-K71A has a weaker phenotype than est3-K71E, Est3-K71A was not included; similarly, only Est3-R110A was tested as est3-R110A and est3-R110E have essentially identical LOF and ODN phenotypes). Consistent with this prediction, the spectra for five of these mutant Est3 proteins were
Identifying separation-of-function mutations

comparable to that of the wild type protein (Figures 1B, 4C and S2), with a thermal denaturation pattern that was either highly similar (Est3-K71E, Est3-R110A, Est3-D164A and Est3-D166R) or only slightly reduced at 36°C (Est3-K68A), relative to the wild type Est3 protein. The spectra for the sixth protein in this group (Est3-K3E) also closely resembled that of the wild type Est3 protein at temperatures up to 36°C (Figures S2 and S8), indicating that the Est3-K3E protein exhibited a secondary structure which was also highly similar to that of the wild type protein under conditions which were permissive for yeast growth. Curiously, however, the thermal denaturation pattern for Est3-K3E protein from 45°C to 75°C indicated that this mutant protein unfolded into an anomalous structure (Figure S8). The biological relevance of this deviation from the wild type denaturation pattern is unclear, since it was only observed at temperatures which were well above laboratory growth conditions for \textit{S. cerevisiae}. This demonstrates that these six mutations encoded proteins with secondary structure content that was highly similar to that of the wild type Est3 protein, as predicted by their comparable \textit{in vivo} LOF and ODN phenotypes (summarized in Figure 5).

Figure 4 also identified a second category of mutations in which the magnitude of the ODN phenotype was less than that predicted by the LOF phenotype. The first example was the \textit{est3}-D164R strain, which exhibited a more pronounced telomere length defect in the LOF assay than in the ODN assay (compare telomere length indicated by the medium size box in Figures 4A and 4B). This predicted that the \textit{in vivo} defect displayed by the \textit{est3}-D164R strain could be attributed at least in part to a partially unstable mutant protein, which was consistent with its thermal denaturation profile (Figures S2 and S3). The most notable contrast between LOF and ODN phenotypes in Figure 4 was exhibited by the \textit{est3}-E104R mutation. In the LOF assay, the \textit{est3}-E104R strain displayed a null phenotype, with telomeres reaching a critically short length after a limited period of growth (small box in Figure 4A). However, the ODN phenotype conferred by the \textit{est3}-E104R mutation was notably less severe, as
Identifying separation-of-function mutations
telomeres were only shortened to an intermediate length by over-expression of this mutant protein (small box, Figure 4B). This contrasts with the est3-R110A mutation (which like est3-E104R exhibited a null phenotype in the LOF assay; Lee et al. 2008), as over-expression of the Est3-R110A mutant protein resulted in the appearance of senescence and the recombination-dependent telomeric rearrangements which are a hallmark of critically short telomeres (Figure 4B and data not shown). The incomplete correlation between the LOF and ODN phenotypes therefore suggested that the Est3-E104R mutant protein was partially destabilized in vivo. To test this prediction, the in vitro properties of the bacterially expressed mutant protein were examined. Although expression and solubility of His10-SUMO tagged Est3-E104R protein were not affected (Figure S1), the Est3-E104R spectra indicated reduced stability, as evidenced by unfolding of the Est3-E104R protein at a temperature (35°) that did not affect the wild type protein (Figure 4C). Both the reduced in vitro structural stability and the attenuated ODN phenotype argues that the magnitude of the LOF phenotype displayed by the est3-E104R strain was due in part to a loss of structural integrity of the mutant protein, rather than solely due to loss of a single biochemical property. This example illustrates how a diminished ODN phenotype can be used to eliminate certain alleles as candidate sof mutations for future studies.

DISCUSSION
Reverse mutagenesis of a protein is usually driven by two technical assumptions: (i) target highly conserved residues and (ii) introduce alanine substitutions. In this study, we have tested these two assumptions with the Est3 protein by conducting a systematic in vivo analysis of how two different categories of amino acids – hydrophobic and charged residues – respond to mutation to alanine vs. a residue with a high potential to disrupt protein folding. The results, summarized in Figures 3 and S9, demonstrate that mutation to a charged residue has a higher probability of generating a severe LOF
phenotype; of the 18 amino acids that conferred short or very short telomeres when mutated, 15 had a more pronounced telomere replication defect in response to a charged residue substitution than mutation to alanine. We subsequently used two complementary assays to assess whether the resulting phenotypes were simply due to destabilization of protein structure, by examining in vivo over-expression dominant negative phenotypes in yeast as well as in vitro structural stability of proteins expressed in E. coli. This second aspect of the analysis indicates that an ODN assay can be used to identify structurally stable mutant Est3 proteins with defined biochemical defects (summarized in Figures 5 and 6). Below we consider several implications of this study and suggest a strategy based on the data presented here that may be useful for designing sof− mutations in other proteins.

Using ODN phenotypes to eliminate hypomorphic mutations due to unstable proteins

Classically, separation-of-function alleles have been defined based on in vivo characteristics, as mutations that confer a restricted subset of the full range of phenotypes displayed by the null mutation. This is often accompanied by the assumption that such mutations have eliminated a single biochemical property (such as a protein binding surface) without impairing overall protein stability. If so, such alleles can be powerful genetic reagents for subsequent studies designed to identify the interacting partner. However, defining sof− alleles based solely on the range of LOF phenotypes potentially fails to identify those mutations that encode (partially) destabilized proteins. One aspect of this study, therefore, was to examine the potential correlation between in vitro protein stability and in vivo phenotypes, as a means of asking whether an ODN phenotype could predict structural stability. Towards this goal, 16 mutant Est3 proteins, corresponding to 7 mutations in hydrophobic residues and 9 in charged residues, were examined for structural stability following expression in E. coli, as summarized in Figure 5. This collection of mutant proteins represented the full range of in vivo phenotypes for est3− mutations: null
LOF but no ODN (3 mutations); moderate LOF but no ODN (1 mutation); an attenuated ODN relative to LOF (2 mutations); and null, strong or moderate LOF with comparable ODN (2, 4 and 2 mutations, respectively). As Figure 5 illustrates, the correlation between mutations with comparable ODN and LOF phenotypes and wild type in vitro protein stability was striking. In contrast, for est3\textsuperscript{−} mutations that conferred an ODN phenotype that was less severe than the corresponding LOF phenotype, the corresponding proteins exhibited a degree of structural instability that reflected the discrepancy between the ODN and LOF phenotypes.

These observations also suggest that ODN potentially provides a far more sensitive means of assessing whether a protein is properly folded than monitoring steady state protein levels in yeast extracts. In the case of Est3, only those variants that behaved as completely unfolded proteins following expression in E. coli (Est3-W21A and Est3-D86A; Figure S1) displayed readily detectable reductions in protein levels from yeast extracts (3- and 10-fold, respectively; Lee et al. 2008). In contrast, measurements of steady-state levels of the Est3-E104R protein from yeast extracts did not uncover any evidence of protein instability, even though the CD spectra and melting curves for the bacterially expressed Est3-E104R protein demonstrate that this mutant protein does not retain a wild type secondary structure. This indicates that while monitoring protein levels in extracts can be a useful tool in excluding substantially destabilized mutant proteins, it fails to discriminate against partially unstable proteins which would be less-than-ideal reagents for subsequent analyses.

**Separation-of function mutations in EST3**

This study, as well as a prior one (Lee et al. 2008), has identified 11 amino acids in EST3 that display ODN and LOF phenotypes of comparable magnitude when mutated. These 11 residues can be divided into three categories, based on their response to either alanine substitution or mutation to a charged
residue (Figure 6). Class I residues exhibit the same degree of impairment in vivo when mutated, regardless of whether the amino acid change is an alanine substitution or a charge swap. In contrast, the majority category (Class II) contains both hydrophobic and charged amino acids which result in a strong phenotype when mutated to a charged residue (as indicated by red bars) but are unaffected (or only modestly impaired, in the case of V75) by an alanine substitution (as indicated by grey bars). The third category contains two residues that are severely impaired by mutation to alanine, whereas a charge swap results in an attenuated ODN phenotype indicative of a partially destabilized protein. This distribution suggests that the simple notion of substitution with alanine as a side chain “null” is overly simplistic. Instead, the effectiveness of an alanine substitution to reveal the role of the targeted amino acid is context-dependent based on the structural environment.

Mutations in these 11 amino acids define two discrete functions of EST3 and thus constitute two different sets of sof¯ alleles, as summarized in the last column of Figure 6. One set of residues (E114, N117, D166 and V168) promotes the association of the Est3 protein with the telomerase complex, as previously shown by immunoprecipitation, whereas a second group performs at least one other activity which is not required for either interaction with telomerase or enzyme activity (Lee et al. 2008; Lee et al. 2010). Excluded from this group of sof¯ mutations is E104, based on the in vivo and in vitro analysis indicating that est3-E104A and est3-E104R encode proteins that are partially unstable (Figures 4 and S10). The importance of determining whether a mutant protein is structurally intact becomes apparent when assessing the ability of mutant Est3 proteins to associate with the telomerase complex. Our prior data indicated that association of the Est3-E104A and Est3-E104R proteins with telomerase was reduced (Lee et al. 2008); however, the results presented in this study suggest that this may simply be an artifact of how an unstable Est3 protein behaves in a telomerase co-immunoprecipitation assay. The same rationale applies to charge swap mutations in K68 and D164 (residues in Class III, Figure 6), as the
decreased interaction of the Est3-K68E and Est3-D164R mutant proteins with telomerase may be subject to the same caveat.

**A rapid reverse genetics protocol for identification of separation-of-function mutations**

The conventional approach for the identification of sof alleles has relied on forward genetics, which has most frequently employed LOF phenotypes as a screening strategy. We propose instead a reverse mutagenesis strategy which incorporates the concept that any mutation that confers LOF and ODN phenotypes of comparable magnitude is a potential sof mutation. This definition has the advantage that it does not rely on characterization of the particular biochemical property in question as a precursor to recovery of this class of alleles. We further propose that limiting the mutagenesis to charge swap mutations in invariant or highly conserved charged residues for ODN phenotypes may provide a rapid protocol for identification of sof mutations. In the case of the 181 amino acid Est3 protein, charge swap mutations in three of the six invariant/highly conserved amino acids conferred strong in vivo phenotypes and encoded structurally stable proteins (est3-K71E, est3-R110E and est3-D166R). In unpublished data from our laboratory, this approach has also been effective in identifying sof mutations in genes encoding the Est1 and Est2 subunits of telomerase as well as a wider set of genes involved in DNA replication and recombination, suggesting that this protocol may be widely applicable. This method obviously will not be comprehensive in identifying every functional residue on the surface of a protein, if only conserved charge residues are targeted, and it is not applicable to proteins that confer lethality when over-expressed. However, even a limited subset of new sof mutations, especially in essential genes, could be highly instructive, as this class of genetic reagents has a long history of uncovering previously unanticipated functions of proteins.
Acknowledgements

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Literature cited


Identifying separation-of-function mutations


FIGURE 1.— Est3 mutant protein stability in vitro correlates with the ability of mutant proteins to confer a dominant negative phenotype in vivo. (A) LOF phenotypes were assessed by monitoring telomere length of est3-Δ strains with single copy plasmids expressing wild type EST3 or the indicated mutations from the native EST3 promoter, whereas ODN phenotypes measured telomere length of wild type strains containing high copy plasmids expressing the same set of wild type or mutant alleles from the constitutive ADH promoter. (B) Spectra of the wild type Est3 protein collected from 30° to 50° with a step size of 2° (upper right), and spectra of the wild type Est3, Est3-R110A and Est3-V168E proteins.
Identifying separation-of-function mutations collected from 5° to 75° with a step-size of 10°; the temperature color-code is shown in the inset of the wild type Est3 protein plot. Proteins were expressed and purified as shown in Figure S1.

**FIGURE 2.**—Analysis of mutations introduced into 18 highly conserved hydrophobic amino acids in Est3. (A) LOF assay monitoring telomere length of est3-Δ yeast strains bearing single copy plasmids expressing mutations in hydrophobic residues (mutation to alanine, upper panels; mutation to glutamic acid, lower panels) from the native EST3 promoter. The broad telomeric restriction fragment that corresponds to ~2/3 of the 32 yeast telomeres is shown; the intact Southern blots can be viewed in...
Figure S5. Telomeres of wild type EST3 strains (boxed in white) provide multiple reference points. The genotypes of mutations that conferred a strong phenotype in the ODN assay are also boxed. (B) An ODN assay showing telomere length of wild type EST3 strains containing high copy plasmids expressing mutations of the indicated genotype from the constitutive ADH plasmid. (C) Summary of the comparison between in vivo and in vitro properties of missense mutations in four hydrophobic residues. Spectra for Est3-V168E and Est3-L6E are shown in Figures 1 and S8, respectively, and expression and solubility levels for the His10-SUMO-Est3-I22E and His10-SUMO-V157E proteins in Figure S1. ODN phenotypes for Est3-L6E are shown in Figures S6 and S7.
FIGURE 3.— Conservation of charged residues predicts the likelihood of generating a sof− allele in EST3. Results from Figure 4, Lee et al. 2008 and data not shown (corresponding to residues that did not have a telomere defect when mutated) are summarized. For LOF phenotypes, “medium short” and “short” correspond to 100 – 130 or 140 – 160 bp reduction in telomere length, respectively, and “null” refers to mutants with telomeres that are >175 bp shorter than wild type with an accompanying senescence phenotype. For ODN phenotypes, “moderate”, “strong” and “severe” correspond to the “medium short”, “short” and “null” LOF phenotypic categories. The two different nomenclatures

<table>
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<tr>
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<th>Alanine mutation; loss of function</th>
<th>Alanine mutation; ODN</th>
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emphasize that the LOF and ODN phenotypes are not directly comparable (since the ODN assay measures telomere length in a strain expressing both wild type and mutant variants of EST3, the phenotypic lag until steady state telomere length is reached is different in the LOF and ODN assays). Degree of amino acid conservation was determined based on an alignment of 20 Est3 protein sequences encompassing the *Saccharomyces*, *Kluveromyces* and *Candida* clades (Figure S4), where “high”, “moderate” or “low” correspond to 75 – 95%, 50 – 75% or < 50% identity and/or highly similar amino acid structure (i.e. arginine vs. lysine or aspartic acid vs. glutamic acid); “invariant” corresponds to 100% amino acid identity in all 20 Est3 proteins.
FIGURE 4.— Comparison of LOF and ODN phenotypes displayed by a panel of mutations in charged residues in EST3. (A) and (B) LOF and ODN assays were performed as described in Figure 1, except that strains were propagated for an additional ~25 generations. As a result, the est3-E104R strain exhibited a more severe telomere length defect in the LOF assay than the comparable null mutants shown in Figure 1A; similarly, the ODN phenotype of the est3-R110A mutation was also more pronounced, as indicated by the amplification of sub-telomeric elements (white arrows) which is a characteristic feature of extensively propagated telomerase-defective strains (Lundblad and Blackburn)
A second assay to measure ODN phenotypes, which monitors synthetic lethality in a yku80-Δ background, is shown in Figure S10. (C) CD spectra of wild type and the indicated mutant Est3 proteins were collected from 5° to 75°, in parallel with the spectra shown in Figure 1.

<table>
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* exhibited an atypical thermal denaturation pattern at ≥ 45°

**FIGURE 5.** — The severity of the ODN phenotype for an est3− mutation, relative to the severity of the LOF phenotype, correlates with *in vitro* structural stability.

The LOF and ODN phenotypes corresponding to the 16 mutant Est3 proteins tested for stability following expression in *E. coli* are indicated as two sub-groups, based on protein stability; subjecting
these two sets of data to a correlation test resulted in Pearson correlation coefficients of 0.32 and 1.0, respectively.

**FIGURE 6.**—Three categories of sof mutations in the EST3 gene

Mutations in 11 Est3 amino acids confer in vivo ODN and LOF phenotypes of comparable magnitude, indicated by colored bars, with red corresponding to the more severe set of phenotypes for each residue (note that the absolute magnitude of each phenotype among mutations is not conveyed; for example, est3-E114A imparts a far more modest phenotype than est3-R110A). Telomerase association is based
on prior observations (Lee et al. 2008) or unpublished data (J.W.L. and V.L., data not shown). The term “unstable” indicates those mutations that exhibit an attenuated ODN phenotype relative to the respective LOF phenotype, indicating that the resulting mutant protein is partially destabilized \textit{in vivo}.

The \textit{est3}-N117 mutation, which was analyzed in our prior study (Lee et al. 2008), is included in order to provide a more complete summary of sof$^-$ mutations in \textit{EST3}.