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Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits

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Phenotypic plasticity and the exposure of hidden genetic variation both affect the survival and evolution of new traits[1–3], but their contributing molecular mechanisms are largely unknown. A single factor, the yeast prion [*PSI*$^+$], may exert a profound effect on both[4]. [*PSI*$^+$] is a conserved, protein-based genetic element that is formed by a change in the conformation and function of the translation termination factor Sup35p[5], and is transmitted from mother to progeny. Curing cells of [*PSI*$^+$] alters their survival in different growth conditions and produces a spectrum of phenotypes in different genetic backgrounds[4]. Here we show, by examining three plausible explanations for this phenotypic diversity, that all traits tested involved [*PSI*$^+$]-mediated read-through of nonsense codons. Notably, the phenotypes analysed were genetically complex, and genetic re-assortment frequently converted [*PSI*$^+$]-dependent phenotypes to stable traits that persisted in the absence of [*PSI*$^+$]. Thus, [*PSI*$^+$] provides a temporary survival advantage under diverse conditions, increasing the likelihood that new traits will become fixed by subsequent genetic change. As an epigenetic mechanism that globally affects the relationship between genotype and phenotype, [*PSI*$^+$] expands the conceptual framework for phenotypic plasticity, provides a one-step mechanism for the acquisition of complex traits and affords a route to the genetic assimilation of initially transient epigenetic traits.

There are three possible explanations for the diversity of phenotypes that are produced when different [*PSI*$^+$] strains are cured of the prion by growth on guanidine hydrochloride (GdHCl)[4]. Firstly, several yeast prions have now been identified[5] (and others probably exist)[6,7], each with different potential biological consequences. As growth on GdHCl cures all known naturally occurring prions in *Saccharomyces cerevisiae*, the diverse phenotypes we observed may have arisen from eliminating different combinations of prions present in different strains. Secondly, the reduction in translation–termination activity that is associated with [*PSI*$^+$] may cause ribosomes to read through naturally occurring stop codons[8–10]. This could alter message stability and/or promote the translation of sequences that are prone to accumulating genetic variation, such as pseudogenes and 3′ untranslated regions (see Supplementary Data and Supplementary Fig. 1). Thirdly, [*PSI*$^+$] prion formation is accompanied by protein aggregation. Therefore, the acquisition and removal of such aggregates could have diverse effects on protein homeostasis and produce distinct phenotypes in different strains[11].

We systematically tested these hypotheses by creating three sets of strain derivatives in several different genetic backgrounds that uncoupled the effects of other prions (set one), translational read-through (set two) and protein aggregation (set three) (Supplementary Table 1). For set one, prions were cured by two different general methods that should eliminate most, if not all, prions (growth on GdHCl and deletion of Hsp104; ref. 5) or by two highly selective methods (mutating the prion-determining domain of the *SUP35* gene (NM) to eliminate [*PSI*$^+$][12,13] and deleting the *RNQ1* gene to eliminate the other known prion present in some of the strains, [*RNQ*$^+$][14]). For set two, we recreated the effects of translational read-through in cells that did not contain the prion ([*psi*$^-$]) either by introducing partial loss-of-function mutations into *SUP35*[15,16] or by introducing mutations that alter the stability of messenger RNAs containing nonsense codons (Δ*upf1*, Δ*ski7*)[17,18]. For set three, prion-like aggregates were recreated in a strain immune to their effects on translation termination. The strains were selectively cured of [*PSI*$^+$] by chromosomal deletion of the prion-forming domain (*sup35*$^{\Delta NM}$) and NM–green fluorescent protein (GFP) was expressed from a strong promoter. We also created other strains that retained Sup35p prion aggregates but had efficient translation termination. This was accomplished by introducing a form of Sup35p that is immune to capture by [*PSI*$^+$] aggregates (extra-chromosomal expression of *sup35C* (without NM)[12] or antisuppressor (ASU) *sup35* variants)[19].

The majority of the phenotypes tested proved to be a simple and direct consequence of [*PSI*$^+$]-mediated nonsense suppression (Table 1; Fig. 1; Supplementary Fig. 2; and data not shown). For example, resistance to 3 mM paraquat was greater in the [*PSI*$^+$] derivative of strain 5V-H19 than in the [*psi*$^-$] derivative (Fig. 1, centre). The [*psi*$^-$] cells acquired resistance to paraquat in the absence of the prion when wild-type *SUP35* was replaced with a mutant that enhanced nonsense suppression (*sup35*^{C653R}). [*PSI*$^+$] cells lost paraquat resistance when [*PSI*$^+$] was selectively cured by deleting the prion-forming domain (ΔNM). Reducing nonsense

suppression with the expression of either *Sup35C* or the anti-suppressor (ASU) mutant *sup35*^{Q15R} in the original [*PSI*$^+$] derivative masked the phenotype. However, re-introducing NM aggregates in the [*psi*$^-$] derivative did not restore paraquat resistance (*sup35*$^{\Delta NM}$ with the NM–GFP construct, data not shown).

Similar observations were made in cases where [*PSI*$^+$] conferred a growth disadvantage. The 5V-H19 [*PSI*$^+$] derivative displayed a greater sensitivity to 100 mM hydroxyurea (HU) than the [*psi*$^-$] derivative (Fig. 1, right). Simply increasing nonsense suppression in the [*psi*$^-$] derivative (with *sup35*^{C653R}) recapitulated HU sensitivity. Either eliminating [*PSI*$^+$] selectively (ΔNM) or decreasing nonsense suppression in a [*PSI*$^+$] strain (with extra-chromosomal *sup35C* or *sup35*^{Q15R}) restored HU resistance to levels similar to those of the [*psi*$^-$] derivative.

Some phenotypes were dependent on read-through, but could not be fully attributed to this (Table 1). For example, the phenotypes were lost when cells were selectively cured of [*PSI*$^+$] or when translation termination efficiency was increased in [*PSI*$^+$], but they were not restored in [*psi*$^-$] cells simply by increasing translational read-through. In order to increase read-through in [*psi*$^-$] strains we used different *sup35* mutations[15,16] to approximate the different levels of read-through that characterized the original [*PSI*$^+$] derivative in each genetic background (verified by nonsense suppression assays[20]; Supplementary Table 2; and data not shown)[4]. Either more precise levels of read-through are required to reproduce some phenotypes, or additional factors (such as other prions or the presence of aggregates) contribute to these traits.

Notably, neither other prions nor aggregation alone could account for any of the phenotypes tested. The selective curing of [*PSI*$^+$] eliminated each phenotype as effectively as curing by general methods. Selectivity in the curing of [*PSI*$^+$] was verified by the retention of [*RNQ*$^+$] in strains known to carry it (Supplementary Table 1; data not shown). Moreover, re-establishing NM-containing aggregates by overexpressing NM–GFP in cells cured of [*PSI*$^+$] (*sup35*$^{\Delta NM}$) did not restore any of the phenotypes. Finally, we analysed diploids created by outcrosses between the [*PSI*$^+$] strains of various genetic backgrounds and found that the phenotypes were recessive in nature. If they had been a direct consequence of the presence of different prions or protein aggregates in different genetic backgrounds they would be predicted to be dominant (Supplementary Table 3; data not shown). Taken together, the data strongly suggest that most [*PSI*$^+$]-associated phenotypes are due to the interaction of particular read-through events with the genetic architecture of the original strain, rather than the presence of other prions or protein aggregation *per se*.

Our data suggest that all phenotypes are associated with translational read-through, but they may be acquired through a variety of molecular mechanisms. A probable source of the phenotypic diversity is the expression of information in previously silent regions of the genome, which are free to acquire genetic variation such as pseudogenes and 3′ sequences at the ends of normal mRNAs (Table 1; Supplementary Data and Supplementary Fig. 1). Translational read-through by [*PSI*$^+$] may also affect other pathways, such as the system that turns over mRNAs when ribosomes read through the last stop codon at the end of a message (the non-stop decay pathway[18] through *SKI7*), or the system that turns over mRNAs when ribosomes stop prematurely (the nonsense mediated decay pathway[17] through *UPF1*). Indeed, some traits (but not all) were recapitulated by the deletion of *UPF1* in [*psi*$^-$] cells, which stablizes transcripts containing premature stop codons (Table 1, Δ*upf1*).

To investigate the genetic complexity of [*PSI*$^+$]-dependent phenotypes, strains were outcrossed to genetic backgrounds that did not have the same [*PSI*$^+$]-dependent phenotype, and meiotic progeny were analysed. Among the many phenotypes assessed, we did not find a single case of simple 2:2 segregation of the trait (Supplementary Table 3; and data not shown). Moreover, the progeny invariably presented a broad range of phenotypes of variable strengths, including some with stronger phenotypes than the parental strains (Fig. 2; Supplementary Table 3). For example, to analyse the resistance to 3 mM paraquat of the [*PSI*$^+$] derivative of the strain 5V-H19, we outcrossed it to D1142-1A. D1142-1A does

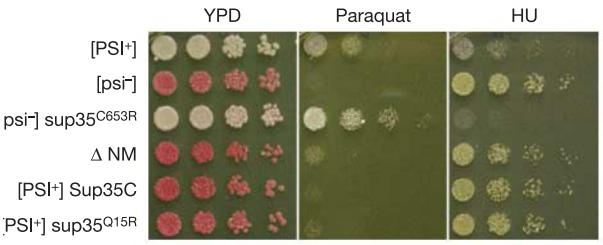

Figure 1 [*PSI*$^+$]-related phenotypes are dependent on read-through. Nonsense suppression enhances resistance to 3 mM paraquat and increases sensitivity to 100 mM hydroxyurea (HU) in the strain 5V-H19 (in [*PSI*$^+$] and [*psi*$^-$] sup35^{C653R}). Elimination of nonsense suppression (in [*psi*$^-$], ΔNM, [*PSI*$^+$] Sup35C and [*PSI*$^+$] sup35^{Q15R}) results in sensitivity to paraquat and increased resistance to HU.

Table 1 **[*PSI*$^+$]-dependent phenotypes across genetic backgrounds**

Phenotype	Strain background	Resistance	Sensitivity	Suppression method(s)	Anti-suppression method(s)
Phenotypes solely dependent on read-through					
Caffeine 10 mM	5V-H19	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35*^{C653R},Δ*upf1*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Caffeine 10 mM	74-D694	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35-R8*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Caffeine 5 mM	SL1010-1A	[*PSI*$^+$]	[*psi*$^-$]	Δ*upf1*[*psi*$^-$]	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Paraquat 3 mM	5V-H19	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35*^{C653R},Δ*upf1*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Paraquat 4 mM	Bsc 783/4c	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35*^{R320I}	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
LiCl 100 mM	5V-H19	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35*^{C653R}	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Benomyl 1 μg ml^{-1}	10B-H49	[*PSI*$^+$]	[*psi*$^-$]	Δ*upf1*[*psi*$^-$]	N/A
Ethanol 10%	5V-H19	[*PSI*$^+$]	[*psi*$^-$]	[*psi*$^-$]*sup35*^{C653R},Δ*upf1*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Ethanol 10%	D1142-1A	[*PSI*$^+$]	[*psi*$^-$]	Δ*upf1*[*psi*$^-$]	N/A
Hydroxyurea 100 mM	5V-H19	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]*sup35*^{C653R}	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
$ZnCl_2$ 7.5 mM	74-D694	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]*sup35-R8*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
LiCl 400 mM	74-D694	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]*sup35-R8*	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
Phenotypes dependent on read-through but may involve other factors*					
CsCl 150 mM	Bsc 783/4c	[*PSI*$^+$]	[*psi*$^-$]	N/O ([*psi*$^-$]*sup35*^{R320I},Δ*upf1*)	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
$CdCl_2$ 75 μM	5V-H19	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]*sup35*^{C653R},Δ*upf1*	P ([*PSI*$^+$]Sup35^{Q15R})
Paromomycin 200 μg ml^{-1}	5V-H19	[*psi*$^-$]	[*PSI*$^+$]	N/O ([*psi*$^-$]*sup35*^{C653R},Δ*upf1*)	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
$ZnCl_2$ 7.5 mM	SL1010-1A	[*psi*$^-$]	[*PSI*$^+$]	N/O (Δ*upf1*[*psi*$^-$])	[*PSI*$^+$]Sup35C,Sup35^{Q15R}
$ZnCl_2$ 5 mM	5V-H19	[*psi*$^-$]	[*PSI*$^+$]	N/O ([*psi*$^-$]*sup35*^{C653R},Δ*upf1*)	[*PSI*$^+$]Sup35C,Sup35^{Q15R}

*Phenotypes partially effected in suppression strains and/or not able to recapitulate with nonsense suppression alone denoted in parentheses.
N/A: not available in the given strain background.
N/O: intended suppression effect is not observed in respect to the given phenotype.
P: partial effect observed.

not grow on 3 mM paraquat in either the [*PSI*$^+$] or the [*psi*$^-$] state. Segregation of paraquat resistance was 2:2 for only two of the sixteen tetrads analysed (Supplementary Table 3) and progeny in many tetrads displayed intermediate phenotypes (Fig. 2; Supplementary Table 3). Similar analyses were completed for the [*PSI*$^+$]-dependent caffeine-resistant traits of both 74-D694 and 5V-H19 (Supplementary Table 3). Each strain was outcrossed to Bsc783/4c and W303-1A, neither of which is able to grow in the presence of 10 mM caffeine. None of the four outcrosses yielded a simple 2:2 pattern of segregation for caffeine resistance and many progeny of intermediate phenotypes were obtained. These and other data (Supplementary Table 3; data not shown) suggest that the [*PSI*$^+$]-dependent phenotypes can be complex traits acquired through a combination of genetic changes (some of which are hidden but are uncovered by translational read through events). Thus, [*PSI*$^+$] provides a route to the acquisition of complex traits in a single step.

Both the acquisition of these heritable new states (when cells become [*PSI*$^+$]) and the loss of those states (when cells revert to the [*psi*$^-$] state) are accomplished without changes in the genome, providing a transient mechanism for survival in fluctuating environments (Supplementary Fig. 3). However, the potential impact of the genetic variation accessed in the [*PSI*$^+$] state would be much greater if it were susceptible to fixation and maintenance in the absence of the prion. Indeed, further analysis of the outcross progeny showed that fixation of the complex [*PSI*$^+$]-mediated traits occurs readily. In fact, it can occur as a result of a single cross. The original phenotypes we examined were dependent upon [*PSI*$^+$], but in many outcross progeny the phenotypes were less dependent on (or even independent of) the prion. For example, the phenotype was maintained after cells were cured of [*PSI*$^+$] by either growth on GdHCl or by deletion of *HSP104*. Also, after 5V-H19 [*PSI*$^+$] (Fig. 3a) and 74-D694 [*PSI*$^+$] (Fig. 3b) were outcrossed to the caffeine sensitive (caffeine^S) strain W303-1A, many progeny maintained caffeine resistance after curing. In each of the phenotypes analysed to this extent (Supplementary Table 3), approximately 50% of the progeny exhibiting the trait maintained it in a [*PSI*$^+$]-dependent manner, whereas the other 50% maintained it in the absence of [*PSI*$^+$]. That is, the trait was fixed. Subsequent analysis demonstrated that there were multiple mechanisms for fixation of the same trait. Moreover, fixation was not due to the simple appearance of global nonsense suppressors (Supplementary Data). Rather, the phenotypes were fixed by virtue of the re-assortment of other genetic polymorphisms, by the appearance of new mutations or by a combination of the two.

Conceptually, [*PSI*$^+$] presents a new framework for phenotypic plasticity. Because [*PSI*$^+$] is a metastable element that is both gained and lost at a low spontaneous rate (10^{-5} to 10^{-7}), large populations of yeast are expected to contain both [*PSI*$^+$] and [*psi*$^-$] derivatives[21]. Individuals at a local peak of an adaptive landscape can suddenly cross to a new adaptive peak and survive when the environment changes. This change in state may be influenced by environmental conditions[22], but also occurs in a stochastic fashion through a self-perpetuating change in the folding of a single protein. Reduced translational efficiency in general, as well as many of the individual genetic variants it uncovers is likely to be detrimental (see Supplementary Data), but the capacity to acquire the [*PSI*$^+$] state has been conserved in yeast for at least a hundred million years[23–26]. The rarity of the change in state[21] ensures that the detrimental impact of the lower translational fidelity is negligible with respect to the survival of the population as a whole[27]. However, the ability to switch to the [*PSI*$^+$] state enables cells to benefit from a hidden potential to adapt and survive in particular circumstances. This increases the population size of the organisms with that genome and in turn, increases the chances for assimilation of the trait. Assimilation could occur by genetic re-assortment through mating (as shown here), by the mutation of stop to sense codons or by the acquisition of mutations that alter the stability of mRNAs (Supplementary Data and Supplementary Fig. 3).

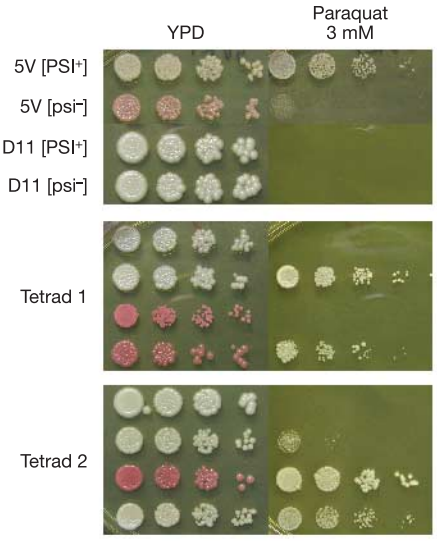

Figure 2 Variable strength phenotypes are observed with progeny from outcrosses. Shown here is the variability of resistance in two tetrads from the cross of 5V-H19 [*PSI*$^+$] to D1142-1A [*PSI*$^+$] on YPD with 3 mM paraquat. Note that suppression of *ade2-1* by [*PSI*$^+$], in combination with the suppressor transfer RNA *SUQ5*, results in an alteration of pigment of colonies grown on standard media, such that [*PSI*$^+$] colonies are white with the combination of these markers. Therefore, the absence of co-segregation of *ade2-1* with *SUQ5* results in some red [*PSI*$^+$] colonies in outcross progeny.

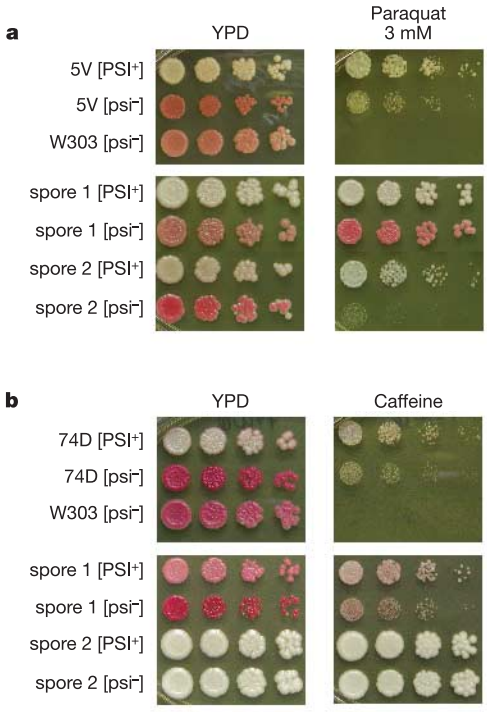

Figure 3 Progeny from an outcross of 5V-H19 show [*PSI*$^+$]-independent caffeine resistance. **a**, [*PSI*$^+$]-dependent resistance to 10 mM caffeine in YPD in the parental strain 5V-H19 and caffeine sensitivity in W303-1A is shown. Some progeny were resistant to caffeine irrespective of the [*PSI*$^+$] state, whereas other progeny maintained [*PSI*$^+$]-dependent resistance. The [*psi*$^-$] variants of spores were derived by either guanidine hydrochloride curing of [*PSI*$^+$] spores, or by deletion of *HSP104*. **b**, [*PSI*$^+$]-dependent resistance to 10 mM caffeine in YPD in the parental strain 74-D694 and caffeine sensitivity in W303-1A is shown along with two progeny in both the [*PSI*$^+$] and [*psi*$^-$] states.

For organisms to survive in the short term and evolve in the long term they must endure fluctuating environments, and then assimilate genetic changes that produce new traits. Must they wait for new mutations to occur in order to produce new traits? Or are there mechanisms that allow organisms to draw on pre-existing genetic variation? We have previously shown that environmental stress can reveal hidden genetic variation in a global genome-wide fashion by altering the activities of a protein chaperone, Hsp90[28,29], which functions in multiple signal transduction pathways. Here we demonstrate that a very different mechanism, a self-perpetuating change in the function and conformation of Sup35p, can also reveal previously hidden genetic variation in a combinatorial fashion on a global scale. In contrast to Hsp90, it has the capacity to do so in a single step that is immediately heritable but not yet fixed. Taking these observations together, we suggest that protein folding mechanisms are likely to have important and unexpected roles in translating genotypes to phenotypes and are likely to influence evolution in ways that could not have been foreseen. □

Methods

Strain construction

All yeast strains used in this study are given in Supplementary Table 1. All yeast genetic and molecular biology manipulations were done as previously described[30]. In the [*PSI*$^+$] state, translation termination efficiency is reduced and the loss of function can be detected by the suppression of nonsense codon mutations in selectable markers[5]. All original [*psi*$^-$] variants were obtained from their isogenic [*PSI*$^+$] counterparts by curing on 3 mM GdHCl in complete rich medium (YPD). Δ*rnq1* mutants were generated independently in both [*PSI*$^+$] and [*psi*$^-$] backgrounds, Δ*upf1* and Δ*ski7* in [*psi*$^-$] and Δ*hsp104* in [*PSI*$^+$] according to standard protocols. All open reading frame (ORF) disruptions were verified by polymerase chain reaction (PCR). The Δ*rnq1* and Δ*hsp104* strains tested negative for Rnq1p and Hsp104p respectively, as assayed by western blot analysis. Conservation of [*RNQ*$^+$] prion was verified by solubility assays as described[14].

To selectively cure [*PSI*$^+$], deletion mutants were generated in [*PSI*$^+$] backgrounds (*sup35*$^{\Delta NM}$ and *sup35*$^{R\Delta 2\text{-}5}$; Supplementary Table 1) by a replacement of wild type *SUP35* with pSup35C and pSup35RΔ2-5 (ref. 13) respectively using a pop-in/pop-out method. Both sets of mutants were verified by PCR, and *sup35*$^{\Delta NM}$ strains tested negative for the presence of the NM region of Sup35p by western blot analysis. The pTEF-NM-GFP construct was integrated into *sup35*$^{\Delta NM}$ background at *URA3*, creating ΔNM/NM-GFP. The presence of NM–GFP aggregates was confirmed *in vivo* by fluorescence microscopy.

The *sup35* mutants (C653R (ref. 16), R320I (ref. 16) and *sup35-R8*(ref. 15)) were introduced into [*psi*$^-$] strains by replacement of the wild type gene. Exogenous *sup35C* was introduced by integration of pTEF-sup35C into the genome of [*PSI*$^+$] variants at *URA3*. The anti-suppressor mutants *sup35*^{Q15R} and *sup35*^{S17R} were integrated into the [*PSI*$^+$] strains at *LEU2* using pASU^{Q15R} and pASU^{S17R}, respectively. The presence of [*PSI*$^+$]-like aggregates in the anti-suppressor strains was verified by *in vivo* aggregates observed with overexpression of NM–GFP. The suppression levels of the strains described above were assayed using the read through assay[20] and by growth on solid minimal media (SD) lacking adenine. The data shown in Supplementary Tables 2 and 4 are an average of two independent transformants grown and assayed on the same day. The absolute numbers varied from day to day, but the trend was always reproducible.

DNA preparation

DNA cloning and PCR were performed using standard methods. Oligonucleotides were obtained from Operon. Sequencing was done by Northwoods DNA Sequencing.

Plasmid construction

The *sup35* mutants (C653R, R320I and *sup35-R8*) were cloned from previously characterized strains[15,16] and inserted into pRS306. pSup35C was constructed by PCR amplification of the carboxy-terminal region of *SUP35*[12] as a *Bam*HI/SacI fragment and inserted into a pRS306 cassette containing the endogenous *SUP35* promoter at *Eco*RI/*Bam*HI. PCR primers used were as follows: 5′Sup35EcoI: 5′-CCGGAATTCATGTCGG ATTCAAACCAAGGC-3′; 3′Sup35XhoI: 5′-CCGCTCGAGTTACTCGGCAATTTTAA CAATTTTACC-3′; 3′GFPXhoI: 5′-CCGCTCGAGTCATTTGTATAGTTCATCCATG-3′. The plasmid pTEF-Sup35C was constructed by substituting the *SUP35* promoter of pSup35C with the TEF promoter (SacI/*Bam*HI) from p416TEF at PmeI/*Bam*HI by blunt-end ligation. To construct pNMG, NM-GFP was amplified by PCR as an *Eco*RI–XhoI fragment and inserted into a pRS306-TEF vector containing the promoter from p416TEF at SacI and XbaI. Anti-suppressors pASUQ15R and pASUS17R were obtained by cloning Sup35Pr-ASU-EF from original vectors[19] as XhoI–SacI fragments into a pRS306 vector.

Phenotypic testing

To test strain sets under various stress conditions, cells were grown to late log phase in YPD and fivefold serial dilutions were spotted on solid media containing appropriate treatments, using a replicator as described previously[4]. The phenotypes shown are reproducible using specific media in the concentrations indicated for various compounds. Alterations in strength and appearance of phenotype are typical with different media and different concentrations of the same additives.

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