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Introduction of Premature Stop Codons as an Evolutionary Strategy to Rescue Signaling Network Function

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Keywords: Signaling Network, Evolution, Premature Stop Codon

ABSTRACT

The cellular concentrations of key components of signaling networks are tightly regulated, as deviations from their optimal ranges can have negative effects on signaling function. For example, overexpression of the yeast mating pathway mitogen-activated protein kinase (MAPK) Fus3 decreases pathway output, in part by sequestering individual components away from functional multi-protein complexes. Using a synthetic biology approach, we investigated potential mechanisms by which selection could compensate for a decrease in signaling activity caused by overexpression of Fus3. We overexpressed a library of random mutants of Fus3 and used cell sorting to select variants that rescued mating pathway activity. Our results uncovered that one remarkable way in which selection can compensate for protein overexpression is by introducing premature stop codons at permitted positions. Because of the low efficiency with which premature stop codons are read through, the resulting cellular concentration of active Fus3 returns to values within the range required for proper signaling. Our results underscore the importance of interpreting genotypic variation at the systems rather than at the individual gene level, as mutations can have opposite effects on protein and network function.

INTRODUCTION

Cells receive stimuli from their surroundings and process them into physiological responses through signal transduction pathways [1]. As protein-protein interactions are crucial for the proper function of signaling pathways, it is not surprising that expression levels of individual components within signaling complexes are tightly regulated [2]. In fact, synthetic attempts at rewiring signaling networks need to consider how changes in the level of individual components might disrupt the precise interactions needed for function [3]. Deleterious consequences of changes in the expression levels of one component of a complex were first observed in studies of transcriptional regulators [4]. An increase in the cellular concentration of a transcription regulator can result in transcriptional inhibition, due to the sequestration of limiting components, a phenomenon known as squelching [4-5]. Though one could draw easy parallels between the effects of changes in the concentration of individual components in gene regulatory complexes and signaling complexes, still little is known about how overexpression of signaling pathway components may affect signaling interactions and consequently function. Furthermore, as mutations could affect steady state levels of signaling components, it is also interesting to explore how natural selection might compensate for such a perturbation.

Saccharomyces cerevisiae, the budding yeast, is an excellent model system to study signaling networks. Budding yeast has five pathways mediated by the canonical mitogen activated protein kinase (MAPK) cascade [6]. The three sequentially activated kinases in these pathways are conserved across diverse

species. In *S. cerevisiae*, the mating specific MAPK, Fus3, acts in a complex with the scaffold protein, Ste5, the MAPK Kinase (MAPKK) Ste7, and MAPK Kinase Kinase (MAPKKK), Ste11. In addition, Fus3 interacts with several other proteins to propagate the signal, as well as to regulate it [Figure 1A].

Fus3 overexpression has been shown to result in signal dampening by displacing the stoichiometric balance of the complex [7-9] [Figure 1B]. Excess Fus3 can bind to its partners individually, sequestering them away and therefore decreasing the levels of fully functional signaling complex. Overexpression of Fus3 could lead to the accumulation of both inactive Fus3 and mono-phosphorylated Fus3 (Fus3-P), both of which dampen mating pathway signal transduction. Inactive Fus3 inhibits mating pathway activity by binding to Gpa1, the α subunit of the heterotrimeric G-protein [10]. Unlike dually phosphorylated Fus3 (Fus3-PP), Fus3-P dampens mating pathway activity [11].

Here, using the yeast mating pathway as a model system, we use a synthetic biology approach to explore potential evolutionary mechanisms that could overcome the negative effects that overexpression of one component of a protein complex has on signaling pathway function. We find that mutations that create stop codons and are subsequently read through *in vivo* restore wt-like expression levels of the MAPK leading to normal signaling function. We therefore propose stop-codon readthrough is an unexpected compensatory mechanism to correct for over expression of signaling components.

RESULTS

Overexpression of Fus3 reduces pathway output

To confirm the deleterious effect of Fus3 overexpression on mating pathway activity, we expressed Fus3 from promoters of varying strengths, in a strain where a fluorescent reporter is fused to a mating pathway responsive promoter (pFUS1-GFP) [12]. As shown in Figure 1C, pathway output, as represented by GFP fluorescence, is strongly affected by Fus3 expression levels: GFP levels are higher when Fus3 is expressed from a weak promoter (pCYC1) than when Fus3 is expressed from a strong promoter (pTEF1), confirming that overexpression of Fus3 decreases mating pathway activity.

While a trivial way to restore pathway activity would be to lower the strength of the promoter, we are interested in identifying mutations in the coding region that increase pathway activity. These mutations would represent potential mechanisms that evolution could employ to overcome squelching. Thus, we focus our attention in mutational changes exclusively affecting Fus3 coding sequence. To identify Fus3 variants that rescue pathway activity, we overexpressed a library of random mutants of Fus3 in a pFUS1-GFP reporter strain, in which wild type (WT) Fus3 and its homolog Kss1 (which can also activate the mating pathway) had been deleted (Figure 2A). We then used fluorescence activated cell sorting (FACS) to isolate yeast cells expressing GFP levels higher than the WT strain (i.e., capable of rescuing mating pathway

activity). We recovered the plasmids from the sorted cells and retransformed them into the Fus3ΔKss1Δ strain to confirm activity. Next, we sequenced these variants to determine the mutations responsible for the mating pathway rescue phenotype.

Overexpressed Fus3 mutants can rescue pathway activity in different ways

We grouped the sorted mutants into two categories based on the type of mutations they carried: variants encoding substitutions of one or more amino acids, and, to our surprise, variants encoding premature stop codons [Figure 2B]. Among the variants encoding amino acid substitutions, three carried the previously-characterized Fus3 activating mutations L63P, D227 and C28Y [13], indicating that our selection is capable of isolating variants with pathway activating mutations. These mutants demonstrate that increasing activity of the kinase is a potential mechanism to overcome the effects of squelching.

Strikingly, eight of the selected Fus3 variants able to rescue mating pathway activity contain premature stop codons (PSCs). If translated correctly, many of these variants would result in significant truncations eliminating large protein fragments that often include many conserved residues vital for function (e.g. all of the active site). An extreme example would be the Fus3 variant truncated at residue G36, as a stop codon at that position would eliminate ~90% of the protein, including the ATP binding site (K42) [13], the activation loop (135-138 HRD) [14], the active site (155-158 DFGL) [14], and the common docking

site into which upstream activators and downstream substrates bind (D314, D317) [15] [Figure 3A]. Given the intriguing role that PSC may play in restoring pathway function, we focused our attention exclusively on the PSC-containing Fus3 variants. The rest of the sorted variants will be analyzed in an independent study.

Further analysis of PSC containing variants suggests readthrough

To ensure that the PSC containing variants were active and that the assignment of the PSC was not an error in sequencing analysis, we introduced a PSC in Fus3, by site-directed mutagenesis, at one of the residues found to have a PSC among the sorted variants – Y119. Analysis of this mutant by flow cytometry confirmed that this PSC containing variant is capable of rescuing mating pathway activity (Figure 3B), suggesting the PSC might be being read through. To further explore this hypothesis, we introduced a mutation, K42R, known to abolish Fus3 activity upstream of the PSC. As shown in Figure 3B, this mutant, Fus3 K42R Y119*, is unable to rescue mating pathway activity in a Fus3ΔKss1Δ strain, confirming that the Y119* mutation is necessary and sufficient for rescue of mating pathway activity. To further determine that the selected PSC containing Fus3 variants were effectively being readthrough, we deleted the region of Fus3 downstream of the PSC at position 119, therefore mimicking the protein product that would result from the accurate translation of the stop codon at that position. As before, we overexpressed this mutant, truncated Fus3, in the Fus3ΔKss1Δ pFus1-GFP strain and measured mating

pathway activity by flow cytometry. As shown in Figure 3B, Fus3 truncated at residue 119 cannot activate the mating pathway. Our integrated results so far suggest that translation is not stopping at the PSC, but rather may be reading through the PSC, resulting in the correct mRNA transcript and ultimately in an active kinase.

Readthrough of PSC leads to full-length active kinases

To confirm that the PSCs are being readthrough, resulting in full-length active kinases, we measured Fus3 protein levels for all eight variants containing PSC in the Fus3ΔKss1Δ strain by western blots. As shown in Figure 3C, all PSC containing variants express full length Fus3, thus demonstrating that the PSCs are read through leading to full-length kinases. Moreover, to determine if the expressed kinases are properly-folded substrates of their upstream MAPKK activator Ste7 and also able to interact with the mating scaffold Ste5, as Ste7-mediated activation occurs only when Fus3 is bound to Ste5, we measured the levels of phosphorylated Fus3 using an anti-phospho-Fus3 specific antibody. As shown in Figure 3C (and Supplementary Figure 1, showing the full picture of the western blot), all eight PSC-containing variants are phosphorylated by Ste7 *in vivo*, indicating that they are properly folded and capable of interacting with Ste7 and Ste5. Together with the flow cytometry experiments that demonstrate pFus1-GFP expression mediated by the PSC-containing Fus3 variants, our results confirm that readthrough of PSC leads to Fus3 variants fully capable of mediating the activation of the mating pathway.

Readthrough of PSCs has been shown to occur naturally in yeast, though at a low rate [16], as readthrough occurs as a result of a decrease in the efficiency of translation termination [17]. In agreement, our results show that, while different PSC-containing Fus3 variants are present in the cell at different levels, all of them are present at levels lower than that of WT Fus3 (Figure 3D). Indeed, our results suggest that the intrinsically low efficiency of readthrough compensates for the high levels of expression of the pTEF1 promoter, and, as a result, restore pathway activity. As shown in Supplementary Figures 2 and 3, similar PSC readthrough is observed when Fus3 variants are expressed in different *S. cerevisiae* strains, indicating that the observed effects are not an anomaly of a peculiar strain.

PSC containing variants can mediate a full mating response

To determine if a PSC containing variant is capable of mediating a full mating response, we assessed the ability of an “a” mating type strain carrying Fus3 Y119* to mate with an “α” mating strain encoding WT Fus3. In our experiment, each of the two mating strains was auxotrophic for a distinct marker, so that only the diploids resulting from the mating of the two strains can grow on minimal media. As shown in Figure 4A, the Fus3 Y119* mutant is able to mediate a full mating response in a Fus3ΔKss1Δ strain. Furthermore, as mating can occur due to cell-to-cell proximity even in the absence of pheromone-induced polarized growth (also called “shmooing”), we also measured the ability of the PSC

containing mutant to shmoo. As shown in Figure 4B, cells expressing Fus3-Y119* in a Fus3ΔKss1Δ strain, can develop shmoos that are indistinguishable from those seen in WT cells. Together, these results confirm that the PSC containing variants can mediate a full mating response.

PSCs are preferentially tolerated at surface residues

In yeast, readthrough of PSCs results in amino acid replacements that depend on the specific identity of the premature stop codon [18-19]. Blanchet *et al.*, and Roy *et al.*, show that, in yeast, the UAA and UAG premature stop codons are most often replaced by Glutamine, Tyrosine, or Lysine, while the premature stop codon UGA is most often replaced by Tryptophan, Cysteine and Arginine, all with distinct frequencies. Roy *et al.* also showed that the specific causes of readthrough (e.g. genetic background or pharmacological agents) can affect these frequencies. Since we are not using pharmacological agents to induce readthrough, and we see strain-independent results confirming readthrough (Supplementary Figures 2 and 3), we assume that our results are due to unprogrammed readthrough inherent to wild-type cells. Based on these measured replacement frequencies, it is possible to hypothesize what the most likely amino acid replacements would be for the PSC present in the sorted Fus3 variants. For instance, for the variant with a PSC at residue 119, the most likely replacement would be a Tyr/Gln, which makes it possible for the substitution to result in the original Tyr present at position 119 in WT Fus3 (Table 1). In contrast, for the mutant with a PSC at residue 36, the wild-type Glycine is not one of the

possible replacement amino acids – the most likely replacement would be a tryptophan. Thus, while it is expected that multiple proteins with different substitutions at the same PSC might co-exist within an individual cell, it is likely that, given the narrow range of possible replacements available, PSC should be tolerated only at defined locations. In particular, we hypothesize that the tolerated locations would be those in which the most likely replacement reverts back to the wild-type amino acid, as in position 119 in Fus3, or those in which the mutated residue would not have detrimental consequences in protein function.

To investigate this hypothesis, we visualized the location of all PSC on the 3D structure of Fus3. Not surprisingly, we found that all the PSC containing mutants occurred at surface exposed residues (Figure 5A), supporting the idea that the resulting amino acid replacements (which most likely lead to Gln, Tyr, Lys, Trp, Cys or Arg substitutions) may be more easily tolerated at locations that do not seriously affect folding and function.

To further understand readthrough tolerance, we introduced PSCs at two residues buried in the Fus3 protein core (A30 and C209) or at two residues exposed on the Fus3 surface (Y228 and G237) (Figure 5B). Moreover, we introduced specific PSCs into these positions, so that they could either be replaced by the wild-type amino acid during readthrough (C209 and Y228) or by non-synonymous substitutions (A30 and G237) (Table 1). As before, we overexpressed these mutants from a TEF1 promoter in a Fus3ΔKss1Δ pFus1-

GFP strain, and measured mating pathway activity by flow cytometry. Yeast cells overexpressing PSCs at the surface-exposed residues Y228 and G237, are able to mediate mating pathway response better than cells overexpressing Fus3 WT, regardless of whether or not the wild-type amino acid is likely substituted for the PSC during readthrough (Figure 5C). PSCs at these residues restore mating pathway activation to levels that are close to that mediated by WT Fus3 expressed from a weak promoter. In contrast, cells overexpressing PSCs at buried residues (A30 and C209) are unable to even mediate a response similar to that of overexpressed WT Fus3, confirming that amino acid substitutions are less tolerated at buried positions. Interestingly, overexpression of Fus3-C209* results in significantly higher pathway activity than overexpression of Fus3-A30* ($P=0.016$, Student's T-test). This could be explained by the fact that, while the PSC at C209 can be substituted by the wild-type amino acid (albeit at a much lower level than Y228*), the PSC at A30 cannot be substituted by an alanine during readthrough. Thus, we conclude that PSCs are most likely to be tolerated when the resulting amino acid replacements occur at exposed locations with lax structural or functional constraints, while at buried residues PSC are more likely to be tolerated when they restore, even if at a low frequency, the wild-type residue. Furthermore, our western blot analysis found that these Fus3 with PSCs express full-length, active kinases (Supplementary Figure 4). In addition, we found that co-expressing Fus3 carrying PSCs with wild-type Fus3 does not affect mating pathway response, indicating that if any truncated species are being expressed, they do not interfere with the function of full-length species

(Supplementary Figure 5).

DISCUSSION

In this work, we used a synthetic biology approach to investigate potential mechanisms by which selection could compensate for the decrease in signaling activity caused by overexpression of a tightly regulated signaling protein – the yeast mating pathway MAPK Fus3. Specifically, we used cell sorting to select a library of random mutants of Fus3 overexpressed from the strong promoter, pTEF1, in a strain where the WT Fus3 (and its paralog Kss1) had been deleted. In this manner, we isolated several Fus3 variants that rescued mating pathway activity. Our selection revealed that, in addition to the predictable way of improving kinase activity, as previously reported for the selected Fus3 variants L63P, D227 and C28Y [13], one unexpected way by which selection could rescue pathway function was through the introduction of PSCs, which are then naturally readthrough.

Readthrough has been shown to occur not just as an error in translation termination, but also as a regulated mechanism of stop codon bypass [20]. Several studies have revealed that readthrough can result in protein extensions. For example, using baker's yeast as a model organism, Namy *et al*. found eight adjacent open reading frames separated only by a unique stop codon to have higher than average stop codon bypass levels [17]. Also in yeast, Artieri and Fraser reported 19 proteins with conserved C-terminal peptide extensions

originating from stop codon read-throughs [21]. Similarly, functionally important protein extensions through readthrough of stop codons were also identified in viral genes [22-26], human genes [27-29], and several other species [28, 30-32]. In addition, programmed readthrough of stop codons is used to change cellular localization of some enzymes, from yeast to humans [33,34]. In yeast, stop codon readthrough has been linked to the unveiling of otherwise cryptic genetic variation [35]. Taken together, these results suggest that stop codon readthrough is a mechanism by which evolution can regulate gene expression.

Our results indicate that the location of the mutated residue in the protein structure, its effect on function, as well as the specific identity of the PSC are all factors that affect the impact that PSC have on protein (and pathway) function. Furthermore, our work demonstrates that, in addition to C-terminal extensions, readthrough can occur at internal PSCs, resulting in proteins with WT length, though with possible amino acid replacements. Thus, readthrough can exert its effects not only by changing the length of a protein, but also by lowering protein levels due to the intrinsically low efficiency of the readthrough process. In other words, our results demonstrate that, at least in the laboratory, selection can compensate for protein overexpression not only by changes in promoter strength, as it would have been easily expected, but also by introducing PSCs at permitted positions.

Interestingly, our results suggest that mutations that one would predict to have a detrimental effect on protein function (e.g. introduction of a PSC) could actually have the opposite effect on network function. This apparent contradiction highlights the importance of understanding genotypic variation at the systems level, rather than at the individual gene level. Studies attempting to predict phenotype from genotype computationally frequently categorize both frameshifting indels and premature stop codons as loss-of-function [36,37]. However, recent work by Tawfik and co-workers showed that the effects of frameshifting insertion and deletions (indels) on the function of a bacterial enzyme can be phenotypically rescued by subsequent slippage of the ribosome [38]. Similarly, our work indicates that PSCs cannot be always considered to detrimentally affect cellular functions.

METHODS

Yeast Strains

Fus3 and Kss1 were targeted for deletion by homologous recombination using Trp and Leu as selectable markers, respectively, in strains with the following genotypes:

W303 *MATa, bar1::NatR, far1Δ, mfa2::pFUS1- GFP, his3, trp1, leu2, ura3*.

W303 *MATa, his3, trp1, leu2, ura3*

Deletions were confirmed by PCR, flow cytometry, and western blots.

Library Construction

Random mutagenesis was carried out PCR-based methods by using Fus3 as a template and the Agilent GeneMorph II Random Mutagenesis Kit. The random mutants were constructed using previously described cloning strategy [6] and expressed from centromeric plasmids with His selection, under the control of a constitutive high expression promoter, Tef1 (pTEF1), and an AdhI transcription terminator. Mutation rate was confirmed by sequencing 18 independent clones from the unselected library.

Site Directed Mutagenesis

Point mutations were introduced into Fus3 by PCR using either Agilent QuikChange II Site-Directed Mutagenesis Kit or by using Pfu Ultra, following the manufacturer's protocols. The PCR products were cloned using the method described under Library Construction under the control of pTEF1 or a constitutive low expression promoter, Cyc1 (pCYC1). Mutations were confirmed by sequencing.

Transformation

Yeast strains were transformed by standard Lithium-Acetate method except libraries which were transformed by specific Lithium-Acetate based high efficiency, large scale transformation method described in [39] with minor modifications.

Fluorescence-Activated Cell Sorting

The Fus3 overexpression random mutant library was sorted by fluorescence-activated cell sorting (FACS) to isolate variants able to elicit a mating pathway response in Fus3 and Kss1 double deletion strains. Specifically, to ensure high library diversity, about 68,000 colonies were harvested after transformation into *Escherichia coli*. PK001 was transformed with library of Fus3 mutants replacing the deleted Fus3 with a random variant. To maintain diversity, about 4,000 transformants were harvested in selective medium and grown overnight at 30°C. Overnight cultures were diluted to OD_{600} between 0.1-0.2 and grown to log phase. Samples were then induced with 1μM α-factor and incubated at 30°C for two hours. After sonication, cells were gated by side and forward scatter and sorted by GFP expression using a BD FACSAria I cell sorter into selective medium. Sorted cells were plated on selective media and cultured.

Flow Cytometry

Double deletion yeast strains were transformed with plasmids expressing Fus3 variants and grown on selective media. Transformants were grown in triplicate selective medium overnight at 30°C. Wild-type strain without the double deletion was grown in complete synthetic dropout medium as positive control and either an empty double deletion strain (grown in complete synthetic dropout medium) or one expressing an empty plasmid (grown in selective medium) was used as negative controls. Overnight cultures were diluted to OD_{600} between 0.1-0.2 and grown to early log phase. Samples were then induced with 1μM α-factor and incubated at 30°C for two hours. Cells were then treated with the protein

synthesis inhibitor, cycloheximide for approximately 30 minutes. The GFP signal of 10,000 cells was measured for each sample with a Miltenyi Biotec MACSQuant VYB. The mean GFP fluorescence and standard deviation of triplicates were calculated using FlowJo.

Western Blots

Western blots were performed as described previously [40] with minor modifications. Overnight cultures were diluted to OD_{600} between 0.1-0.2 and grown to mid-log phase. Samples were then induced with 2μM α-factor and incubated at 30^{o}C for 15 minutes. After freezing the pellet in liquid nitrogen, the cells were then lysed and run in 10% Tris-Gly, SDS polyacrylamide gels at 200V for 50 minutes with the Odyssey® Protein Molecular Weight Marker (928-40000) using the Mini-PROTEAN® Tetra Cell. Bio-Rad Trans-Blot Turbo™ Transfer System was used to transfer the proteins to Bio-Rad low fluorescence PVDF membranes. Membranes were blocked in Odyssey® Blocking Buffer and TBS overnight at +4^{o}C. The membranes were then incubated with primary antibodies (Fus3: (yC-19) #sc6773, goat polyclonal IgG, Santa Cruz Biotechnology, dilution 1:5000; Fus3-PP: rabbit monoclonal phospho- p44/42 MAPK antibody, Cell Signaling Technology, #4370, dilution: 1:2000; PGK (loading control): Invitrogen 459250 mouse monoclonal, dilution: 1:5000) for 2 hours. After washing the primary antibodies with TBS + 0.05% Tween20, membranes were incubated with secondary antibodies (Fus3: donkey anti-goat Licor IRDYE 800 antibody, 926-32214; Fus3-PP: goat anti-rabbit Licor IRDYE 800 antibody, 926-32211; PGK:

goat anti-mouse Licor IRDYE 680LT antibody, 926-68020; all at dilution of 1:10000) for 1 hour. Membranes were then washed and visualized on a Licor Odyssey® CLx Infrared Imaging System.

Mating Assay

Mating assays were performed as described previously [12] with minor modifications. Equal amounts of yeast cells of mating type “a” (W303 *MATa, his3, trp1, leu2, ura3*, Fus3::kanMX4+KIURA3, Kss1::leu) were transformed with either empty vector, Fus3 expressed from pTEF1 or Fus3 Y119* expressed from pTEF1 and yeast cells of mating type “α” were mixed and added to a polycarbonate filter. After incubating at 30^{o}C for 3 hours, cells were extracted by vortexing and aliquots were plated on minimum synthetic media.

Microscopy

Yeast cells expressing WT Fus3 or Fus3 Y119* from pTEF1 were grown to log phase and induced with 1μM α-factor. Cells were imaged using an automated inverted Leica TCS SP8 confocal microscope.

FIGURE LEGENDS

Figure 1. Yeast mating pathway activity depends on Fus3 levels. (A) Yeast mating pathway activity can be measured by flow cytometry using a pathway responsive promoter fused to a fluorescent reporter. Fus3 interacts with many

proteins. (B) Optimal amount of Fus3 is required for mating pathway activity. Deviations from optimal Fus3 expression decreases mating pathway activity. (C) Fus3 overexpression-driven decrease in mating pathway activity can be triggered by expressing Fus3 from the strong TEF1 promoter.

Figure 2. Identification of pathway activating Fus3 variants. (A) Fus3 is overexpressed from a strong promoter in a Fus3Δ Kss1Δ pFus1-GFP background. Pathway activating variants are isolated by FACS. (B) Fus3 variants with higher than WT mating pathway induction ratio (Fold change in pFus1-GFP fluorescence over WT).

Figure 3. Genetic analysis of Fus3 variants carrying PSCs. (A) Functionally important, conserved residues are shown on Fus3 structure (PDB: 2B9F) (B) Fus3 carrying PSC at residue 119 is able to activate the mating pathway. Fus3 with a lost-of-function mutation (LOF) upstream of the PSC cannot activate the mating pathway. Fus3 truncated at residue 119 cannot activate the mating pathway. (C) Western blot analysis of Fus3 mutants with PSCs. All Fus3 mutants with PSC express full-length, active Fus3. (D) Fus3 variants with PSCs express less Fus3 than WT, even when expressed from the same strong promoter, as indicated by the normalized signal intensity.

Figure 4. Functional analysis of Fus3 variants carrying PSCs. (A) Fus3Δ Kss1Δ yeast cells expressing Fus3 WT or Y119* mutant from pTEF1 have normal

mating responses, as indicated by growth on minimum media after mating with a tester strain. (B) Yeast cells expressing Fus3 Y119* from pTEF1 have mating-induced morphological responses similar to WT.

Figure 5. Structural analysis of residues that tolerate PSC. (A) All PSCs map to surface residues. Based on Blanchet *et al.,* [18] we predict the possibility of each of the residues, when mutated to a stop codon to be substituted by the WT residue during readthrough. Residues that cannot revert to WT (red) and residues that could possibly revert to WT (black) are shown. (B) Buried residues, A30 (red) and C209 (blue); and exposed residues Y228 (magenta) and G237 (orange) are shown on the Fus3 3D structure (2B9F). (C) Fus3 with a PSCs at buried residues cannot mediate mating response.

Table 1. Premature Stop Codon containing mutants and their likely amino acid substitutions. List of Fus3 variants with premature stop codons tested in this study. Possible replacements based on premature stop codon identified through sequencing and likely substitutions based on Blanchet *et al.,* [18] and Roy *et al.*, [19].

AUTHOR CONTRIBUTIONS

P. S. K. and S.G.P designed study; P. S. K. performed experiments; P. S. K., A. M. M. and S.G.P. discussed results. P. S. K. and S.G.P. wrote the manuscript with input and commentaries from all authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

The following supporting information is available: Supplementary Methods and Supplementary Figures 1-5.

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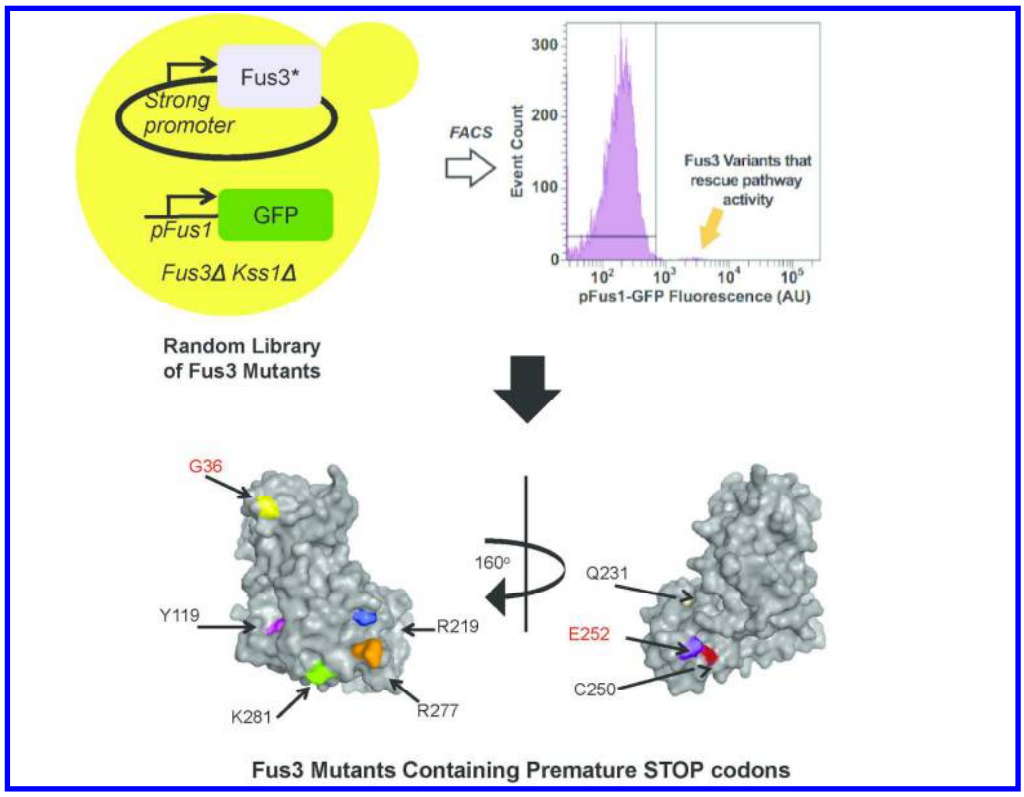

Selection can compensate for protein overexpression by introducing premature stop codons at permitted positions. Because of the low efficiency with which premature stop codons are read through, the resulting cellular concentration of the active MAPK Fus3 returns to values within the range required for proper signaling. Our results underscore the importance of interpreting genotypic variation at the systems rather than at the individual gene level, as mutations can have opposite effects on protein and network function.
graphical abstract
188x145mm (300 x 300 DPI)

Premature Stop Codon Mutants	Location	Wild type Codon	Premature Stop Codon	Wild type Amino Acid	Possible Replacements	Possible change to WT
F13L N146D E252*		GAG	TAG	E	Q, Y, K	No
K67E K281* P323H		AAA	TAA	K		Yes
Y119*		TAC	TAG	Y		
Q231* R249W		CAA	TAA	Q		
G36*		GGA	TGA	G	W, C, R	No
M179L C250* D314E		TGT	TGA	C		Yes
R219*		CGG	TGA	R		
R277*		CGA	TGA	R		
A30*	Buried	GCA	TAA	A	Y/Q/K	No
C209*	Buried	TGC	TGA	C	W/C/R	Yes
G237*	Surface Exposed	GGT	TGA	G	W/C/R	No
Y228*	Surface Exposed	TAT	TAG	Y	Y/Q/K	Yes

Premature Stop Codon containing mutants and their likely amino acid substitutions. List of Fus3 variants with premature stop codons tested in this study. Possible replacements based on premature stop codon identified through sequencing and likely substitutions based on Blanchet et al., [18].

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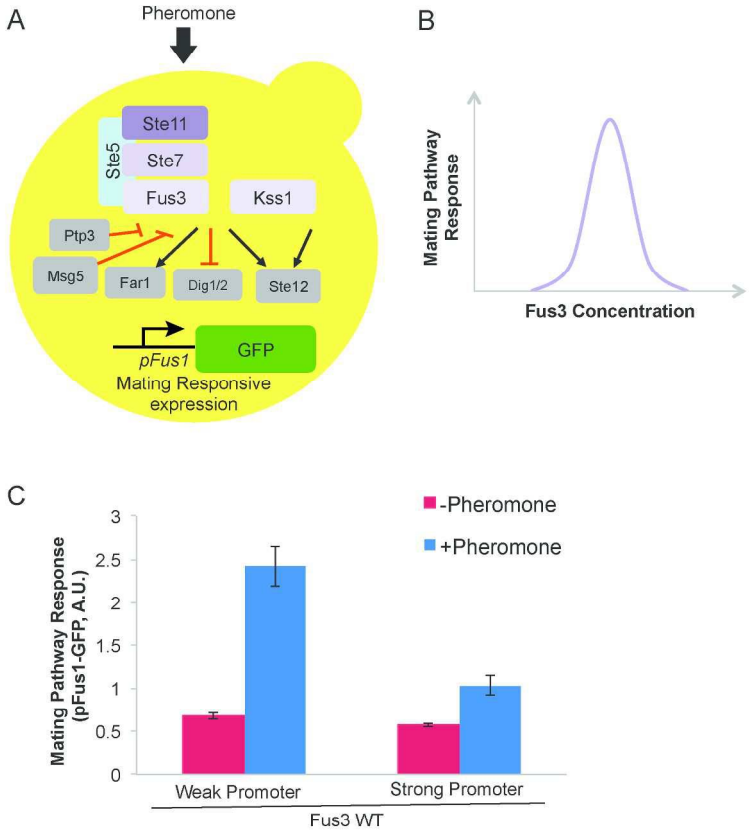

Figure 1. Yeast mating pathway activity depends on Fus3 levels. (A) Yeast mating pathway activity can be measured by flow cytometry using a pathway responsive promoter fused to a fluorescent reporter. Fus3 interacts with many proteins. (B) Optimal amount of Fus3 is required for mating pathway activity. Deviations from optimal Fus3 expression decreases mating pathway activity. (C) Fus3 overexpression-driven decrease in mating pathway activity can be triggered by expressing Fus3 from the strong TEF1 promoter.

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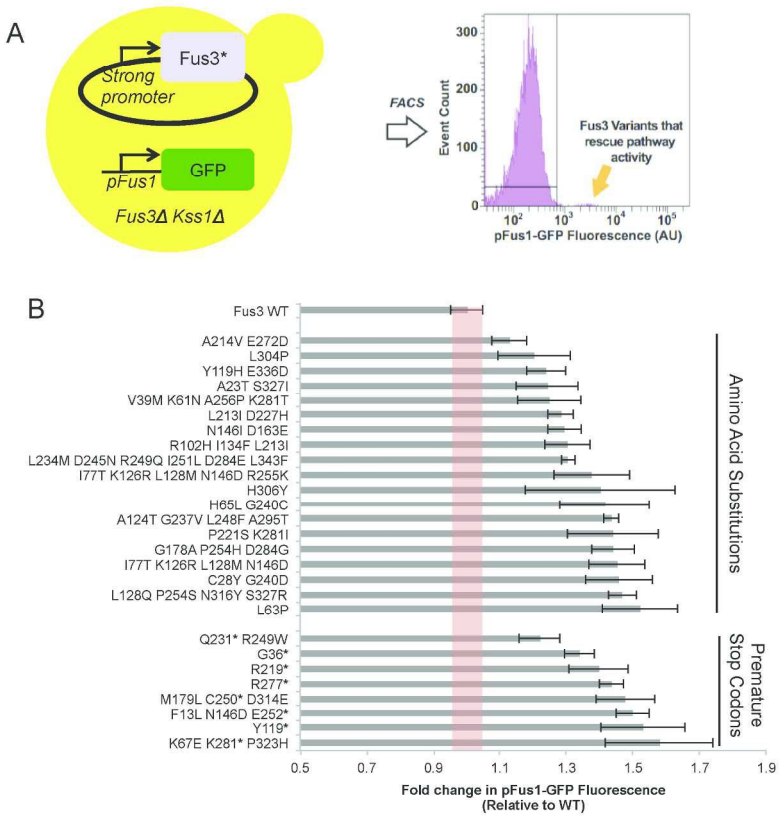

Figure 2. Identification of pathway activating Fus3 variants. (A) Fus3 is overexpressed from a strong promoter in a Fus3Δ Kss1Δ pFus1-GFP background. Pathway activating variants are isolated by FACS. (B) Fus3 variants with higher than WT mating pathway induction ratio (Fold change in pFus1-GFP fluorescence over WT).

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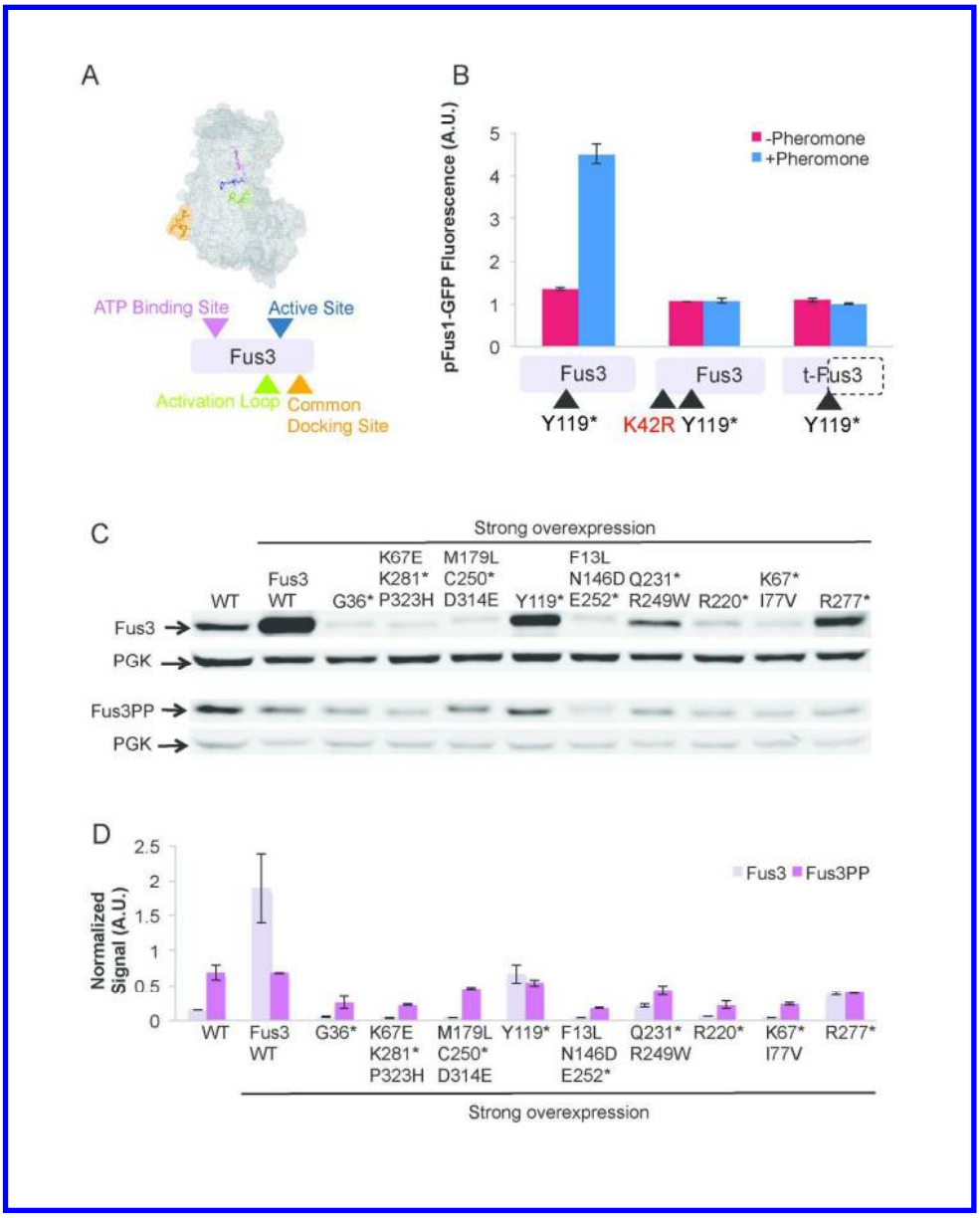

Figure 3. Genetic analysis of Fus3 variants carrying PSCs. (A) Functionally important, conserved residues are shown on Fus3 structure (PDB: 2B9F) (B) Fus3 carrying PSC at residue 119 is able to activate the mating pathway. Fus3 with a lost-of-function mutation (LOF) upstream of the PSC cannot activate the mating pathway. Fus3 truncated at residue 119 cannot activate the mating pathway. (C) Western blot analysis of Fus3 mutants with PSCs. All Fus3 mutants with PSC express full-length, active Fus3. (D) Fus3 variants with PSCs express less Fus3 than WT, even when expressed from the same strong promoter, as indicated by the normalized signal intensity.

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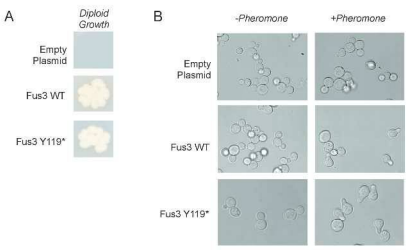

Figure 4. Functional analysis of Fus3 variants carrying PSCs. (A) Fus3Δ Kss1Δ yeast cells expressing Fus3 WT or Y119* mutant from pTEF1 have normal mating responses, as indicated by growth on minimum media after mating with a tester strain. (B) Yeast cells expressing Fus3 Y119* from pTEF1 have mating-induced morphological responses similar to WT.

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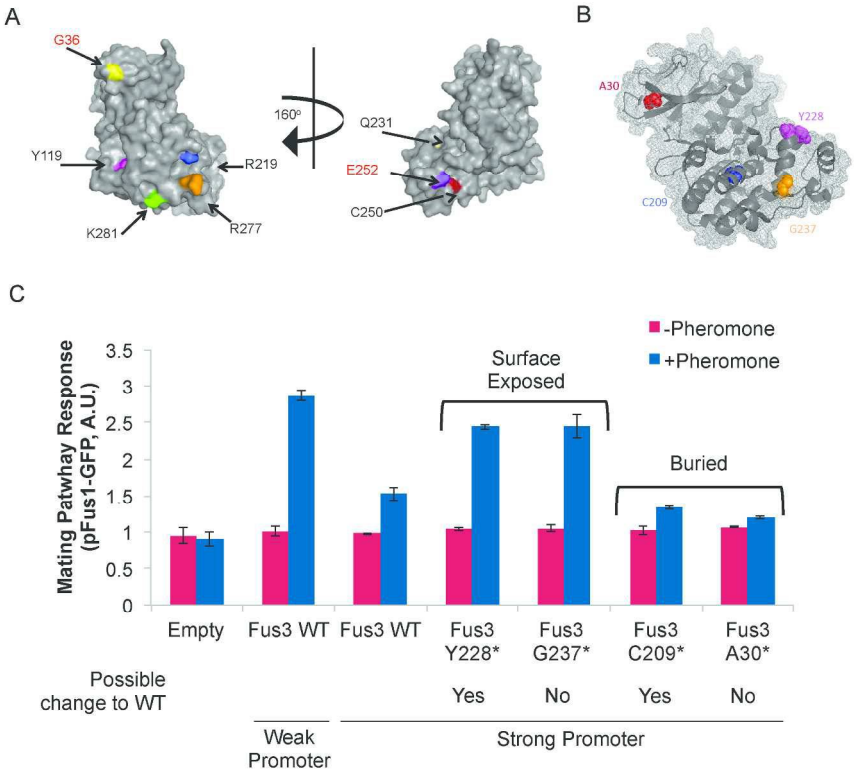

Figure 5. Structural analysis of residues that tolerate PSC. (A) All PSCs map to surface residues. Based on Blanchet et al., [18] we predict the possibility of each of the residues, when mutated to a stop codon to be substituted by the WT residue during readthrough. Residues that cannot revert to WT (red) and residues that could possibly revert to WT (black) are shown. (B) Buried residues, A30 (red) and C209 (blue); and exposed residues Y228 (magenta) and G237 (orange) are shown on the Fus3 3D structure (2B9F). (C) Fus3 with a PSCs at buried residues cannot mediate mating response.

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