Negative feedback confers mutational robustness in yeast transcription factor regulation

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Organismal fitness depends on the ability of gene networks to function robustly in the face of environmental and genetic perturbations. Understanding the mechanisms of this stability is one of the key aims of modern systems biology. Dissecting the basis of robustness to mutation has proven a particular challenge, with most experimental models relying on artificial DNA sequence variants engineered in the laboratory. In this work, we hypothesized that negative regulatory feedback could stabilize gene expression against the disruptions that arise from natural genetic variation. We screened yeast transcription factors for feedback and used the results to establish ROX1 (Repressor of hypO2Va) as a model system for the study of feedback in circuit behaviors and its impact across genetically heterogeneous populations. Mutagenesis experiments revealed the mechanism of Rox1 as a direct transcriptional repressor at its own gene, enabling a regulatory program of rapid induction during environmental change that reached a plateau of moderate steady-state expression. Additionally, in a given environmental condition, Rox1 levels varied widely across genetically distinct strains; the ROX1 feedback loop regulated this variation, in that the range of expression levels across genetic backgrounds showed greater spread in ROX1 feedback mutants than among strains with the ROX1 feedback loop intact. Our findings indicate that the ROX1 feedback circuit is tuned to respond to perturbations arising from natural genetic variation in addition to its role in induction behavior. We suggest that regulatory feedback may be an important element of the network architectures that confer mutational robustness across biology.

Robustness of organismal function in the face of perturbations is critical for fitness. Since the seminal work of Waddington (1), biologists have remarked on the stability of phenotypes against environmental and genetic variation, and understanding how organisms achieve robustness remains one of the major challenges in systems biology (2–4). Much of the search for molecular mechanisms of robustness has focused on gene regulation. Characteristics of regulatory networks that confer robustness include pathway redundancy and master regulatory organization (5), phenotypic capacitors (6–8), paired activating and inhibiting inputs (9), and cooperative and feed-forward regulation (10). Additionally, negative regulatory feedback, in which a biomolecule represses its own abundance, can buffer variation in gene expression (11, 12) and negative feedback loops have been shown to underlie robustness to variable environmental conditions and stochastic intracellular change (13–15). Negative feedback may also confer network stability against the effects of mutations (3, 16), but evidence for negative feedback as a driver of mutational robustness in vivo has been at a premium (17); the relevance of this principle to natural genetic variation remains largely unknown.

In this work, we focused on negative feedback in yeast hypoxia regulation motivated by the extensive evidence for feedback in oxygen response pathways across biology (18, 19). We characterized the feedback loop at the yeast hypoxia regulator ROX1 in molecular detail, and we harnessed this system as a test bed to study how feedback confers stability against naturally occurring mutations. Given the precedent for negative feedback as a determinant of quantitative behaviors of inducible circuits (20–24), we also investigated the role of Rox1 feedback in expression regulation during oxygen response.

Results

We set out to establish a tractable model system for the study of feedback and robustness using yeast transcription factors. For this purpose, we first screened transcription factor genes for feedback on protein abundance. We used fluorescence microscopy (25) to measure protein expression from a single genomic copy of each factor tagged with GFP in a diploid strain (26) while varying levels of an untagged copy of the factor (Fig. L4). To maximize signal to noise, we analyzed the 23 most highly abundant factors in rich medium (Table S1) and found evidence for negative feedback in four cases (Fig. 1B). As expected (27, 28), these screen hits included Rox1 and Swi4; we also found uncharacterized feedback loops by Mbp1 and Mot3. Independent replicate experiments confirmed the ability of each factor to repress its own abundance (Fig. 1C).

To investigate the role of feedback in mutational robustness and systems-level network behavior, we focused on the transcription factor Rox1. This master regulator is repressed in hypoxic conditions and induced under normoxia to regulate biosynthetic pathways that use molecular oxygen as a substrate (29). Anticipating that Rox1 would act directly at the ROX1 promoter (27), we identified four candidate Rox1 binding sites in the 500 bp upstream of the ROX1 coding start site (Fig. S1). Mutagenesis confirmed the role of these sites in ROX1 feedback, with each site contributing incrementally to the strength of autoregulation in an ROX1 transcriptional reporter (Fig. 2). Promoter response to changes in dose of ROX1 was markedly reduced when all four sites were mutated in combination, indicating a near-complete abrogation of feedback (Fig. 2). We used these mutations to engineer a version of ROX1 in which the feedback mutant promoter drove expression of Rox1 fused to GFP; to avoid potentially confounding effects from elevated Rox1-GFP steady-state levels in the presence of the feedback mutations, we manipulated the use of optimal codons (30) in the ROX1-GFP coding region. The suboptimized sequence, in conjunction with mutated Rox1 binding sites in the ROX1 promoter, gave rise to steady-state expression levels comparable with those levels of the WT (Fig. S2). We refer to this version of ROX1 as the suboptimized feedback mutant; a strain harboring this gene grew indistinguishably from the WT across environmental conditions (Fig. S3).

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To analyze the role of feedback during ROX1 induction, we grew WT and feedback mutant strains under hypoxic conditions and measured Rox1-GFP expression during oxygen exposure. For the feedback mutant promoter driving expression of the WT ROX1-GFP reporter, levels during induction far overshot the steady state of the strain harboring a WT promoter (Fig. 3). Such elevated expression was toxic to cells: this strain displayed growth defects under a variety of environmental conditions (Fig. S3). As expected, in the suboptimized feedback mutant, protein levels reached those of the WT at steady state but with slower kinetics owing to the reduced translation efficiency of the reporter construct (Fig. 3). We conclude that the ROX1 locus harbors regulatory information encoding strong activation during oxygen exposure. In WT cells, repression by Rox1 serves as a brake on this induction signal, avoiding the deleterious effects of elevated expression. Thus, the feedback loop tunes the kinetics of ROX1 induction, enabling a rapid approach to a moderate level of steady-state expression during the transition from hypoxia to normoxia.

We next sought to evaluate Rox1 feedback as a mechanism for robustness of gene expression to naturally occurring genetic variation. For this purpose, we developed an assay to interrogate

![Fig. 1. Screening yeast transcription factors for regulatory feedback. (A) Each part of the schematic represents one yeast strain. Green rectangles indicate the coding sequence of GFP, and the oval on the right represents a plasmid carrying an overexpression construct controlled by the GAL1-10 promoter (44). △, whole-gene deletion. (B) Each data point represents the results of two analyses of nuclear abundance of a transcription factor fused to GFP (26) measured by quantitative microscopy in diploid strains. Each analysis, as indicated on the x or y axis, compared fluorescence from a given tagged factor in two strains encoding different doses of the untagged version of the factor with strains named according to the schematic in A. (C) Each set of bars reports nuclear fluorescence measurements of the indicated factor as a fusion with GFP measured by quantitative microscopy and normalized with respect to WT levels. Each bar reports measurements from one strain with names as in A; error bars represent the SD over biological replicates and microscope fields. *Comparisons relative to WT that are significant at Wilcoxon P < 0.001.](image)

![Fig. 2. Transcription factor binding sites in the ROX1 promoter are required for transcriptional feedback. Each set of bars reports expression from one ROX1 transcriptional reporter in a diploid yeast strain measured by flow cytometry. Shading represents the presence or absence of a single untagged copy of ROX1 in the strain background. Each set of bars labeled with Site corresponds to a reporter with the indicated Rox1 binding sites (Fig. S1) mutagenized in the ROX1 promoter; 4 site indicates mutagenesis of all four sites. △, whole-gene deletion of ROX1.](image)

![Fig. 3. The ROX1 locus confers strong induction during oxygen exposure. Each set of points reports fluorescence of a haploid yeast strain bearing an ROX1-GFP fusion reporter gene measured by quantitative microscopy after a transfer of a culture from hypoxia to normoxia. Each data point represents median nuclear fluorescence across cells of one culture at the indicated time after oxygenation. Each color represents one reporter. FB mutant indicates a feedback mutant ROX1 promoter with all four Rox1 binding sites mutagenized, and subopt indicates that the ROX1 and GFP sequences were encoded with suboptimized codons.](image)
the effects on ROX1 expression of the spectrum of variants present in a set of divergent yeast strains of environmental and laboratory origin. For each such tester strain, we crossed it to a laboratory strain bearing the WT ROX1-GFP reporter, and we performed an analogous cross using the suboptimized feedback mutant. Haploid recombinant progeny, each a mosaic of inheritance from the tester and laboratory parents, served as a panel of genetically distinct strains among which ROX1 expression could vary. For a given cross, we measured the median Rox1-GFP levels in a culture of each progeny strain. The results, shown in Fig. 4, revealed deviation in Rox1-GFP abundance of up to eightfold across strain cultures, reflecting the impact of naturally occurring genetic variation on ROX1 expression. Eliminating feedback compromised robustness to these variants, with a wider spread of median Rox1-GFP levels across genetic backgrounds in feedback mutant strains than in WT; the coefficient of variation across strains was two- to fivefold higher in the presence of the feedback mutation (Fig. 4). Control experiments ruled out codon suboptimization as a predominant cause of this effect (Fig. S4). Interestingly, the extent of expression variation across recombinant progeny was a function of the tester strain parent, indicating that some testers harbored alleles with more dramatic consequences for Rox1-GFP expression than others (Fig. 4). We conclude that Rox1 autorepression buffers the effects of natural genetic variation on ROX1 expression, establishing regulatory feedback as a determinant of mutational robustness in this system.

We hypothesized that the ability to buffer gene expression could be a driver of the prevalence of feedback circuits across yeast transcription factors. In particular, we reasoned that evolutionary pressures for buffering expression levels and thus for feedback would be strongest among factors for which deviations from homeostatic expression levels give rise to fitness defects. In the case of Rox1, both increases and decreases in dosage were toxic to yeast cells (Fig. S3). To test the relationship between expression homeostasis and feedback more generally, we used growth rates of yeast strains manipulated to overexpress each transcription factor in turn (31). We first integrated these data with the results of our reporter-based screen for feedback among transcription factors (Fig. 1). Conforming to our model, when overexpressed, factors subject to feedback conferred a fitness defect ~70% more severe than factors with no evidence for feedback (Wilcoxon P = 0.02) (Fig. 5A). We next sought to expand our analysis beyond the transcription factors detectable in our experimental screen. For this purpose, we used a bioinformatic approach (described in Materials and Methods) to predict instances of direct transcriptional feedback by transcription factors based on the presence of their binding sites in promoters of their own encoding genes. Even using this unvalidated set of feedback inferences, evidence for autoregulation was again associated with overexpression toxicity, albeit to a less dramatic extent; toxicity effects were 13% more severe for factors with inferred feedback relative to the remainder of the set (Wilcoxon P = 0.04) (Fig. 5B). To address the possibility that computationally predicted binding sites were better specified for factors with stronger overexpression toxicity, we tested the relationship between overexpression growth rate for a factor and the number of its predicted targets genome-wide, and we found no effect (regression P = 0.2). Taken together, our results highlight feedback by transcription factors as a correlate of the toxic effects of overexpression, lending credence to the notion that many such feedback loops function in vivo as a control against misregulation.

Discussion

Landmark studies have identified negative feedback loops that tune the quantitative properties of gene circuits (20–24, 32). Negative feedback has also been implicated in the robustness of gene networks to environmental and stochastic change (13–15), but the role of native feedback circuits as buffers against natural genetic variation has remained unknown. Addressing the question requires detailed molecular analysis of feedback regulation and its impact across genetically heterogeneous populations, for which we have established an experimental paradigm using yeast Rox1 as a model system.

Rox1 regulates the expression of genes involved in oxygen-dependent sterol biogenesis and respiratory pathways. We have shown that cell growth is remarkably sensitive to changes in this activity even in normoxic conditions, with overexpression and deletion of Rox1 each conferring distinct defects. Consistent with a requirement for tight regulatory control of Rox1, our analysis has revealed two related ways in which Rox1 feedback limits deviations from steady-state expression optima. In a constant genetic background, negative feedback enables rapid ROX1 activation during oxygen exposure, minimizing the time spent in intermediate expression states and avoiding toxic effects of overexpression in a manner that dovetails with similar roles for autorepression in other networks (11, 33). Additionally, across genetic backgrounds, Rox1 feedback serves as a buffer against the perturbations arising from naturally occurring sequence changes. A primary implication of our findings is thus that the Rox1 negative feedback circuit is tuned to respond quantitatively to subtle up- and down-regulating effects of natural genetic variation as well as dramatic shifts in regulatory input when conditions change. In each case, trans-acting input that up-regulates ROXI would be counterbalanced by increased Rox1 occupancy and repression at its own encoding locus, and
input that represses ROX1 would be counterbalanced by a reduction in Rox1 occupancy at its own gene.

Will regulatory feedback prove to underlie mutational robustness as a general mechanism across biology? Pathway-level feedback is common in yeast; in many cases, the network can detect the perturbation of an artificially introduced genetic lesion and up-regulate functionally related genes to compensate (17). Whether gene-level feedback will be of similar importance for robustness on a genomic scale depends, in part, on the prevalence of autoregulation in gene networks. Most highly expressed genes in yeast are not subject to complete dosage compensation when mutated (34), but feedback at the gene level may be particularly common among transcription factors (35, 36). In light of our evidence that feedback may serve to constrain the deleterious effects of misregulation among transcription factors, a model invoking especially strong pressures for such feedback control would be consistent with the extreme overexpression toxicity observed across transcription factors in yeast (31). The emerging picture is one in which both gene- and pathway-level feedback, at transcription factor genes and elsewhere in the yeast network, may be key elements of the architecture that mediates buffering of genetic change. Understanding which gene circuits are buffered and how will be of critical interest in the effort to interpret the extent and phenotypic penetrance of regulatory variation (37–40) and the evolution of robustness mechanisms (16, 41).

Addressing these questions and applying the emergent principles to bioengineering (42) and human disease treatment (43–45) will serve as continued motivation for the study of feedback and robustness in regulatory circuits.

### Materials and Methods

**Yeast Strain Construction and Analysis of Genetic Variation.** For each of 67 yeast transcription factors (Table S1), strains bearing one copy of a GFP protein fusion of the respective factor gene (26) (Invitrogen) were mated to strains deleted for the respective gene (46) (Invitrogen), and they were imaged by microscopy as described below. For the 23 factors of highest abundance (nuclear fluorescence > 30,000 arbitrary units) (Table S1), GFP-tagged haploid strains were then mated to strains bearing URa3 carrying (URa3)-marked plasmids encoding each ORF under the GAL1,10 promoter (47) for the complete screen as shown in Fig. 1. All plasmid transformations and yeast cell matings were performed by standard techniques (48). Construction of reporters and mutagenesis protocols are described in detail in SI Materials and Methods. The method for generating ROX1-GFP expression is provided in Fig. S5.

Crosses for analysis of genetic variation were generated by mating ROX1-GFP strains with BY4741 (Open Biosystems), SK1 (49), YPS606 (49), and 22:3:8 (50). In the case of YPS606 and SK1, homothallic switching endonuclease was replaced with URa3 by cloning and transformation as above. For each cross, hybrid diploids were sporulated on solid minimal sporulation medium (48), haploids were isolated by spore enrichment (51), and 60–90 GFP+ recombinants were selected by growth on Yeast Peptone Dextrose (YPD) medium (48) supplemented with G418. To compare the variances of the feedback-mutant and WT distributions for a given cross, we first calculated the F statistic for differential variance using the var.test function in R (www.r-project.org).

We then permuted mutant and WT strain expression values 10,000 times, repeated the F calculation for each permuted dataset, and evaluated the statistic from the real data against this null distribution to yield a one-sided empirical P value.

**Feedback Assays.** For the feedback screen, one culture of each yeast strain was inoculated into complete supplemented media (CSM) minus uracil (MP Biomedicals) with 2% raffinose and grown in 2-ml well 96-well plates at 30 °C with shaking to saturation. Strains were back-diluted into CSM minus uracil with 2% galactose to an OD of 0.1. Strains were imaged after ~6 h of growth having reached OD of 0.5–1.0. On preparation of 96-well plates (see below), 100 µL cell suspension were added to each well and allowed to settle and bind for 30 min before imaging as described below. In the screen, only transcription factors with putative feedback effects consistent between overexpression and deletion assays were considered for additional study (Fig. S5). For confirmation of screen hits in Fig. 1C, three independent cultures of each strain were grown and prepared for imaging as above.

**Hypoxia Time Course.** For induction experiments, a culture of each yeast strain was grown in CSM with 2% galactose, back-diluted into deoxygenated media at OD of 0.002, and grown for 40 h at 30 °C in a 16 × 12-mm Hungate tube with septum stopper and screw cap (Belco). From each culture, a 10-ml aliquot was spun down, and cells were resuspended in 200 µL oxygenated media; 100 µL were added to concanavalin-treated, glass-bottomed plate wells and allowed to settle for 1–5 min before imaging as described below at the time points indicated in Fig. 3. To deoxygenate media, 10 mL were added to a stoppered tube and boiled for 5 min with a syringe through the stopper. After boiling, the syringe was removed, and each tube of media was cooled to <50 °C and purged with N2.

**Fluorescence Microscopy.** Glass-bottomed 96-well plates (Falcon) were coated with 100 µL of a 10 µg/mL solution of Con A type V (Sigma-Aldrich), incubated at 1 h at room temperature, and then washed three times with water. Once cells were added and allowed to adhere, wells were then washed and resuspended with media immediately before imaging. For imaging, we used a 60x PlanApo objective (N.A. = 1.4) under oil immersion in a Nikon TE2000 inverted microscope equipped with a mercury lamp, motorized stage, and 512/BFT MicroMax cooled CCD camera (Photometrix). We imaged three to six observation fields per strain, with each field containing ~100 cells. For each field, we acquired a 0.5-s GFP exposure and a defocused bright-field image for cell identification and boundary determination using Metamorph 7.0 software (Universal Imaging Corporation). Using Cell-ID 1.0 (25), for each cell, we estimated nuclear fluorescence by calculating average pixel intensity in the area of a 3-pixel radius around the brightest point. We estimated cytoplasmic fluorescence by calculating the average intensity of pixels immediately inside the cell’s edge. We then calculated normalized nuclear fluorescence by subtracting the estimated cytoplasmic fluorescence from the estimated nuclear fluorescence.

**Flow Cytometry.** Cultures of each strain were grown in CSM with 2% galactose to saturation and back-diluted to OD of 0.1–0.5 h before measurement. Fluorescence measurements of ~10,000 cells/sample were acquired with an LSRSFortesa BD Biocytex Analyzed in R with Bioconductor (www.bioconductor.org). A given strain was eliminated from analysis if the majority of side scatter values across cells in its culture sample did not fall within a range typical of a nonflocculant laboratory strain growing at log
phase. The median background fluorescence from cells of a strain bearing no GFP gene was subtracted from the median across cells of each culture of interest.

**Growth Condition Screen.** Strains were grown overnight in CSM with 2% glucose at 30 °C to saturation and then reincubated and grown for >2 doublings before plating; 1:10 serial dilutions were performed in microtiter plates. Dilutions were transferred to plates using a multipronged inoculating device (frogger). Plate media were CSM with one of the following additions: 2% glucose, 2% galactose, 3% glycerol, or 3.2% ethanol; 2% glucose with 20 μg/mL fluconazole (Sigma-Aldrich); or VPD (48).

**Bioinformatic Predictions of Feedback.** Sequences corresponding to the 1,000 bp upstream of coding start for each of the 5,922 yeast genes were downloaded (52). Position weight matrices to score binding preferences for each yeast transcription factor were downloaded from http://faenza.mit.edu/.