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Efflux Pump Control Alters Synthetic Gene Circuit Function

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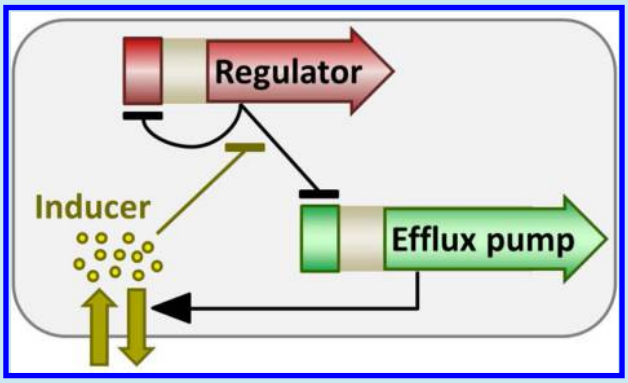
Supporting Information

ABSTRACT: Synthetic biology aims to design new biological systems for predefined purposes, such as the controlled secretion of biofuels, pharmaceuticals, or other chemicals. Synthetic gene circuits regulating an efflux pump from the ATP-binding cassette (ABC) protein family could achieve this. However, ABC efflux pumps can also drive out intracellular inducer molecules that control the gene circuits. This will introduce an implicit feedback that could alter gene circuit function in ways that are poorly understood. Here, we used two synthetic gene circuits inducible by tetracycline family molecules to regulate the expression of a yeast ABC pump (*Pdr5p*) that pumps out the inducer. Pdr5p altered the dose−responses of the original gene circuits substantially in *Saccharomyces cerevisiae*. While one aspect of the change could be attributed to the efflux pumping function of Pdr5p, another aspect remained unexplained. Quantitative modeling indicated that reduced regulator gene expression in addition to efflux pump function could fully explain the altered dose−responses. These predictions were validated experimentally. Overall, we highlight how efflux pumps can alter gene circuit dynamics and demonstrate the utility of mathematical modeling in understanding synthetic gene circuit function in new circumstances.



KEYWORDS: *synthetic gene circuit, efflux pump, stochastic simulation, feedback*

Synthetic biology aims to build biological devices for predefined purposes.[1−5] One important goal for synthetic biologists is to construct synthetic gene circuits[6,7] that function as switches, oscillators, logic gates, dimmers, or counters.[8−16] Small molecule inducers that bind to the protein components of such gene circuits are often used to control them externally. The hope is that by placing specific genes under the control of such inducible synthetic gene circuits, users can deliver precise stimuli to cell populations.[17−20] For example, synthetic gene circuits could enable the controlled secretion of drugs or biofuel compounds for clinical or industrial purposes.[21,22] Secreting drugs and biofuels requires efflux pumps that actively move them across the cell membrane. ATP-binding cassette (ABC) family multidrug resistance pumps are prime candidates to fulfill this function. Highly conserved across bacteria, fungi, and mammals, ABC family efflux pump proteins cause microbial resistance to antibiotic treatment and chemoresistance in tumors by pumping a wide range of compounds out into the extracellular medium.[23] Importantly, in addition to their intended substrates, efflux pumps can also drive out the intracellular inducer and thereby reduce its concentration. This creates a feedback loop[24] that may alter the function of synthetic or natural gene networks that control efflux pump protein expression. Understanding the effect of this implicit feedback is important if efflux pumps are to be used as parts of synthetic gene circuits. Yet, the effect of efflux pumps on synthetic gene circuit function has not been thoroughly investigated.

Our goal here was to study the interaction between an efflux pump and two synthetic gene circuits that regulate it. To achieve this, we modified two TetR-based synthetic gene circuits, called "negative regulation" (NR) and "negative feedback" (NF), both inducible by tetracycline analogues. We have previously characterized versions of these gene circuits that controlled a passive target gene (yEGFP::zeoR), which does not affect its

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upstream transcriptional regulator TetR. Here, we replaced this passive target gene with the yeast pleiotropic drug resistance (PDR) pump-fluorescent reporter fusion gene *PDR5::GFP*. Once the Pdr5p::GFP protein is expressed, it pumps out the inducer,[25−27] altering the activity of its transcriptional regulator, and thereby creating a feedback loop. We studied by experiment and mathematical modeling whether this implicit feedback loop altered the dose−response of the original gene circuits as intuitively expected from efflux pump function. Surprisingly, we observed an additional and unexpected dose−response change after introducing the gene that encoded the efflux pump. Using a combined mathematical-experimental approach, we identified reduced TetR expression as the cause underlying this additional change. These findings shed light on how efflux pumps can alter gene network function and highlight the importance of mathematical modeling in elucidating unexpected consequences of gene circuit modifications.

RESULTS AND DISCUSSION

Efflux Pump Alters the Dose−Response of the NR Synthetic Gene Circuit. To understand how an efflux pump affects the function of its transcriptional regulatory network, we modified the previously characterized[16] "negative regulation" (NR) gene circuit to create a new gene circuit, NRpump. Both NR and NRpump consisted of two parts: a *target gene* and a *regulator gene* (Figure 1A,B). The regulator gene in both gene circuits was the *tetR* repressor under the control of the natural *PGAL1* promoter,[16] which is constitutively active in galactose-containing media. The TetR protein can repress gene transcription by binding to *tetO2* operator sites in the target promoter. In the NR gene circuit, the target gene was *yEGFP::zeoR* (*yEGFP* fused to *zeoR*) under the control of *GAL1-D12*, a *GAL1* promoter modified to contain two *tetO2* operator sites[28] (Figure 1A). In the NRpump gene circuit, the target gene was *PDR5::GFP* (Figure 1B), which can remove the inducer doxycycline from the cell interior.[29−31] To avoid potential confounding interference with the native copy of *PDR5*, both gene circuits were chromosomally integrated into endogenous *PDR5*-knockout yeast cells (Figure S1). Overall, the NR and NRpump gene circuits differed only in their target gene: NR contained the passive target gene *yEGFP::zeoR*,[16] whereas NRpump contained the active target gene *PDR5::GFP*. Throughout the paper, the term "pump" will indicate the inclusion of *PDR5* into gene circuits and the implicit feedback generated through inducer removal.

To determine how the efflux pump alters NR gene circuit function, we measured experimentally (by flow cytometry) the dose−responses of fluorescence intensity in both NR and NRpump with respect to the same inducer, doxycycline (Figure 1C−F). Flow cytometry is a quantitative, highly reproducible method for measuring protein levels in yeast,[32] producing results consistent with those of other measurement techniques, including Western blotting.[32,33] Plotting mean reporter (yEGFP::ZeoR or Pdr5p::GFP) fluorescence intensity at increasing doxycycline concentrations (Figure 1E) revealed a sigmoidal dose−response of mean target gene expression in both gene circuits, with a steep increase at intermediate inducer concentrations. However, compared to NR, the reporter in NRpump began to express at lower doxycycline concentrations. Phrased differently, NRpump was more inducer-sensitive at low induction. The opposite was true at high doxycycline concentrations: the NRpump gene circuit required higher doxycycline concentrations to reach saturation, meaning that it was less inducer-sensitive at high induction.

The experimentally measured gene expression noise, characterized by the coefficients of variation (CVs) (standard deviation normalized by the mean), peaked at a slightly lower doxycycline concentration and reached a lower maximum for NRpump compared to NR (Figure 1F). The CV peaks (Figure 1F) corresponded to the broad histograms at intermediate doxycycline concentrations (Figure 1C and 1D), indicating heterogeneous reporter expression in the cell population. The lower CV peak for NRpump could be due to pump-mediated negative feedback, which is known to reduce noise.[34−36]

In summary, we observed two dose−response changes in NRpump compared to NR: increased inducer-sensitivity at low induction and decreased inducer-sensitivity at high induction. The latter observation (decreased inducer-sensitivity in high doxycycline) could be intuitively explained by the Pdr5p::GFP pump removing some doxycycline molecules from the cell interior, thereby lowering inducer-sensitivity. On the other hand, the increased inducer sensitivity at low inducer concentrations was counterintuitive.

A Mathematical Model of the Pump-Mediated Dose−Response Change. To better understand the dose−response changes in the NRpump gene circuit relative to NR, we turned to computational modeling. We previously established a computational model to simulate the NR dose−response.[16] Here, we verified that stochastic simulations based on this earlier model were able to reproduce our experimental NR dose−response observations (Figure 2A,B and Figure S2). Then we introduced, using Michaelis−Menten kinetics, an efflux pump term into this earlier model to simulate the changes in the NRpump dose−response relative to NR (see the Methods). However, the modified model could only partially reproduce the dose−response change in the NRpump gene circuit relative to NR (Figure 2A,B). Specifically, the addition of the pump term reproduced decreased inducer-sensitivity at high doxycycline concentrations, in agreement with experimental results. However, it failed to reproduce the increased inducer-sensitivity at low doxycycline concentrations.

To restore agreement with the experimental data, we analyzed the parameter sensitivity of the model (Table S1). This analysis revealed that both aspects of the dose−response change in NRpump could be reproduced only if the repressor protein (TetR) production rate was reduced in addition to introducing the pump term. Similar effects from altered regulator expression have been observed before.[37] A new model that included this effect successfully reproduced both increased inducer-sensitivity at low inducer concentrations and decreased inducer-sensitivity at high concentrations, as in the experimental NRpump dose−response data (Figure 2C,D).

Efflux Pump Alters the Dose−Response of the NF Synthetic Gene Circuit. To test the predictive power of the computational model, we set out to also predict the pump-mediated dose−response change for a related gene circuit,[16] called "negative feedback" (NF) or "linearizer". The NF gene circuit is similar to NR, except for the promoter driving *tetR* expression, which is replaced with the TetR-repressible promoter that also controls target gene expression (Figure 3A). Therefore, NF incorporates negative feedback compared to NR, because TetR represses its own expression (in addition to the target gene). Remarkably, the mean NF dose−response for yeast[16] and mammalian[19] cells carrying the NF gene circuit was linear nearly up to saturation.

To predict how efflux pump protein regulation may affect the dose−response of the NF gene circuit, we first introduced TetR negative feedback into the NR mathematical model as

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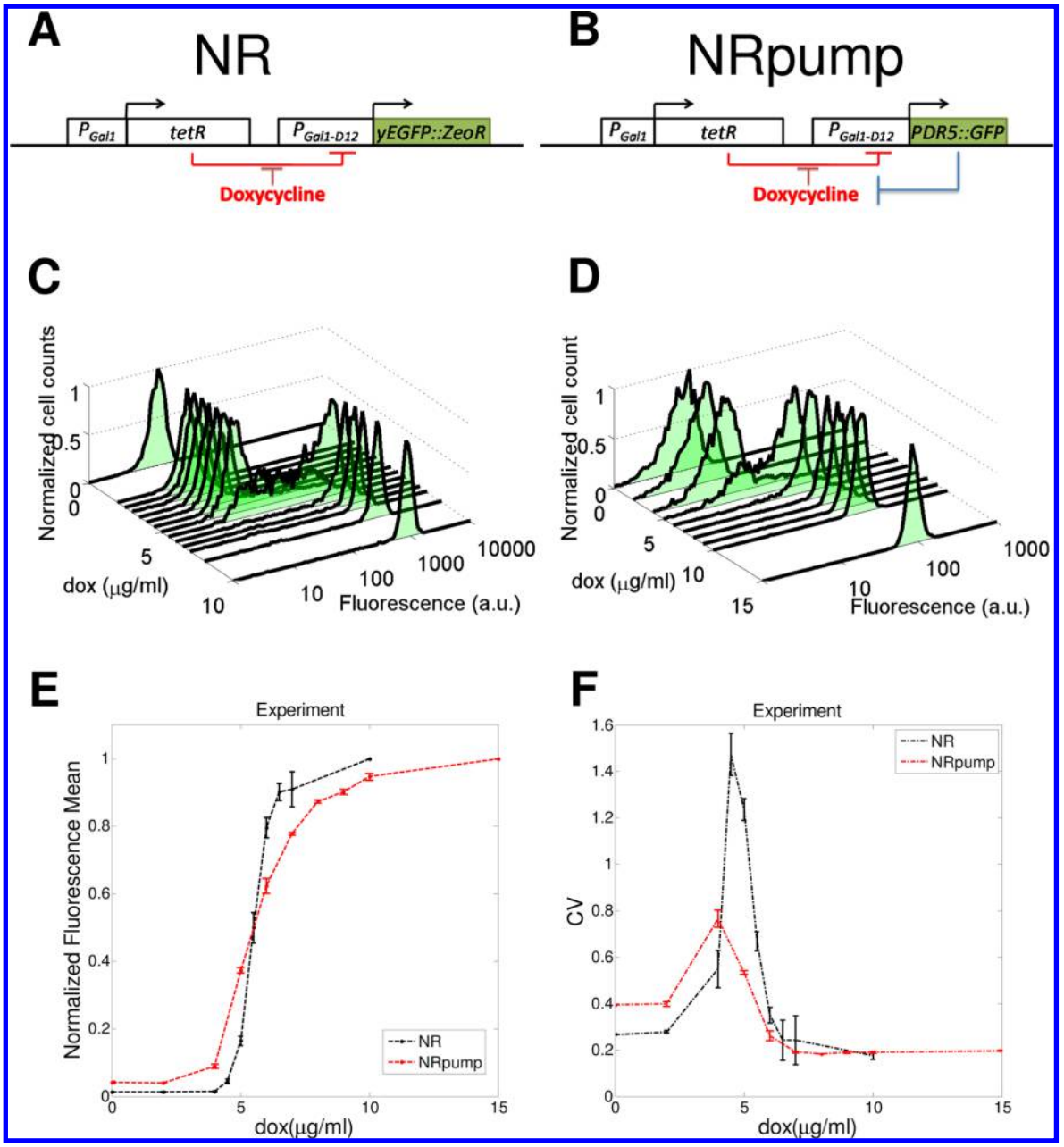

Figure 1. Experimentally measured dose−responses of the NR and NRpump strains. (A) Negative Regulation (NR) gene circuit design. This gene circuit consists of a regulator gene (*tetR*) that represses target gene (*yEGFP::zeoR*) expression in a doxycycline concentration-dependent manner. Red blunt arrows indicate repression. (B) Negative Regulation pump (NRpump) gene circuit design. This gene circuit is identical to NR, except for the target gene (*PDR5::GFP* in this case). Note that the Pdr5p efflux pump transports doxycycline out of the cell, creating an implicit feedback loop (blue blunt arrow) in NRpump, which does not exist in NR. (C) Experimentally measured histograms of fluorescence intensity for the NR gene circuit, indicating yEGFP::zeoR expression at increasing doxycycline concentrations. (D) Experimentally measured histograms of fluorescence intensity for the NRpump gene circuit, indicating PDR5::GFP expression at increasing doxycycline concentrations. (E) Experimentally measured dose−responses of NR (black) and NRpump (red) mean fluorescence intensity. These and all subsequent mean dose−responses were normalized by the maximum fluorescence intensity such that normalized fluorescence values fall between 0 and 1. (F) Experimentally measured dose−responses of NR (black) and NRpump (red) Coefficient of Variation (CV).

previously,[16] and confirmed that the dose−response became linear before saturation. Then we introduced the same pump term as in the NRpump model, and found that the simulated NFpump dose−response became concave, curving downward while staying always below the NF dose−response curve (Figure S3). Finally, to predict the additional effect(s) of reducing TetR synthesis on the NFpump gene circuit's dose−response, we changed the same parameter as in the NRpump model to reduce TetR synthesis rate. The numerical solutions of the latter NFpump model predicted a dose−response that was still concave, but was above the NF dose−response, indicating increased inducer-sensitivity for NFpump (Figure 3C). On the other hand, the simulated CVs of both NF and NFpump were similarly low at all inducer levels (Figure 3D).

To test these computational predictions experimentally, we constructed the NFpump gene circuit in a manner similar to NRpump. Then we measured by flow cytometry the dose−responses of fluorescence intensity for the NF and NFpump gene

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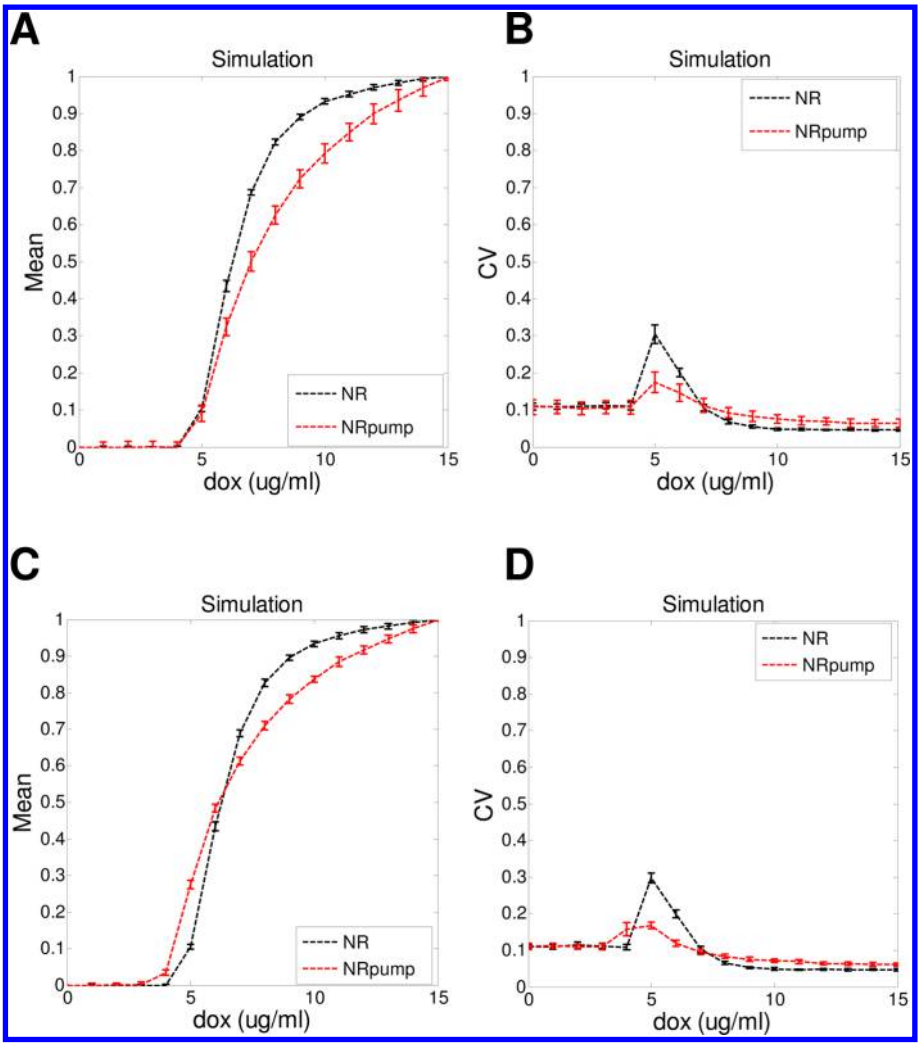

Figure 2. Stochastic simulation of pump-mediated dose–response change. (A) Stochastic simulation of NR (black) and NRpump (red) mean dose–responses. The simulations for NR are based on previous models,[16] with only an efflux pump term added for NRpump. (B) Stochastic simulation of NR (black) and NRpump (red) CV dose–responses, with only an efflux pump term added to previous models, as described above. (C) Stochastic simulation of NR (black) and NRpump (red) mean dose–responses with an efflux pump term added and TetR synthesis rate reduced compared to previous models. (D) Stochastic simulations of NR and NRpump CVs with an efflux pump term added and TetR synthesis rate reduced compared to earlier models. Most parameters in (A) and (B) were based on a previous model[16] (except for l_z, K, h, and k, which were obtained by fitting the data) as follows: $a_x = a_z = 50$ nM h^{-1}, $l_z = 10$ nM h^{-1}, $b = 10$ nM^{-1}, $C = 10$ [dox] h^{-1}, $d_x = d_z = 0.12$ h^{-1}, $d_y = 1.2$ h^{-1}, $\theta = 0.44$ nM, $n = 4$, $K = 50$ nM, $h = 3.5$, and $k = 0$ h^{-1} for NR strain. All parameters were identical for the NRpump strain, except enabling pump function by setting $k = 200$ h^{-1}. The same parameters were used in (C) and (D) as in (A) and (B), respectively, except for lowering $a_x = 43$ nM h^{-1} for the NRpump strain.

circuits at increasing concentrations of doxycycline (Figure 3E–H). The results confirmed that NF had a linear fluorescence mean dose–response between doxycycline concentrations 0 μg/mL and 4 μg/mL. By contrast, the mean dose–response for NFpump was concave (not linear) (Figure 3E), and lay above the NF dose–response (Figure 3E), as predicted computationally when TetR synthesis rate was reduced in addition to introducing the pump term (Figure 2C). Gene expression noise for both NF and NFpump was low at all levels of induction (Figure 3F). Accordingly, the fluorescence histograms of both NF and NFpump were narrow and uniform at all doxycycline concentrations (Figure 3G and 3H).

In summary, introducing the efflux pump altered two aspects of the NF gene circuit's mean dose–response, causing loss of dose–response linearity and increased inducer-sensitivity. This agreed with computational models that had reduced TetR synthesis besides including the efflux pump term.

Efflux Pump Mutants Reveal That Pump Function Is Insufficient to Explain Dose–Response Differences. Our findings so far indicated that the shapes of the mean dose–response curves in both NRpump and NFpump changed in two different ways compared to NR and NF, respectively. Computational simulations predicted that the most likely causes of these changes were the efflux pumping function of Pdr5p combined with lower TetR expression. Therefore, we set out to test experimentally if Pdr5p efflux pump function alone was indeed insufficient to produce both aspects of NRpump and NFpump mean dose–response change.

To test the effect of efflux pump function on the dose–response separately from any other effects, we constructed two Pdr5p functional mutants, Pdr5p-S558Y and Pdr5p-G312A. Both mutants should disrupt the association between Pdr5p and ATP, thereby significantly compromising the efflux pumping function of Pdr5p.[25,26] Thus, we replaced the wild-type *PDR5* gene with each of these mutant genes in the NRpump and NFpump gene circuits and integrated the new gene circuits separately into the parental yeast genome to create two new

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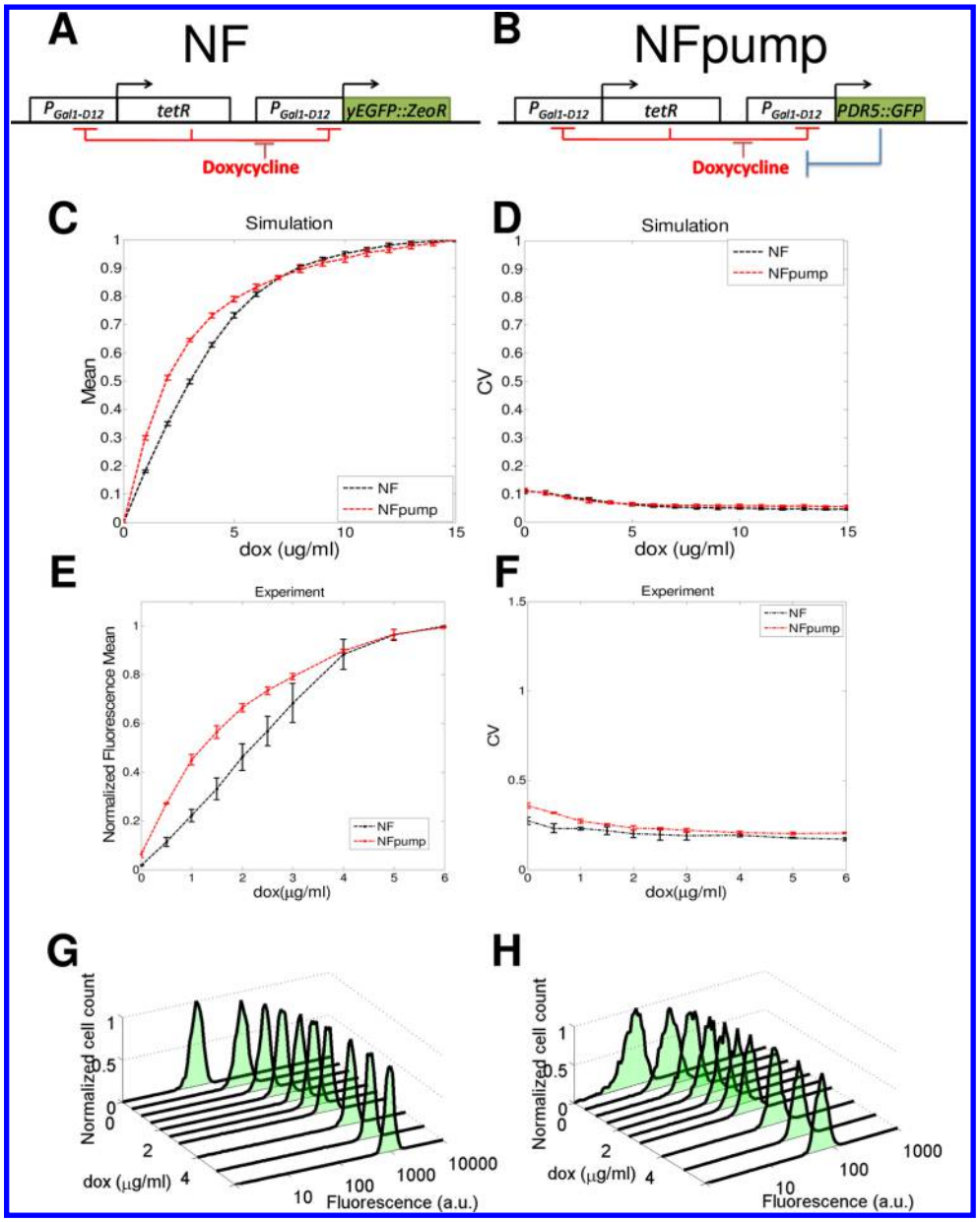

Figure 3. Computationally predicted and experimentally measured dose−responses of the NF and NFpump strains. (A) NF gene circuit design. This gene circuit differs from NR in a single aspect: TetR autorepression (negative feedback). (B) NFpump gene circuit design. This gene circuit differs from NRpump in a single aspect: TetR autorepression (negative feedback). It differs from NR in two aspects, each creating a feedback loop: TetR autorepression and an implicit feedback through Pdr5p::GFP pump function. (C) Stochastic simulation of NF (black) and NFpump (red) mean dose−responses with an efflux pump term added and TetR synthesis rate reduced compared to previous models.[16] (D) Stochastic simulation of NF (black) and NFpump (red) CV dose−responses with an efflux pump term added and TetR synthesis rate reduced compared to previous models. (E) Experimentally measured NF (black) and NFpump (red) mean dose−responses. (F) NF and NFpump CV dose−responses (experimental data). (G) Experimentally measured histograms of fluorescence intensity for the NF gene circuit, indicating yEGFP::zeoR expression at increasing doxycycline concentrations. (H) Experimentally measured histograms of fluorescence intensity for the NFpump gene circuit, indicating PDR5::GFP expression at increasing doxycycline concentrations. Parameters for the stochastic simulations were the same as described in the legend of Figure 2, and were set to $a_x = a_z = 50\ \text{nM h}^{-1}$, $l_z = 10\ \text{nM h}^{-1}$, $b = 10\ \text{nM}^{-1}$, $C = 10\ [\text{dox}]\ \text{h}^{-1}$, $d_x = d_z = 0.12\ \text{h}^{-1}$, $d_y = 1.2\ \text{h}^{-1}$, $K = 50$ nM, $h = 3.5$, $\theta = 0.44$ nM, $n = 4$, $k = 0\ \text{h}^{-1}$ for the NF strain, and similarly, with the exception of $a_x = 43\ \text{nM h}^{-1}$ and $k = 200\ \text{h}^{-1}$, for the NFpump strain.

"NRpumpmutant" and "NFpumpmutant" strains: NRpump-558, NRpump-312, NFpump-558 and NFpump-312.

Next, we measured experimentally the mean dose−responses and CV dose−responses of NRpump-312 and NRpump-558, and compared them with NR and NRpump. The experimental results showed that NRpumpmutant strains responded to doxycycline concentrations as low as 4 μg/mL (Figure 4A−C and Figure S4A). This resembled the increased inducer-sensitivity of NRpump (Figure 1E). On the other hand, the mean dose−response curves of NRpumpmutant strains increased as steeply as for NR at intermediate induction (4 μg/mL to 6 μg/mL doxycycline), contrasting the gradual dose−response of NRpump (Figure 4C). The dose−responses of CVs were similar for all strains, except that the CV of NR peaked at slightly higher doxycycline concentration than the other three strains (Figure 4D), according to the decreased inducer-sensitivity of NR. These experiments confirmed that the efflux pumping function of Pdr5p caused the dose−response change at high

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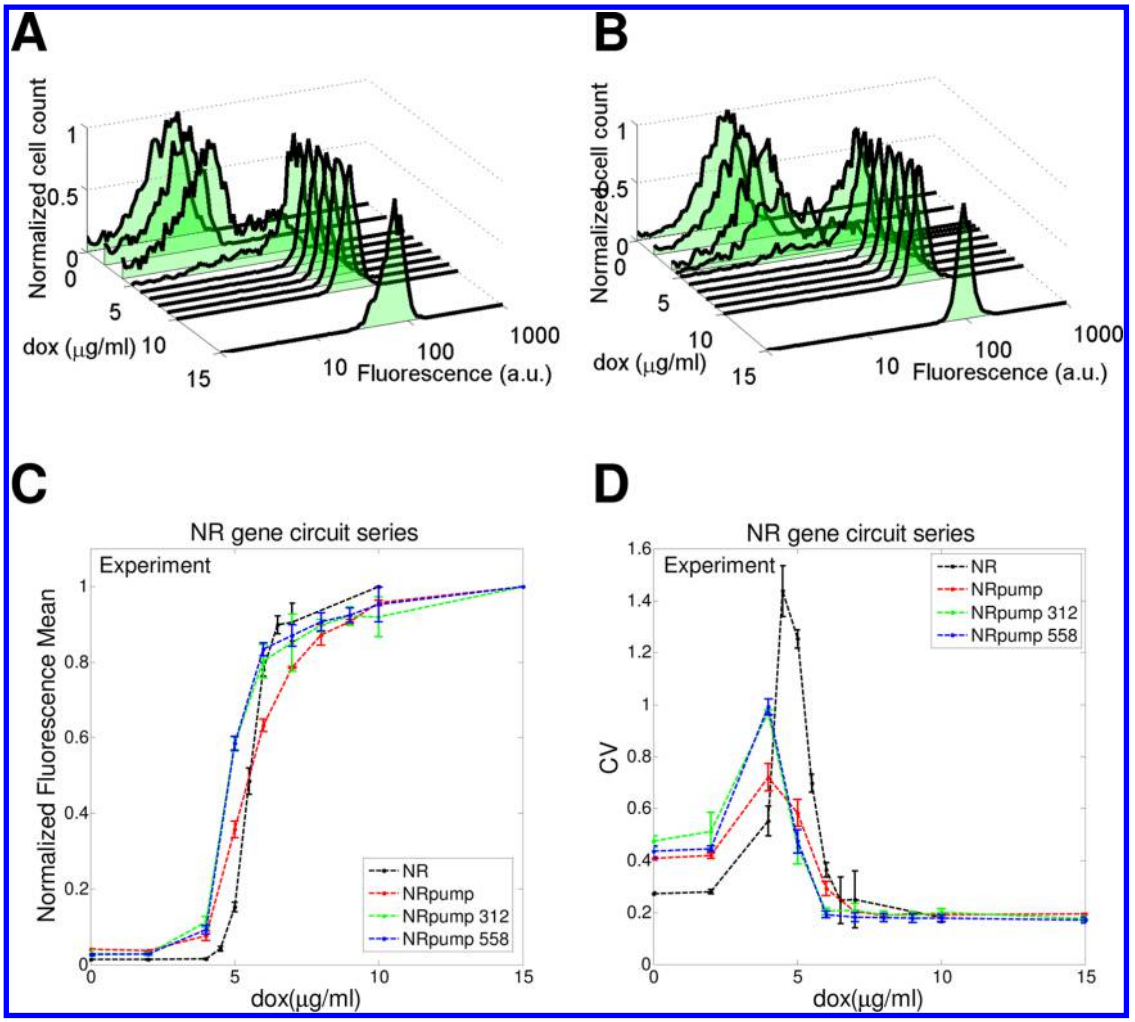

Figure 4. Experimentally measured dose−responses of the NR, NRpump and NRpumpmutant strains. (A) Experimentally measured histograms of fluorescence intensity for the NRpump-312 strain, indicating Pdr5p-312::GFP expression at increasing doxycycline concentrations. (B) Experimentally measured histograms of fluorescence intensity for the NRpump-558 strain, indicating Pdr5p-558::GFP expression at increasing doxycycline concentrations. (C) Experimentally measured dose−responses of NR (black), NRpump (red) and NRpumpmutant (green, blue) fluorescence means indicate that eliminating the pump function is insufficient to recover the NR dose−response. (D) Experimentally measured dose−responses of NR (black), NRpump (red) and NRpumpmutant (green, blue) fluorescence CVs.

doxycycline concentrations, but not at low doxycycline concentrations.

Afterward, we measured the experimental dose−responses of the reporter means and CVs of NFpumpmutant strains. Both NFpumpmutant strains had linear dose−responses between doxycycline concentrations 0 and 2 μg/mL similar to NF, and saturated at 3 μg/mL (Figure 5A−C and Figure S4B). Recovering linearity in the NFpumpmutant strains indicated that the dose−response concavity in NFpump was most likely due to the efflux pumping function of Pdr5p::GFP. However, the slopes of the linear ranges in both NFpumpmutant strains were still higher than that in NF. This suggested that the non-functional pump gene somehow still increased the sensitivity of the NFpumpmutant gene circuits to doxycycline concentration, similar to NFpump.

Accordingly, as we lowered efflux pumping rate in NRpump and NFpump computational models with reduced TetR synthesis, we obtained dose−responses similar to the experimentally observed NRpumpmutant and NFpumpmutant dose−response curves (Figure 6A and 6C), which still differed from those of NR and NF.

Both the experimentally measured and simulated CVs for NFpumpmutant strains were similarly low and slightly decreasing as for the NF and NFpump gene circuits, consistent with the narrow and uniform distribution of reporter expression observed by single cell-level measurements (Figures 5D and 6D). Likewise, the doxycycline concentration at which the CV peaks occurred in the NR, NRpump, and NRpumpmutant strains, as well as the relative size of the peaks, were consistent between computational models and experimental observations (Figures 4D and 6B).

Overall, we found that there was still a difference between the mean fluorescence dose−responses of NRpumpmutant and NR as well as NFpumpmutant and NF strains, whether or not the pump was functional (Figure S4 shows mean dose−responses without normalization). Thus, we confirmed the computational predictions that the efflux pumping function of Pdr5p is insufficient to fully explain the dose−response change. Specifically, it cannot cause the increased inducer-sensitivity observed in NRpump and NFpump (although it most likely causes the decreased inducer-sensitivity of NR high doxycycline and concavity of NFpump dose−response).

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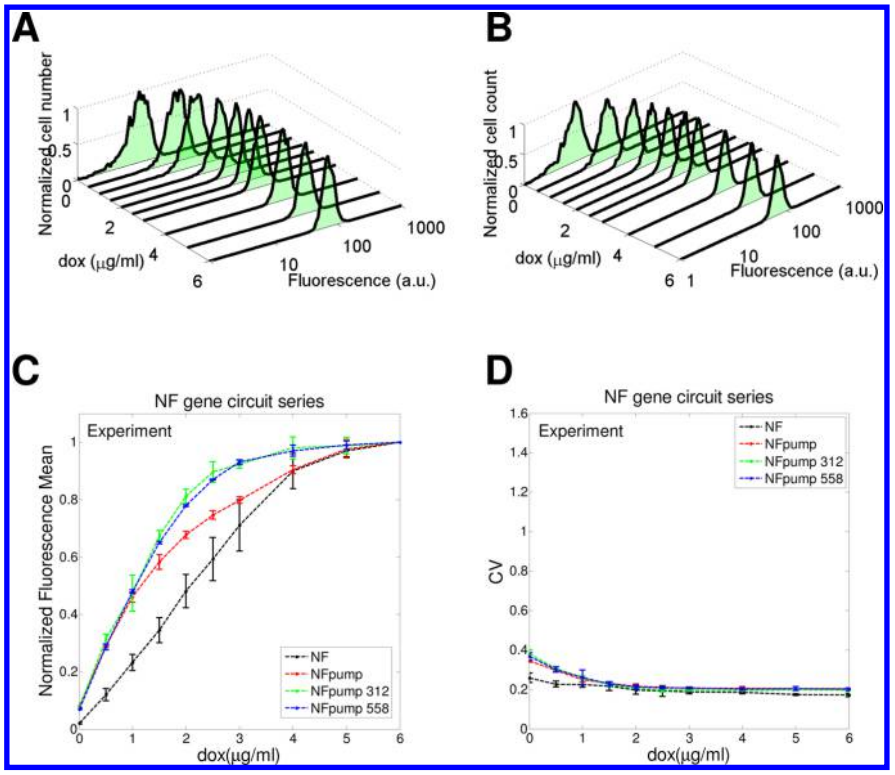

Figure 5. Experimentally measured dose−responses of the NF, NFpump and NFpumpmutant strains. (A) Experimentally measured histograms of fluorescence intensity for the NFpump-312 strain, indicating Pdr5p-312::GFP expression at increasing doxycycline concentrations. (B) Experimentally measured histograms of fluorescence intensity for the NFpump-558 strain, indicating Pdr5p-558::GFP expression at increasing doxycycline concentrations. (C) Experimentally measured dose−responses of NF (black), NFpump (red) and NFpumpmutant (green, blue) fluorescence means indicate that eliminating the pump function is insufficient to recover the NF dose−response. (D) Experimentally measured dose−responses of NF (black), NFpump (red) and NFpumpmutant (green, blue) fluorescence CVs.

Experimental Confirmation of Lower TetR Expression in NRpump and NFpump Strains. So far we have shown that efflux pump activity of Pdr5p only explains one aspect of the dose−response changes in NRpump and NFpump. Computational models suggested lower TetR expression causing the other aspect (increased inducer-sensitivity). To confirm whether regulator gene expression was indeed lower in all other strains compared to NR and NF, as predicted computationally, we fused *tetR* with the fluorescent reporter, *mCherry*, and then replaced the original *tetR* with this bifunctional fusion in all of the 8 synthetic gene circuits mentioned above. Thus, by combining *tetR::mCherry* with *yEGFP::zeoR* for NR and NF, and *tetR::mCherry* with *PDR5::GFP* for all other strains, including the efflux pump mutants, we created eight 2-color (2c) gene circuits (Figure 7). Importantly, all of the experiments described above could have been done with these two-color gene circuits. Therefore, if the dose−response changes in the 2-color strains are consistent with those in the corresponding single-color strains, then the 2-color strains are appropriate means to investigate the causes of any dose−response changes in single-colored strains.

We induced all the 2-color strains with the same doxycycline concentrations as for the corresponding single-color counterparts. The dose−response changes of fluorescence means for the target gene in all 2-color strains were consistent with those of the corresponding single-color strains (compare, for example Figure 8A and 8B with 3E, and see Figure S5). Moreover, the TetR::mCherry regulator expression's dose−responses in ^{2c}NF, ^{2c}NFpump and ^{2c}NFpumpmutant strains were also consistent with the reporter expression in the same strain and the corresponding single-color strains (Figure 8A and 8B). On the other hand, TetR::mCherry expression level at all doxycycline concentrations remained the same in the ^{2c}NR, ^{2c}NRpump and ^{2c}NRpumpmutant strains (Figure 8E), as expected because the wild-type *GAL1* promoter driving *tetR::mCherry* expression in these strains is constitutively active in galactose-containing media, and should not respond to doxycycline. Thus, the 2-color strains are suitable to reveal all the causes of dose−response changes in single-colored strains.

To test if regulator expression levels were altered by introducing the efflux pump-encoding target gene, we measured the red fluorescence intensity of the regulator in all of the 2-color strains. Interestingly, by investigating TetR::mCherry expression levels in this way, we observed clone-dependent variation (Figure 8C,D), causing large error bars in Figure 8E and 8F. In addition, there were some minor strain-dependent differences in TetR levels (Figure 8C and 8D). Nevertheless, TetR::mCherry expression was always higher in NR and NF clones than in clones carrying any of the other gene circuits (Figure 8C,D), confirming our computational predictions. Furthermore, TetR::mCherry mean dose−response measurements indicated that the repressor had higher expression level in NR compared to NRpump and NRpumpmutant strains at all doxycycline doses (Figure 8E), as expected. In a similar agreement with computational predictions, TetR::mCherry expression level in NF was higher than that in NFpump and NFpumpmutant strains at all doxycycline doses (Figure 8F).

Overall, these experimental results from 2-colored strains confirmed the computational prediction that reduced regulator expression, rather than the efflux pumping function of Pdr5p, caused the unexpected aspect of dose−response changes

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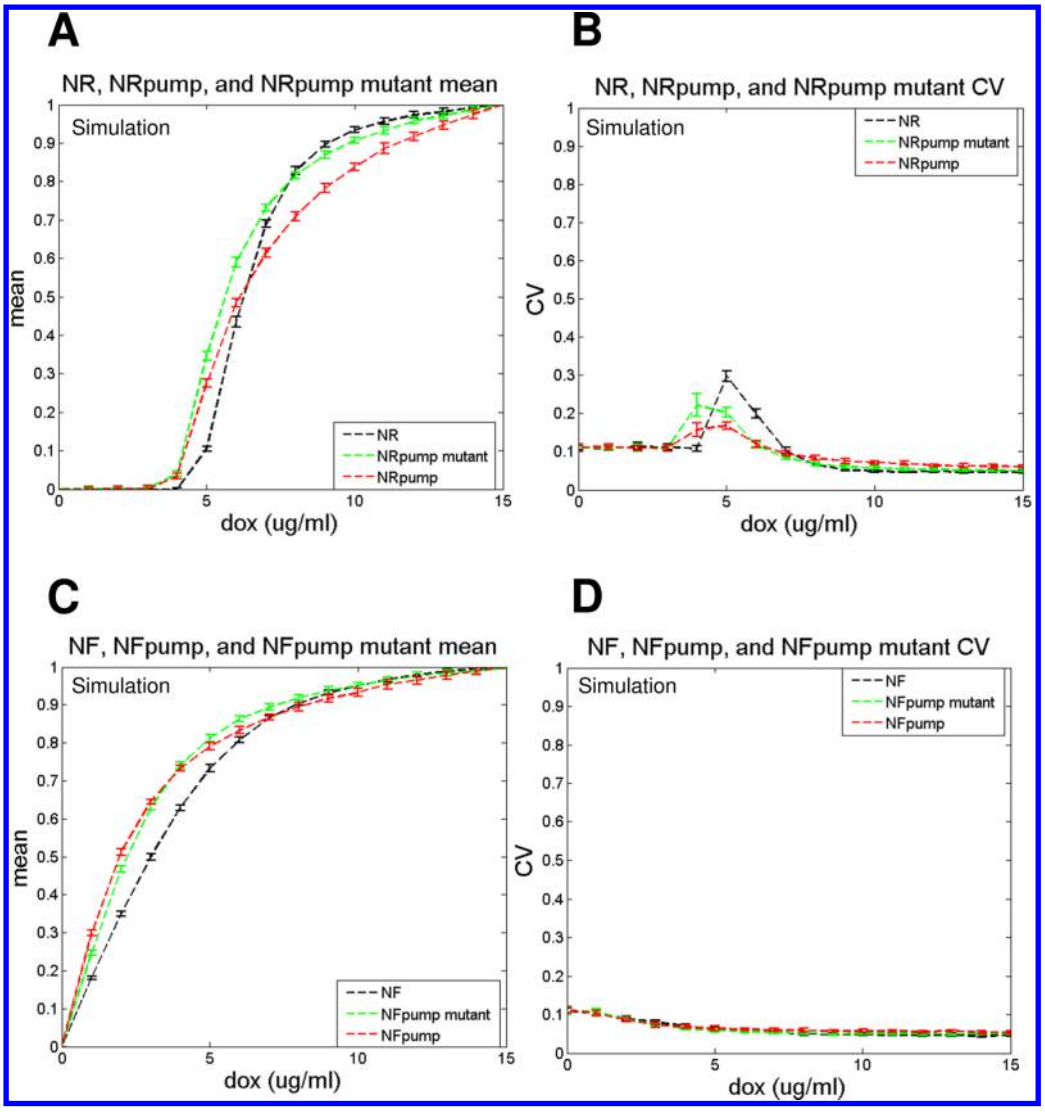

Figure 6. Stochastic simulation of dose−response change in pumpmutant and nonmutant pump strains. (A) Simulated dose−responses of NR (black), NRpump (red), and NRpumpmutant (green) fluorescence means. (B) Simulated dose−responses of NR (black), NRpump (red), and NRpumpmutant (green) CVs. (C) Simulated dose−responses of NF (black), NFpump (red), and NFpumpmutant (green) fluorescence means. (D) Simulated dose−responses of NF (black), NFpump (red), and NFpumpmutant (green) CVs. Parameters were set to $a_x = a_z = 50$ nM h^{-1}, $l_z = 10$ nM h^{-1}, $b = 10$ nM^{-1}, $C = 10$ [dox] h^{-1}, $d_x = d_z = 0.12$ h^{-1}, $d_y = 1.2$ h^{-1}, $K = 50$ nM, $h = 3.5$, $\theta = 0.44$ nM, $n = 4$, $k = 0$ h^{-1} for NR and NF strains, $a_x = 43$ nM h^{-1} and $k = 200$ h^{-1} for NRpump and NFpump strains, and similarly, with the exception of $a_x = 43$ nM h^{-1} and $k = 10$ h^{-1}, for NRpumpmutant and NFpumpmutant strains.

(increased inducer-sensitivity). Thus, both effects together (efflux pump function and reduced TetR expression) fully explain all of the observed the dose−response changes.

Discussion and Outlook. We studied the effect of introducing an efflux pump into the previously characterized,[16] tetracycline analogue-inducible negative regulation (NR) and negative feedback (NF) synthetic gene circuits in *Saccharomyces cerevisiae*. We found that introducing *PDR5::GFP*, a multidrug resistance pump-reporter gene fusion, changed the dose−responses of the two gene circuits in both intuitively expected and unexpected ways. Namely, we intuitively expected that both NRpump and NFpump should be less inducer-sensitive than their nonpump counterparts, considering that Pdr5p can exclude tetracycline family molecules from the cell and thereby decrease intracellular inducer concentration.[25−27] This was indeed true at high induction, but not in general. These surprising results implied the existence of additional causes that modulate the dose−responses of NRpump and NFpump in unexpected ways.

To uncover the causes of the unexpected effects, we turned to computational modeling. Interestingly, when we reduced the regulator synthesis rate, our simulations fully reproduced the dose−response changes we observed experimentally (Figures 2C and 3C).

Therefore, we identified two potential causes that jointly resulted in the NRpump and NFpump dose−response changes: efflux pump function and regulator level reduction. To determine the effect of efflux pump function, we created NRpumpmutant and NFpumpmutant strains bearing *PDR5* mutants with compromised efflux pump function. The dose−responses of NRpumpmutant and NFpumpmutant strains only captured one aspect of each dose−response change, indicating the insufficiency of pump function to fully explain the observations. Thus, to confirm the computationally predicted role of altered TetR levels, we measured regulator expression in all the strains by fusing mCherry to TetR. The experimental measurements confirmed that TetR had lower expression level in all other

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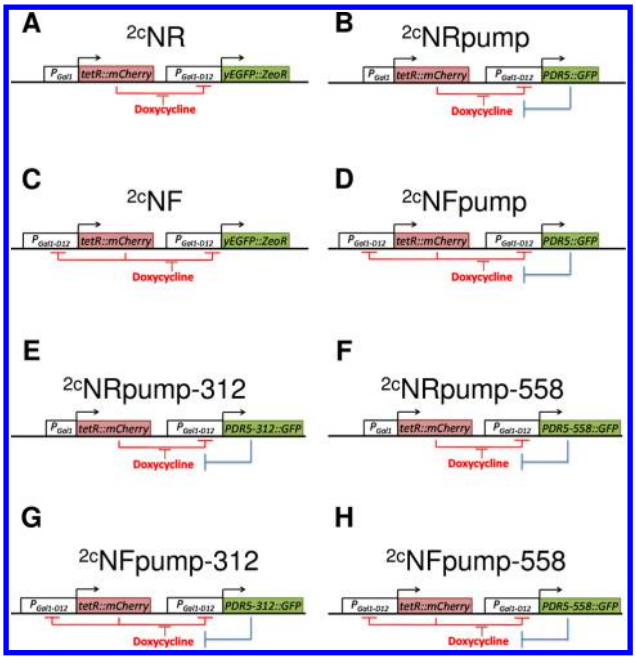

Figure 7. Regulation schemes for the 2-color (2c) gene circuits. These strains are identical to the corresponding single-color versions (NR, NRpump, NRpumpmutant, NF, NFpump, NFpumpmutant), except for the regulator gene, which is a bifunctional *tetR::mCherry* fusion for two-color strains. Design schemes for (A) ^{2c}NR; (B) ^{2c}NRpump; (C) ^{2c}NF; (D) ^{2c}NFpump; (E) ^{2c}NRpump-312; (F) ^{2c}NRpump-558; (G) ^{2c}NFpump-312; and (H) ^{2c}NFpump-558.

strains (whether or not Pdr5p was functional) compared to NR and NF strains, in agreement with computational predictions. Lower TetR level in these strains weakened the repression of *PDR5* transcription, causing increased inducer sensitivity, in agreement with previous observations.[37]

The mechanism reducing TetR protein expression (Figure 8) is still unclear and needs further investigation. Pdr5p protein levels did not affect regulator expression since TetR::mCherry level remained the same, although Pdr5p protein levels increased with doxycycline in ^{2c}NRpump strains (Figure 8). Likewise, fluorescence measurements in NRpumpmutant and NFpumpmutant strains demonstrated that Pdr5p efflux activity did not reduce TetR levels. One possibility is that the integration of the *PDR5* gene upstream of *tetR*'s promoter impaired *tetR* transcription. This may have occurred only in *PDR5*-containing strains because the *PDR5* gene is over 5 kb long while the *zeoR* gene (in NR and NF) is only ~1 kb long. Another possibility is that epigenetic modifications (for example, methylation) of *PDR5* affects general transcription factor binding to *tetR*'s promoter. Finally, growth rate differences between NR and NRpump as well as NF and NFpump strains may have also altered TetR concentrations[38−42] (Figure S6).

To our knowledge, how an efflux pump alters the behavior of its synthetic or natural regulatory network has not been characterized experimentally or mathematically in a eukaryote. A related study[43] used mathematical models to simulate the dynamics of natural interlinked negative and positive feedback loops that control an efflux pump in *E. coli*. Therefore, our findings and methods may be useful for predicting and understanding expected and unexpected consequences of modifying natural and synthetic gene networks that regulate an efflux pump and/or other active target genes. In general, we expect that our methods will facilitate quantitative understanding of how the dose−responses and dynamics of efflux pump-regulating gene circuits will change. For example, genetic toggle switches might require higher inducer concentration to flip when they are controlling an efflux pump. Efflux pumps may also alter genetic oscillator dynamics, by creating secondary feedback on top of the primary delayed feedback needed to generate oscillations.[14] On the other hand, introducing *PDR5* into bistable networks with positive autoregulation may result in a new way to build oscillators.

Our findings suggest that the presence of multidrug resistance pumps in natural or synthetic gene networks can have predictable and unexpected consequences. First, pump terms similar to the ones we used should be generally applicable to model the effect of inducer removal from the cellular interior. In many cases, adding such terms to existing gene network models should be sufficient to predict their altered behavior. However, as the yeast gene circuits indicate, pump-encoding genes can have additional effects that are difficult to predict in advance. Nonetheless, even in these cases, mathematical modeling should help identify the causes of such additional changes. The methodology should be analogous to what we used above: first, test if including a pump term is sufficient to capture the altered behavior. If not, then perform a parameter scan to identify where the additional changes originate.

Besides new insight into altered gene circuit behavior, this study has numerous applications. The ability to precisely control Pdr5p expression using synthetic gene circuits with low noise (such as NF) may allow novel applications and quantitative studies of biological processes that involve efflux pumps. For example, recent work has shown that an efflux pump increased biofuel production in *Escherichia coli* by exporting biofuel molecules outside of the bacteria and reducing their toxicity.[21] Also, negative feedback regulation of an efflux pump was found to increase the tolerance to a certain biofuel molecule, thereby improving its production rate.[44] Similarly, NFpump can regulate the secretion of intracellular biofuel molecules precisely, maintaining optimal balance between cellular fitness and productivity. Furthermore, combining synthetic gene circuits with biosensors could help cells detect certain molecules and respond automatically. For example, a recently engineered biosensor in yeast cells is able to detect steroid hormones.[27] An automatic system for production of steroids could be built by adding a biosensor to synthetic gene circuits with pumps to introduce feedback into hormone systems. As a result, different concentrations of intracellular steroids will automatically result in appropriate PDR5 expression levels to maintain hormone production at the desired level.

In addition to regulating biomaterial production, *PDR5*-containing gene circuits can also be used to advance basic research. A group of Pdr proteins in *Saccharomyces cerevisiae* has been shown to affect aging.[45] The NFpump gene circuit can tune the expression of such genes to study the aging process more precisely. Our system can also facilitate research on pump-mediated multidrug resistance (MDR). Earlier studies showed that transcriptional noise aided survival in stressful environments/conditions.[46−50] Thus, one cause for drug resistance could be MDR gene expression noise. Since the NF gene circuit reduces transcriptional noise, using NFpump and NRpump to independently control MDR pump levels and variability enables studying how cells survive drug treatment, how drug resistance evolves, and possibly, how to prevent it.

METHODS

Synthetic Gene Circuit Construction. Each synthetic gene circuit we used consisted of two parts originating from separate

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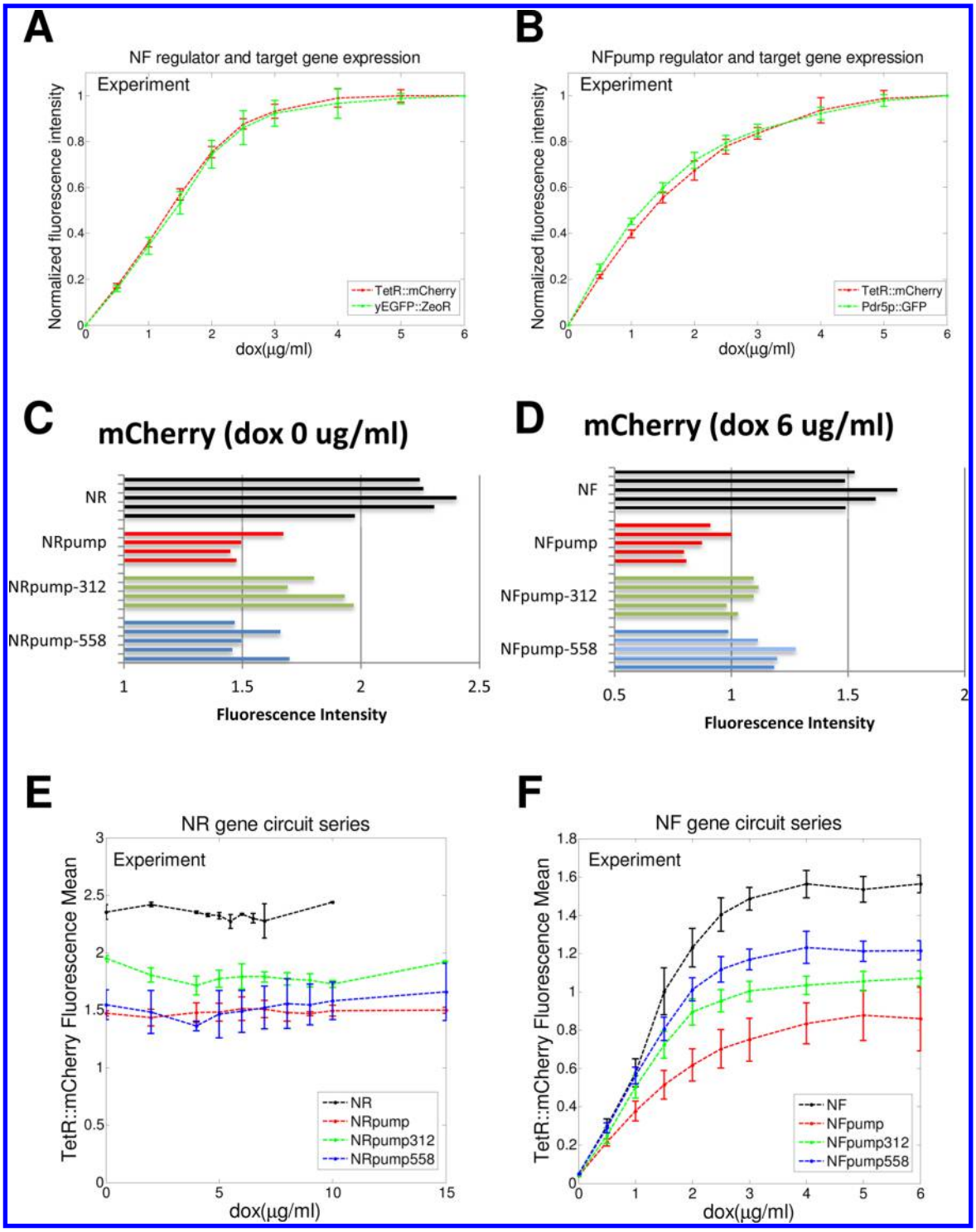

Figure 8. Experimentally measured TetR::mCherry expression levels in the NR and NF clones is higher than in other clones. (A) Experimentally measured mean TetR::mCherry and yEGFP::ZeoR fluorescence intensity dose−responses in the NF strain. (B) Experimentally measured mean TetR::mCherry and PDR5::GFP fluorescence intensity dose−responses in the NFpump strain. (C) Experimentally measured TetR::mCherry fluorescence means of NR, NRpump and NRpumpmutant strains for individual clones. These data indicate that repressor expression is consistently lower in "pump" strains, as predicted computationally. (D) Experimentally measured mean TetR::mCherry fluorescence intensities of NF, NFpump and NFpumpmutant strains for individual clones. These data indicate that repressor expression is consistently lower in "pump" strains, as predicted computationally. (E) Experimentally measured dose−responses of mean NR, NRpump and NRpumpmutant TetR::mCherry fluorescence intensities. (F) Experimentally measured dose−responses of mean NF, NFpump and NFpumpmutant TetR::mCherry fluorescence intensities. Data shown in panels (E) and (F) was an average of 3 replicates, indicating that repressor expression was higher in NR and NF clones than in any other clones at all doxycycline doses.

plasmids: the target gene (a bifunctional reporter-efflux pump fusion) and the regulator gene (Figure 1). We obtained the *PDR5::GFP* gene fusion (the target gene) by PCR amplification from whole-genome extraction of the GFP-tagged yeast library[51]

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and cloned it into the pRS4D1 integrative yeast plasmid, which was used to build the NRpump and NFpump gene circuits,[46,49] and chromosomally integrated into the *GAL1-GAL10* locus as previously described.[16,52] In each NRpump558/312 and NFpump558/312 version, a single nucleotide mutation was introduced into the *PDR5* gene sequence before following the same procedure for yeast integration. In the S558Y mutant, the C was changed to A at nucleotide position 1673 in the *PDR5* gene. In the G312A mutant, the G was changed to C at nucleotide position 935 in the *PDR5* gene.

Strains and Medium. The haploid *S. cerevisiae* strain YPH500 (*α*, *ura3−52*, *lys2−801*, *ade2−101*, *trp1*Δ63, *his3*Δ200, and *leu2*Δ1) (Stratagene) was used as a parental strain. The reporter plasmid was integrated into the native *GAL1-GAL10* locus first. Then the regulator plasmid was integrated into the *ampR* gene in the reporter plasmid by homologous recombination. The transformation procedure was described previously.[53] Strains with single integration were selected by PCR and flow cytometry. All cell cultures were grown in synthetic drop-out (SD) medium with appropriate selection markers and 2% galactose.

Reporter Gene Expression Measurement. Strains were streaked on agar plates with SD medium and 2% glucose, and grown at 30 °C for 2 days. Single colonies were selected from the plates and incubated overnight in liquid SD medium supplemented with 2% galactose and appropriate selection markers at 30 °C. Then the cell cultures were suspended into fresh SD medium of the same composition with starting cell density of 5×10^5 cells/ml (determined using a NexCelom Cellometer Auto M4). Cells were resuspended regularly every 12 h with the same starting cell density into fresh medium of the same composition over the entire length of the experiment. In dose−response experiments, cells were suspended in SD medium with 2% galactose, appropriate selection markers and increasing doxycycline concentrations, which varied from strain to strain. Flow cytometry was used to measure reporter gene expression every 24 h after reporter gene expression became stable (the fluorescence intensity histogram did not change from 1 day to the next). A gate based on Forward Scatter (FSC) and Side Scatter (SSC) was used to filter out nonliving cells, doublets and cell debris.

Data Analysis. Flow cytometry data was processed in Matlab by the Mathworks, Inc. FSC, SSC and fluorescence intensity for all living cells were extracted and used to assign gates to select the most concentrated cell population in each sample to exclude external noise, such as difference in cell size and shape. Then fluorescence intensity for gated cells was extracted and read within the specified gate. Cells with log fluorescence deviating >3 standard deviations from the arithmetic mean were considered outliers and were discarded from the analysis (based on our experience, these were rare cells left over from previous samples). Arithmetic mean and coefficient of variation (CV), defined as the standard deviation divided by the mean of fluorescence intensity, were calculated for gated cells for each sample. Mean and CV was then plotted for each dose−response assay.

Computational Modeling and Data Fitting. On the basis of the previously published mathematical model,[16] we established differential equations to simulate the dose−response of cells with an NR gene circuit regulating a pump as follows:

$$\frac{dx}{dt} = a_x F_x(x) - bxy - dx$$

$$\frac{dy}{dt} = C - bxy - fy - \frac{ky^h z}{(K^h + y^h)}$$

$$\frac{dz}{dt} = l_z + a_z F_z(x) - dz$$

Here the variables x, y, and z correspond to free intracellular repressor (TetR), inducer (doxycycline), and pump/reporter (Pdr5p::GFP) protein concentrations, respectively. C is a control parameter proportional to extracellular inducer concentration. Repressor protein synthesis rate is a_x, pump/reporter protein synthesis rate is a_z, leaky pump/reporter protein synthesis rate is l_z, inducer−repressor association rate is b, the rate of dilution due to cell growth is d, the combined rate of inducer dilution and degradation is f, the inducer concentration at which pump activation is half-maximal is K, the pump Hill coefficient is h, and rate of Pdr5p mediated inducer efflux is k ($k = 0$ for the NR circuit, and $k > 0$ for the NRpump circuit).

The functions $F_x(x)$ and $F_z(x)$ are Hill functions that respectively describe the repressor dependence of protein synthesis from the upstream and downstream promoters:

$$F_x(x) = F_z(x) = \frac{\theta^n}{\theta^n + x^n}$$

Here, θ is the induction threshold and n the corresponding Hill coefficient ($F_x = 1$ for NR and NRpump circuits, and $F_x = F_z$ for NF and NFpump circuits).

These parameters were obtained by fitting the model to experimental data. An analytic solution for z as a function of the control parameter C is available for the NR circuit case (see ref 28 for details):

$$z(C) = \frac{l_z + a_z F_z(x(C))}{d}$$

where

$$x(C) = \frac{(a_x b - df - bC) + \sqrt{(a_x b - df - bC)^2 + 4bda_x f}}{2bd}$$

The analytic equation was fit to the experimental data for the NR strain using Matlab's curve fitting application (fit options: Nonlinear Least Squares, algorithm: Trust-Region, $r^2 = 0.9991$) guided by biologically realistic parameter lower and upper bounds (Figure S2). Fitting for the other strains was based on these parameters, tuning additional parameters by hand to fit the experimental data. Computational results for the ODE model were obtained using Malab's ode45 differential equation solver and were used together with experimental data to guide stochastic simulations.

The system of ordinary differential equations was converted to the following reaction equations and simulated using the Gillespie stochastic simulation algorithm:[54,55]

$$\varnothing \xrightarrow{\kappa_x} x$$

$$x \xrightarrow{\delta_x} \varnothing$$

$$\varnothing \xrightarrow{\kappa_y} y$$

$$y \xrightarrow{\delta_y} \varnothing$$

$$x + y \xrightarrow{\lambda} \varnothing$$

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$$\varnothing \xrightarrow{\kappa_z} z$$

$$z \xrightarrow{\delta_z} \varnothing$$

where $\kappa_x = a_x F_x$, $\delta_x = d$, $\kappa_y = C$, $\delta_y = f + ky^{-1}(y^h/K^h + y^h)z$, $\lambda = b$, $\kappa_z = l_z + a_z F_z$, and $\delta_z = d$. All simulation results were obtained from 20 realizations of 1000 cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00154.

Supporting figures and tables. (PDF)

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Author Contributions

JD, DC, CP and GB designed research; JD, DN, and ZB performed experiments; JD analyzed the data; DC developed mathematical models and performed simulations; and JD, DC and GB wrote the paper.

Notes

The authors declare no competing financial interest.

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