Engineering Biology

Research Article

IET Journals
The Institution of Engineering and Technology

Engineered gene networks enable non-genetic drug resistance and enhanced cellular robustness

eISSN 2398-6182
Received on 21st April 2019
Revised 29th July 2019
Accepted on 17th September 2019
doi: 10.1049/enb.2019.0009
www.ietdl.org

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Abstract: Drug resistance complicates the treatment of cancer and infectious diseases, and often arises from the elevated expression of a gene that neutralises or reduces drug activity. To investigate this and other expression-based mechanisms of drug resistance, the authors engineered a set of gene regulatory networks in the eukaryotic model organism *Saccharomyces cerevisiae* to control a homologue of the cancer-related human multidrug resistance gene *MDR1*. Using this system, they explored experimentally how different gene regulatory network features, also called genetic network motifs, contribute to gene expression dynamics and cellular fitness. They observed that coherent feedforward and positive feedback motifs enable rapid and self-sustained activation of gene expression, and enhance cell survival in the presence of a cytotoxic drug. These observations underscore that genetic network motifs can be critical for drug resistance and that genetic network engineering can be used to enhance cellular tolerance to cytotoxins or other environmental stresses.

1 Introduction

Non-genetic drug resistance is linked to mechanisms of phenotypic plasticity and diversification, and is advantageous in fluctuating or unpredictable environments [1, 2]. A better understanding of these mechanisms could not only help circumvent resistance in clinical contexts, but also inform the engineering of industrial microorganisms to better tolerate cytotoxins and environmental stress.

Different drug resistance mechanisms benefit populations in different contexts [3]. Generally, mechanisms involving pre-existing phenotypic diversification are thought to be favoured when environmental conditions change frequently and unpredictably, and when stresses are more severe [3, 4]. Alternatively, mechanisms involving changes in gene expression in response to an environmental signal are favoured when conditions change less frequently, and when stresses are less severe [5].

Bet-hedging is one example of a mechanism for phenotypic diversification and drug resistance in fluctuating environments [6]. It involves a cell population that 'hedges its bets' by randomly relegating a small fraction of cells to a specialised phenotypic state that is resistant to adverse growth conditions [7, 8]. In this scenario, the cells that have switched to the resistant state are able to withstand the environmental assault and reseed the original population once conditions improve. Stochasticity, or 'noise', in gene expression, is a source of the phenotypic variability [9, 10] that is a critical component of bet-hedging [8, 11].

Gene expression noise can also contribute to drug resistance in the absence of clearly distinct phenotypic states [12–16]. In this case, minute differences in the expression of a resistance gene provide a broad spectrum of resistance phenotypes upon which selection can act. Cells in a gene expression state that allows them to survive may then seed a drug-resistant population provided that the beneficial levels of gene expression are heritable to a sufficient degree [13, 17].

The non-genetic and epigenetic factors underlying the formation, maintenance, and inheritance of gene expression states are not fully understood. There are, however, many diverse mechanisms that can, and likely do, contribute to non-genetic heterogeneity and thus non-genetic drug resistance [12, 13, 18–20]. We believe this to be true because only two conditions need to be met: (i) a cell must be able to temporarily express a phenotype with enhanced drug tolerance, and (ii) this state must be at least transiently heritable. Moreover, mechanisms leading to non-genetic heterogeneity would likely be subject to positive selection pressure and, therefore, conserved and refined on evolutionary time-scales.

One of the most common mechanisms of drug resistance found in nature involves transmembrane efflux pumps. These pumps recognise a wide range of compounds inside cells and export them to the extracellular environment [21, 22]. Among several classes of transmembrane pumps, members of the ATP-binding cassette (ABC) family are highly conserved [23, 24] and relevant to human disease [25]. For example, a member of the ABCB subfamily, ABCB1 (MDR1/p-glycoprotein), is responsible for multiple drug resistance (MDR) of tumours to chemotherapy [25–31].

The expression of *MDR1* is regulated by a myriad of factors, making it difficult to use this system to study how non-genetic mechanisms might contribute to drug resistance. To address this issue, past studies have focused on the expression of the *MDR1* homologue, the pleiotropic drug resistance (PDR) gene *PDR5*, in the eukaryotic model organism *Saccharomyces cerevisiae*. Critically, it has been predicted that the transcriptional regulatory network controlling the expression of *PDR5* and *MDR1* contains certain basic building blocks, or genetic network motifs [32], that may enable the development of non-genetic resistance [33].

To test this prediction, we here use genetic network engineering to recreate and isolate motifs found in the *PDR5* and *MDR1* transcriptional regulatory networks. This allows us to investigate if and how these motifs might enable non-genetic drug resistance and enhanced robustness to cytotoxins and environmental stress. Specifically, our investigations demonstrate that a network containing a feedforward loop (FFL) and a positive feedback loop (PFL) enhances the cells' ability to subsist following drug treatment. While the FFL enables the rapid induction of *PDR5* expression necessary for short-term survival, the FFL and the PFL allow prolonged 'memory' of a transient activating signal to enable persistent drug resistance. These observations confirm how gene regulatory network 'wiring' impacts cellular drug resistance, thus

revealing design principles that can be exploited by synthetic biologists, or contribute to a better understanding of the evolution of drug resistance networks.

2 Methods and materials

2.1 Yeast strains

Yeast strains were created in the BY4742 background [34], lacking the native *PDR5* gene. The synthetic constructs expressing GEV (Gal4 DNA binding domain, estradiol receptor, VP16 activation domain) [35], and rtTA (reverse tetracycline-controlled transactivator) [36] were integrated into the yeast genome at the *GAL4* and the *ADE4* loci, respectively. A yEGFP (yeast-enhanced green fluorescent protein) reporter expression cassette or a PDR5-yEGFP fusion reporter expression cassette was integrated into the *ADE2* locus.

Strains were created by integrating synthetic cassettes into the genome through a combination of polymerase chain reaction (PCR), DNA splicing by overlap extension PCR, and transformation-associated recombination (TAR) [37, 38]. Briefly, DNA sequences were cloned by PCR to add overhangs of 20–50 base pairs, homologous to either an adjacent DNA sequence in the synthetic cassette or the region of the yeast genome into which the entire cassette would be inserted. Adjacent DNA pieces were combined into a complete synthetic DNA cassette by overlap extension PCR and completed cassettes were integrated into the yeast genome by homologous recombination following transformation.

Standard PCRs were prepared with 10 μM custom primers (Invitrogen), 200 μM dNTP mix (N04475, New England Biolabs), 1 unit Phusion High Fidelity Polymerase (F-5305, Thermo Scientific), and 1X High Fidelity Buffer (F-518, Thermo Scientific). Approximately 100 ng of genomic DNA or 10 ng of plasmid DNA was used when cloning from a genomic or plasmid template, respectively.

Overlap extension PCR was performed by completing ten pre-cycles of PCR in the absence of primers, using an annealing temperature calculated based on the melting temperature of the overlapping regions of homology, in order to splice adjacent DNA pieces without amplification. This was followed by 20 cycles of PCR using corresponding primers.

Transformations were performed based on the standard Gietz and Schiestl lithium acetate (LiAc) transformation protocol [39]. Briefly, cells in mid-log-phase growth were pelleted, washed once with ddH_2O and then with 100 mM LiAc. Cells were aliquoted into tubes of $\sim 10^8$ cells each and resuspended in the transformation mixture composed of 33% w/v polyethylene glycol (PEG), 100 mM LiAc, 0.1 mg salmon sperm ssDNA, and 200 ng of each DNA part. After a 45 min heat shock at 42°C, cells were either plated immediately on selective media (if transformed with an auxotrophic marker), or pre-incubated in 1 ml YPD for 1 h at 30°C before plating on drug plates (if transformed with a drug resistance marker). Positive transformants were validated by PCR using one primer internal to the inserted DNA part and one primer in the flanking genomic region.

2.2 Growth conditions and media

Cells were grown in liquid YP media (2% w/v yeast extract, 4% w/v bacteriological peptone) supplemented with 2% w/v dextrose (YPD) or galactose (YPgal). Solid media plates were made similarly using YP supplemented with 2% w/v glucose or galactose and 4% w/v agar. For synthetic network induction, doxycycline (D9891, Sigma) was dissolved in ddH_2O for a stock concentration of 1 mg/ml and β-estradiol (E8857, Sigma) was dissolved in ddH_2O for a stock concentration of 5 mM. Both stock solutions were stored at −20°C. Synthetic transcription factors were induced by subculturing cells in mid-log-phase growth in YPD supplemented with the required concentration of either or both small molecule inducers. Unless otherwise indicated, transcription factor activity was induced for 6 h.

For drug treatment, cycloheximide (No. C-6255, Sigma Chemical Company) was dissolved in ddH_2O to a stock concentration of 1 mg/ml. The stock solution was diluted in liquid or solid-phase media to the desired concentration. For multi-day experiments, cultures were maintained in the log phase growth and diluted every 12 h in fresh media. All cultures were grown at 30°C, and liquid cultures were agitated using a tissue culture rotator.

2.3 Spot assays

Cells were grown to reach mid-log phase growth at the time of the experiment. Cultures were diluted to an OD_{600} of 0.1 and then used to prepare four 1:10 serial dilutions in a 96-well plate. The diluted cultures were spotted on agar plates with specific growth conditions using a 48-pronged hand pinner. Plates were incubated until at least one spot had reached full growth, at which time pictures were taken.

2.4 Flow cytometry

Cultures were grown to reach mid-log phase growth at the time of the experiment and diluted 1:10 in 50 mM sodium citrate buffer. Experiments were performed on a BD FACSCelesta (BD Biosciences) flow cytometer, exciting yEGFP with a 488-nm laser and detecting with a 530/40 filter. Flow Cytometry Standard (.fcs) files were analysed with Matlab® 2015a using a custom script to extract and analyse data. Collected events were gated based on forward and side scatter (~40% of events) in order to minimise extrinsic variability. Fluorescence data is presented in arbitrary units and cell count distributions are normalised by dividing the number of cells in each binned population by the total number of cells in the sample.

3 Results

3.1 Genetic network engineering

To investigate the impact of specific regulatory interactions on network dynamics within the natural *PDR5* network (Fig. 1*a*) [40], we dissected the network into its component parts and identified four distinct motifs: a direct activation (DA) motif, a cascade (CAS), an FFL and a PFL [33]. Interestingly, the human *MDR1* gene is similarly regulated by a network that combines the FFL and PFL network motifs [41].

The four network motifs in the PDR5 network were identified previously [33] and arise from the binding of two transcription factor proteins encoded by the genes *PDR1* and *PDR3* to the *PDR3* and *PDR5* promoters [40, 42–44]. The DA motif is produced by the binding of Pdr1 to sites within the *PDR5* promoter, and the CAS motif is produced by a regulatory chain in which Pdr1 activates *PDR3* transcription by binding to the *PDR3* promoter, and Pdr3 activates *PDR5* transcription by binding to the *PDR5* promoter. The FFL motif is produced by combining the DA and CAS motifs, and the PFL is produced by the binding of Pdr3 to the *PDR3* promoter.

The synthetic equivalent of the native *PDR5* network is illustrated in Fig. 1*b*. In this network, the *PDR5* gene is fused to a yeast-enhanced green fluorescent protein (yEGFP), and the expression of this variant, labelled *PDR5** in Fig. 1*b*, is controlled by two synthetic transcriptional activators: the GEV fusion protein (composed of the DNA binding domain from the yeast Gal4 protein, the human oestrogen receptor and the viral VP16 activation domain [35]), and the reverse tetracycline-controlled transactivator, rtTA (composed of the VP16 domain fused to a modified DNA binding domain from the bacterial tetracycline repressor [36]). These synthetic transcription factors are convenient because their DNA binding activity can be modulated by the small molecules β-estradiol and doxycycline, respectively, and because different network variants can be obtained by eliminating the well-defined GEV and rtTA DNA binding sequences from the synthetic promoters driving the expression of *rtTA* and *PDR5**. In the rest of this paper, we will refer to simplified network representations similar to that illustrated in Fig. 1*c*, which represents the full FFL + PFL network.

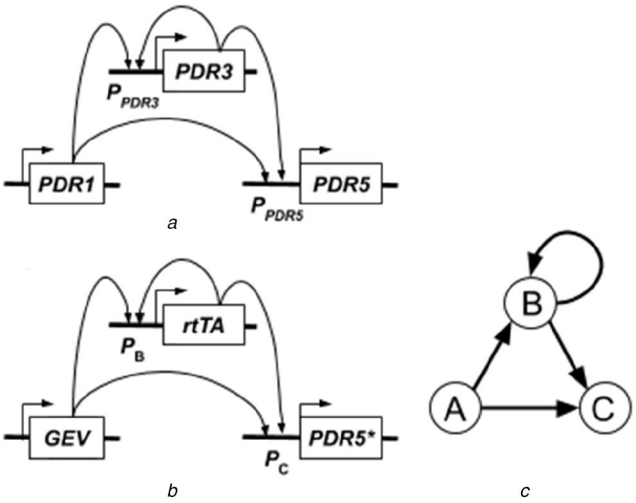


Fig. 1 *Network schematics*

(a) Yeast pleiotropic drug resistance network, ***(b)*** Synthetic drug resistance network constructed for this study, ***(c)*** Simplified representation of network topology. Arrows indicate protein-DNA binding or gene activation. Abbreviations: PDR1, PDR3, PDR5 are the Saccharomyces Genome Database standard pleiotropic drug resistance gene names; P_{PDR3} and P_{PDR5} refer to the promoter regions of the PDR3 and PDR5 genes, respectively; GEV and rtTA are the β-estradiol and doxycycline inducible synthetic transcriptional activators, respectively; PDR5* is a gene variant obtained by fusing the sequences of the PDR5 gene and a fluorescent reporter gene; P_B and P_C refer to the promoter regions of the rtTA and PDR5* genes, respectively

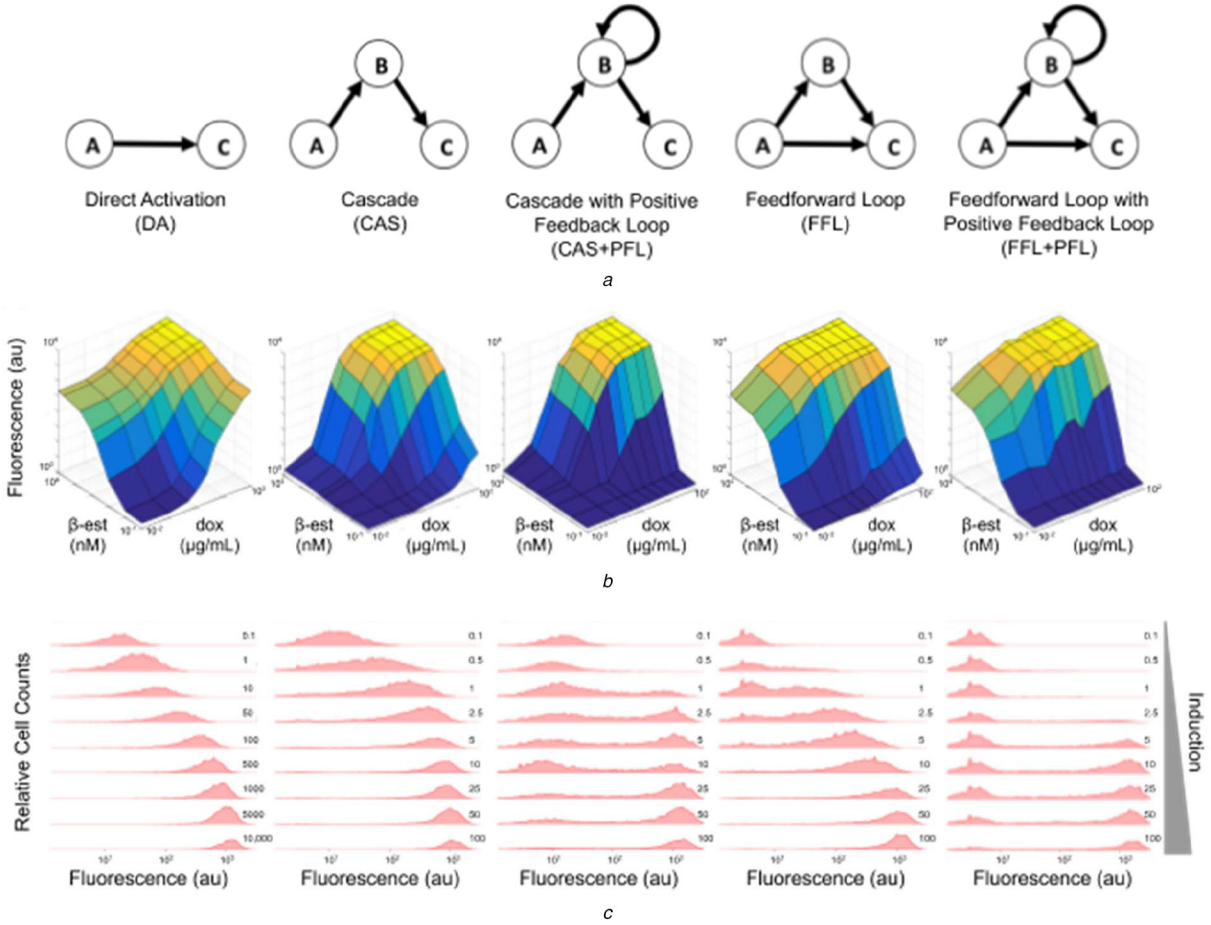


Fig. 2 *Network characterisation*

(a) Simplified network representations and their names. In the diagrams, 'A' represents GEV, 'B' represents rtTA, and 'C' represents yEGFP or PDR5-yEGFP, ***(b)*** Dose-response surfaces corresponding to the networks shown in (a), obtained from population-averaged fluorescent reporter gene expression at different concentrations of β-estradiol (β-est) and doxycycline (dox). The DA surface was obtained from a strain in which GEV and rtTA both directly and independently activate reporter expression. A strain with only the single DA from GEV was used for subsequent experiments, ***(c)*** Representative fluorescence distribution profiles (relative cell counts) illustrating the dose-dependent cell-to-cell variability in reporter gene expression at increasing induction. Profiles for the CAS, CAS + PFL, FFL and FFL + PFL networks are shown for 10 nM β-est and increasing dox concentration (μg/ml). The profiles for the DA network are shown for increasing β-est (nM)

3.2 Network characterisation

To study the effects of individual network connections on reporter gene expression in the absence of selective pressure, we created strains in which the expression of *yEGFP* is controlled by a DA network, a CAS network, a CAS + PFL network, an FFL network, or an FFL + PFL network (Fig. 2*a*).

Reporter gene expression from each network variant was characterised by the induction of GEV and rtTA with varying concentrations of their respective small molecule inducers. The resulting dose-response surface plots, capturing the population-averaged gene expression levels at each combination of two inducer concentrations, are shown in Fig. 2*b*.

The dose-responses in Fig. 2*b* verify the expected properties of the networks. First, the CAS variants, with no DA connection between GEV and yEGFP, show no expression in the absence of doxycycline, as the second transcription factor, rtTA, cannot be activated. Second, the FFL variants, which contain a DA connection, approach saturation at high induction with β-estradiol even in the absence of doxycycline. Third, the PFL-containing network variants exhibit an ultrasensitive switch-like behaviour typical of systems with positive feedback.

The network variants also exhibit theoretically predicted differences in reporter expression at the single-cell level [32] (Fig. 2*c*). For example, networks containing a PFL display switch-like increases in expression in response to increasing inducer level, which is visualised as bimodally-distributed cellular gene expression. In contrast, for networks lacking a PFL, increasing induction causes a gradual shift of a unimodal distribution of cellular gene expression from low to high.

Interestingly, compared to the CAS-containing network variants, the FFL-containing variants display slightly higher reporter expression levels at identical inducer concentrations, presumably due to the combined effect of the direct and indirect activating signals.

3.3 DA enables rapid responses

The ability to express a resistance phenotype rapidly in response to cellular stress could explain the presence of a DA motif in the natural *PDR5* network [40]. To determine if the DA motif enables rapid induction, we compared reporter gene expression at different times following induction (Fig. 3). The induction of population-averaged reporter expression levels in the CAS and CAS + PFL strains was delayed by about 2 h compared to the FFL and FFL + PFL strains (Fig. 3*a*). This delay, which is quite striking when comparing single-cell reporter expression profiles (Fig. 3*b*), is likely due to the requirement for the CAS network variants to accumulate sufficient rtTA to induce reporter gene expression. Interestingly, the presence of a PFL appears to delay the response as well, presumably due to some of the expressed rtTA being sequestered by its own promoter.

3.4 DA facilitates drug resistance

To confirm the biological significance of the rapid transcriptional induction, we examined the effect of *PDR5* expression on cells exposed to the fast-acting cytotoxic inhibitor of protein translation, cycloheximide. A viability spot assay was performed using three strains: the wild-type parental strain, a derivative in which *PDR5* is deleted (*pdr5*Δ), and a *pdr5*Δ strain carrying the *PDR5*-yEGFP fusion gene under the control of the DA network. Uninduced cell cultures were spotted on rich-media agar plates containing cycloheximide and β-estradiol (induced) or cycloheximide alone (non-induced). The relatively low cycloheximide concentration of 0.05 μg/ml was chosen in order to slow growth without preventing it completely.

This spot assay demonstrates that *PDR5* expression is critical for cycloheximide resistance (Fig. 4*a*). While the wild-type strain is able to grow in the presence of the drug, the strain that lacks *PDR5* is unable to grow, and re-introducing *PDR5* under the control of the DA network restores viability only under inducing conditions. This shows that direct estradiol-mediated activation of *PDR5* expression is sufficiently rapid to protect cells against the cytotoxic effects of the drug.

To assess if DA of *PDR5* expression is necessary and sufficient for drug resistance, we repeated the assay, spotting uninduced DA, CAS, CAS + PFL, FFL, and FFL + PFL cells onto plates containing cycloheximide and β-estradiol (induced) or cycloheximide alone (non-induced). In these experiments, the plates also contain doxycycline to ensure that expressed rtTA is always active. Notably, *PDR5* is initially not expressed in any of the strains, as determined by pre-induction fluorescence distribution profiles that are similar to autofluorescence (data not shown), and survival is dependent on the activation of *PDR5* expression to protect cells from the lethal drug exposure.

Fig. 4*b* demonstrates that DA of *PDR5* expression is necessary and sufficient to protect cells. While the DA and FFL strains show normal growth when induced, the growth of the CAS strain is severely impeded. Similarly, when considering the addition of a PFL, only the FFL + PFL is viable, while the CAS + PFL, which lacks the DA connection, is not. None of the strains are able to grow in the absence of network induction (data not shown). It is

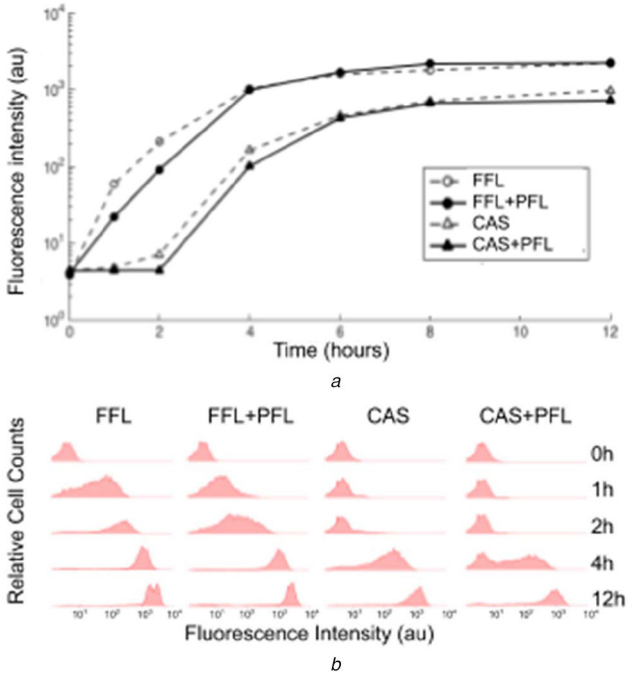


Fig. 3 *DA accelerates network induction*

(a) Time course of population-averaged reporter gene expression, presented in arbitrary units (au), following network induction with 5 μM β-estradiol and 5 μg/ml doxycycline, ***(b)*** Representative reporter expression profiles (relative cell counts) measured at different time points following network induction. Network topologies are outlined in Fig. 2*a*

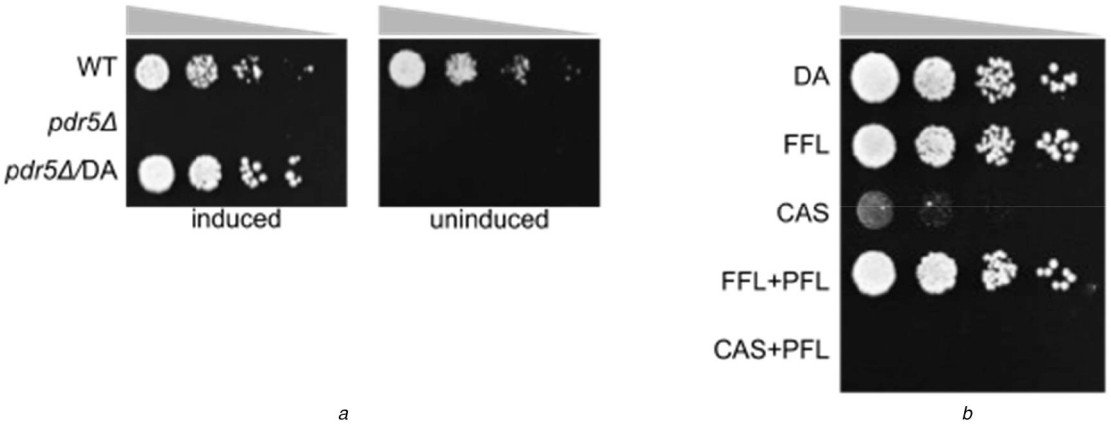


Fig. 4 *DA enhances drug resistance in spot assay experiments*

(a) PDR5 expression is required for resistance to cycloheximide treatment. Plates contain 0.05 μg/ml cycloheximide and 5 μM β-estradiol (induced) or no β-estradiol (uninduced), ***(b)*** DA of PDR5 expression is required for drug resistance from synthetic networks. Plates contain 5 μg/ml doxycycline, 0.05 μg/ml cycloheximide and 5 μM β-estradiol (induced). Abbreviations: Wild-type (WT); PDR5 gene deletion (pdr5Δ); pdr5Δ with a synthetic DA network driving PDR5-yEGFP (pdr5Δ/DA). Grey triangles represent the decreasing density of the spotted cell culture, 1:10 serial dilutions from OD_{600} 0.1 to 1×10^{-4}. Photographs were taken after 3 days of growth. Network topologies are outlined in Fig. 2*a*

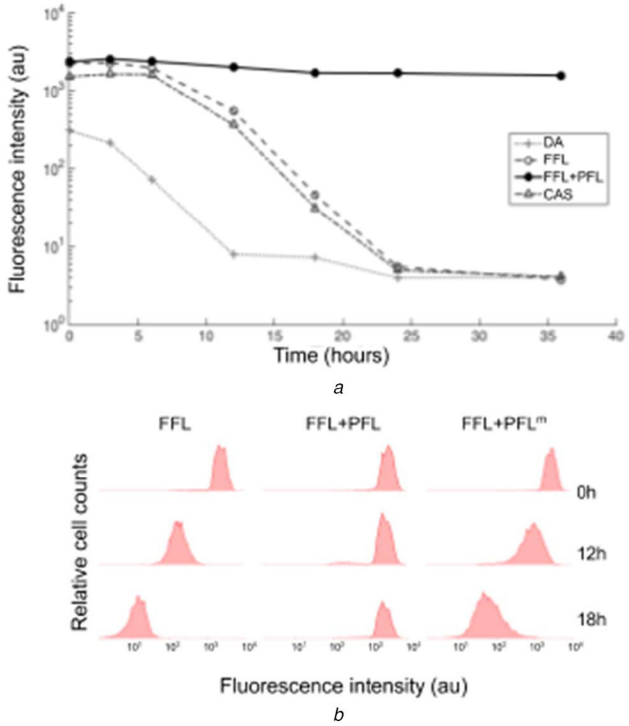


Fig. 5 *Prolonged activation of networks containing indirect activation or positive feedback in the absence of induction*

(a) Time course of fully induced (5 μM β-estradiol, 5 μg/ml doxycycline) population-averaged reporter expression, presented in arbitrary units (au), following the removal of β-estradiol, ***(b)*** Reporter expression profiles (relative cell counts) for FFL networks with or without a PFL at different time points following the removal of β-estradiol. The profiles labelled FFL + PFL^{m} were obtained from a genetic variant of the FFL + PFL network with weakened feedback. Network topologies are outlined in Fig. 2*a*

also interesting to note that the CAS + PFL appears even less viable than the CAS network, which is likely related to the slightly delayed response previously observed with the PFL (Fig. 3).

3.5 Indirect activation and positive feedback prolong cellular memory

An expression state that confers drug resistance must be maintained as long as cells are exposed to the drug. Correspondingly, in the case of prolonged exposure, population survival requires that the drug resistance is transmitted from one generation to the next. One way to ensure this is through mechanisms that endow cells with non-genetic memory [33].

To assess the contribution to non-genetic memory of individual network connections in the absence of drug exposure, we tracked reporter expression in induced DA, CAS, FFL and FFL + PFL strains following the transfer of cells from conditions containing high concentrations of β-estradiol and doxycycline to conditions containing no β-estradiol (Fig. 5*a*). As expected, reporter expression decreased rapidly in the DA strain culture and the yEGFP signal was almost indistinguishable from background fluorescence after 12 h. In contrast, the CAS and FFL strains maintained a high level of reporter expression for at least 6 h after the removal of inducer, with the signal detectable for about 24 h. The FFL + PFL strain maintained high reporter expression for the entire 36-hour duration of the experiment.

To confirm that the PFL endows FFL + PFL cells with long-term memory, we introduced targeted mutations into the promoter that controls the expression of the *rtTA* gene. Specially, we converted the thymine in position four of each *rtTA* DNA binding motif into adenine [45]. This is expected to reduce the strength of the PFL by decreasing the ability of rtTA to bind its own promoter.

The weakening of this binding site has a dramatic impact on memory. Fig. 5*b* illustrates the single-cell reporter expression profiles, obtained at different time points, of strains carrying the

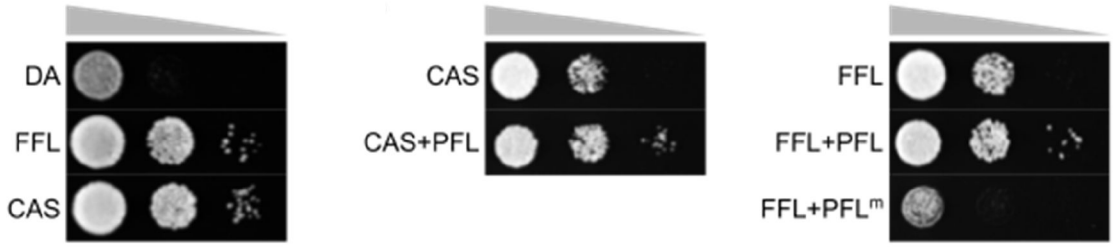


Fig. 6 *Indirect activation and positive feedback enhance drug resistance in spot assay experiments*
(a) Strains with direct and indirect activation networks, ***(b)*** Strains with indirect activation alone or combined with strong positive feedback, ***(c)*** Strains with feedforward activation alone or combined with original or mutated (*m*) positive feedback. Plates contain 5 μg/ml doxycycline, 0.025 (a) or 0.05 μg/ml (b, c) cycloheximide and no β-estradiol. Photographs were taken after 3 days of growth. Grey triangles represent the decreasing density of the spotted cell culture, 1:10 serial dilutions from OD_{600} 0.1 to 1×10^{-3}. Network topologies are outlined in Fig. 2*a*

FFL network, the mutated FFL + PFL^m network and the original FFL + PFL network. While the FFL + PFL strain maintains high reporter gene expression after the inducer, β-estradiol, is removed from the culture, the FFL + PFL^m strain has a noticeable loss of reporter expression after 12 and 18 h of growth without inducer. However, the expression is higher in the FFL + PFL^m strain compared to the FFL strain, suggesting that the feedback loop is weakened but still active.

3.6 Non-genetic memory enhances drug resistance

To confirm the biological significance of network-mediated non-genetic memory for drug resistance, we evaluated the impact of cycloheximide on the viability of the DA, CAS, FFL, FFL + PFL and the FFL + PFL^m strains using spot assays. In these experiments, cells were pre-induced in a condition with high concentrations of doxycycline and β-estradiol to ensure high *PDR5* expression prior to the spotting of these cells onto plates containing cycloheximide and doxycycline but no β-estradiol.

The results of these spot assays are shown in Fig. 6. Notably, strains were spotted onto multiple plates in a manner that allows direct comparison of the viability of the CAS and FFL strains to the DA strain (Fig. 6*a*), the CAS + PFL strain to the CAS strain (Fig. 6*b*), and the FFL + PFL and FFL + PFL^m strains to the FFL strain (Fig. 6*c*). We also note that the different strains had similar fluorescence distribution profiles immediately prior to plating (data not shown).

Comparison of the photographs in Fig. 6*a* shows that the strains carrying a network with an intermediate transcriptional regulatory step (the CAS and the FFL strains) grow faster than the strain where the intermediate step is missing (the DA strain). This provides direct evidence that the delayed reduction in *PDR5* expression in these strains has a biologically significant impact and protects cells from the toxic effects of drug exposure.

Similarly, a comparison of the photographs in Figs. 6*b* and *c* shows that the strains carrying a network with a positive feedback loop (the CAS + PFL and the FFL + PFL strains) grow faster than the strains in which this network motif is missing (the CAS and FFL strains). This provides direct evidence that positive feedback regulation of *PDR5* expression has a biologically significant impact in protecting cells from drug exposure. Interestingly, given the conditions in our experiments, the added protection provided by the PFL appears to be relatively minor compared to that provided by an intermediate transcriptional regulatory step.

A comparison of the photographs in Fig. 6*c* shows that the FFL + PFL^m strain has significantly reduced viability compared to the FFL + PFL strain suggesting that reducing the strength of the positive feedback regulation has a significant impact on drug resistance. While this is the expected result, the FFL + PFL^m strain also has reduced viability compared to the FFL strain. This is surprising because the mutations introduced into the promoter of the *rtTA* gene were expected to reduce viability by weakening the PFL but not eliminate it. The fact that the FFL has higher viability than the FFL + PFL^m strain suggests that the mutations have a detrimental impact on drug resistance beyond their impact on the PFL.

4 Conclusion

Synthetic biology provides tools to study fundamental properties of gene regulatory networks and their building blocks, the network motifs, in ways that are otherwise extremely challenging. As we have shown in this study, it is possible to create libraries of network variations in which connections between nodes are systematically added or removed, or the strength between network nodes is modulated.

In this study, we used synthetic biology to systematically examine how different network connectivities and motifs can contribute to drug resistance. We did this by recreating a gene regulatory network that is similar to the one regulating the expression of *PDR5* in yeast and of *MDR1* in humans [41].

The results of our study are summarised in Fig. 7, which lists the response rate, response duration, level of expression variability, and potential drug resistance mechanism for each network. We define two potential drug resistance mechanisms:

- *Responsive resistance mechanism* allows unprotected cells to respond to the presence of a cytotoxin or environmental stress by changing its phenotypic state to mitigate the negative consequences.
- *Preemptive resistance mechanism* allows protected cells to maintain a phenotypic state that mitigates the negative consequences of a cytotoxin or environmental stress.

With these definitions, networks with DA of a resistance gene can support responsive resistance, while those with indirect activation or positive feedback regulation can support the preemptive resistance mechanism. Networks with both mechanisms may act as substrates for evolutionary strengthening of one strategy over the other, depending on the degree of environmental fluctuation [46].

Preemptive resistance may be sufficient to provide permanent protection of a cell population. Under the right conditions, high variability in gene expression can cause the spontaneous emergence of a resistant subpopulation that is able to survive acute exposure to a drug or an environmental stress. This subpopulation may develop permanent resistance if the beneficial gene expression state is sufficiently long-lived to be passed from one generation to the next [13, 15, 33]. Indeed, the networks that regulate the expression of the drug resistance genes *PDR5* in yeast and *MDR1* in humans are known to involve the feedforward motif and the positive feedback motif [41].

In our opinion, and as pointed out by Brock, Chang and Huang a decade ago [14], networks that control the expression of drug resistance phenotypes may have profound importance for the understanding of chemotherapeutic resistance in cancer treatment. The regulatory network controlling *MDR1* expression contains motifs that facilitate non-genetic drug resistance and it seems plausible, if not likely, that these motifs contribute to the development of drug-resistant tumours in cancer patients. Indeed, in agreement with the Brock hypothesis [14], it was recently

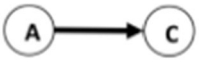
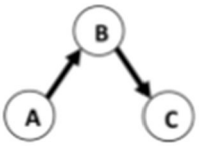
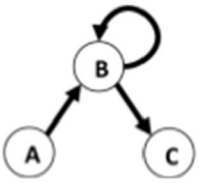
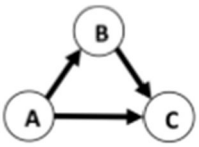
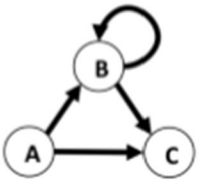
Network Name	Network Topology	Response Rate	Response Duration	Expression Variability	Resistance Mechanism
Direct activation		Fast	Short	Low	Responsive
Cascade		Slow	Medium	Medium	Preemptive
Cascade with positive feedback		Slow	Long	High	Preemptive (long-lived)
Feedforward		Fast	Medium	Medium	Responsive, Preemptive
Feedforward with positive feedback		Fast	Long	High	Responsive, Preemptive (long-lived)

Fig. 7 *Summary of network properties and drug resistance mechanisms*

reported that 'noisy' positive feedback networks in mammalian cells both facilitate the development of drug resistance in the absence of mutation and reduce the frequency of adaptive mutations [16].

We have shown that synthetic gene networks provide the rapid and prolonged responses critical for biologically relevant drug resistance. Although these networks are oversimplified as models of natural gene networks, our study demonstrates the biological relevance of simple gene network design principles, that may be helpful in understanding the evolution of natural gene regulatory systems, and informs approaches to engineer cells for robustness to cytotoxins and environmental stress.

5 Acknowledgments

This work was supported by an NSERC Discovery Grant for M.K., and NSERC Graduate Scholarships for B.C., I.J.R and A.A.

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