Gene regulatory networks are a mechanism for drug resistance

by

Brendan Camellato

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Abstract

Multidrug resistance has become a major issue in the treatment of both microbial infections and cancers. While genetically encoded drug resistance is fairly well understood, it cannot explain all observed cases of resistance, namely the ability of a subset of disease cells to persist in an otherwise susceptible population. This non-genetic resistance requires the heterogeneous expression of a drug resistance phenotype, which can be produced by certain gene regulatory network architectures. Two particular network motifs, the coherent feedforward loop (CFFL) and the positive feedback loop (PFL), have functional properties that implicate them in the development of non-genetic heterogeneity and response to changing conditions. Motivated by the observation that CFFL and PFL motifs are involved in the transcriptional regulation of multiple pleiotropic drug resistance (PDR) genes in yeast, it has been hypothesized that CFFLs and PFLs could contribute to the development of drug resistance. This hypothesis was based on model simulations and has not been tested experimentally. In this thesis, it is demonstrated experimentally that the *PDR5* gene is indeed expressed heterogeneously within an isogenic population of yeast cells, and that this cell-to-cell variability enables a subset of cells to persist drug treatment. While these observations agree with model predictions, it is also observed that the resistant phenotype occurs within a subset of cells that are morphologically distinct. This subpopulation has previously been linked to abnormal mitochondrial function, which cannot be ruled out as a likely cause of the observed drug resistance. To validate the hypothesis that CFFLs and PFLs contribute to drug resistance, the expression of the *PDR5* gene was placed under the control of synthetic gene regulatory networks constructed to contain different combinations of direct activation, indirect activation, and positive feedback. These networks are used to show that direct activation can provide a selective advantage enabling rapid responses, while indirect activation and positive feedback can provide a selective advantage by maintaining favourable gene expression states. These results demonstrate that a gene regulatory network combining CFFLs and PFLs can contribute to the development of drug resistance, and highlight plausible means by which cells can exploit certain network features to gain a fitness advantage.

Acknowledgements

I would like to first and foremost thank Dr. Mads Kaern for providing me with this opportunity to perform meaningful and exciting research and to develop as a scientist, supporting me tremendously the entire time.

My labmates Danny Salem, Hilary Phenix, Ian Roney, Kateland Simmons, and Nada Elnour were great friends as well as colleagues and deserve great thanks for providing me with utmost support, advice, and great discussions. I would also like to thank Afnan Azizi, who I unfortunately did not have the pleasure of working with, for laying much of the groundwork for this project. I am grateful as well to members of the Downey, Rudner, Baetz, Couture, and Blais labs for the friendships I've made and the support they provided.

I would like to thank Mila Tepliakova for her friendship and discussion, her hard work in keeping the lab running, and for teaching me how to do this job almost as well as she did.

I would like to also thank Dr. Adam Rudner and Dr. Alexandre Blais for guiding my work and providing me with very useful insight.

Finally, I would like to thank my family and my friends for supporting me and allowing me to pursue my studies, my interests, and my dreams.

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Introduction

"Strength lies in differences, not in similarities"

– Stephen Covey from "The 7 Habits of Highly Effective People"

1.1 Drug Resistance

Modern medicine largely relies on the use of pharmaceuticals to prevent, treat, and palliate a large number of diseases and disorders. A notable amount of biomedical research is thus focused on developing new drugs to target diseases that have no current treatment or for which the current treatment may be improved, often due to the development of drug resistance. Drug resistance, the means by which disease cells escape the effects of drugs designed to target them, has become a major issue in healthcare, in large part because of the incorrect or unnecessary prescription of drugs to treat most common illnesses (Ventola 2015). Aside from this, even diseases for which drugs are correctly prescribed can develop resistance, which poses a major problem when no other effective treatment exists (J. Davies and D. Davies 2010).

In general, resistance can be achieved by inactivating the drug, altering its cellular target, modifying metabolic pathways to circumvent the drug target, or by decreasing the concentration of the drug inside the cell (Schmieder and Edwards 2012). While each mechanism can effectively confer resistance, altering or circumventing the drug target usually involves mutation of a protein or re-

arrangement of a cellular pathway, which is generally expected to have associated adverse effects. It is thus likely that reducing intracellular drug concentrations is a more common mechanism of drug resistance. Intracellular drug concentration can be effectively decreased by preventing it from entering the cell, or by removing or metabolizing it after it has entered (Blair et al. 2015). While either of these mechanisms could adversely affect the cell by preventing from entering, or actively exporting, metabolites and other molecules required for cellular function, active transportation of molecules out of the cell is typically more selective and so should result in less of a detriment to the cell.

A classic mechanism of drug resistance involves increased active transport of small molecules by transmembrane efflux pumps of the ATP-binding cassette (ABC) transporter family. These transporters, which hydrolyze ATP as a source of energy to move ions and other small molecules against their concentration or electrochemical gradient (Alberts et al. 2002), play a variety of roles in normal cellular function in both prokaryotes and eukaryotes (Davidson and J. Chen 2004; Davidson, Dassa, et al. 2008; Dean, Hamon, and Chimini 2001). The mutation or altered expression of ABC transporters are also implicated in many different diseases (Pohl, Devaux, and Herrmann 2005) and in drug resistance (Glavinas et al. 2004). Notably, the transmembrane efflux encoded by the human ABCB1 (ATP-binding cassette subfamily B member 1) gene is responsible for multiple drug resistance (MDR) by exporting a variety of structurally unrelated compounds that are foreign to the cell (Lin and Yamazaki 2003). This gene encodes the permeability glycoprotein P-glycoprotein 1, also known as MDR1, whose normal physiological role is to remove xenobiotics from cells in metabolic organs, such as the intestine and liver, and into respective lumens or ducts to be excreted (Thiebaut et al. 1987). High expression of MDR1 has been observed in a variety of cancers and is directly linked to increased resistance to chemotherapy (Gottesman, Fojo, and Bates 2002).

Drug resistance, regardless of the underlying mechanism, can be broadly classified as predisposed/primary, acquired, or induced (Fernández and Hancock 2012). In predisposed resistance, cells express a resistance phenotype even in the absence of a drug and are thus minimally affected by the drug when it is encountered. In acquired resistance, cells become resistant through a perma-

nent genetic change affecting a gene implicated in drug resistance. These genetic mutations may occur spontaneously or may be caused by increased stress resulting from the presence of a drug (Venkatesan et al. 2017). In induced resistance, a drug resistance phenotype is expressed upon exposure to the drug, where different pathways may be generally or specifically responsible for sensing the drug, or the stress it causes, and instigating a phenotypic response.

The conventional paradigm is that drug resistance is often acquired and develops slowly and randomly as some cells accumulate mutations that confer increased protection against a particular drug (Woodford and Ellington 2007). The essence of this paradigm is that the development of drug resistance is an evolutionary process in which random mutations provide phenotypic heterogeneity upon which selection can act. While such permanent, genetically-coded resistance is undoubtedly an important issue, drug resistance may in some cases involve, or at least begin with, populations of cells that are genetically identical to one another (Spratt 2004; Merlo et al. 2006; Marusyk, Almendro, and Polyak 2012). For example, drug resistance linked to MDR1 can be caused by spontaneous mutation within the regulatory network that controls the expression of the ABCB1 gene (K. G. Chen and Sikic 2012), but could also be caused by non-genetic mechanisms. Identifying and studying these mechanisms will shed light on important evolutionarily principles underlying non-genetic heterogeneity and population survival, which may contribute to the development of strategies for the prevention or mitigation of drug resistance in the treatment of disease.

1.2 Non-genetic Heterogeneity

There are documented instances of drug resistance in isogenic populations, independent of genetic mutation (Brock, H. Chang, and S. Huang 2009; Niepel, Spencer, and Sorger 2009; Rotem et al. 2010). While the underlying mechanisms are relatively poorly understood compared to those caused by mutation (Levin and Rozen 2006), they involve what is known as non-genetic heterogeneity. Non-genetic heterogeneity generally describes the expression of different phenotypes within a genetically identical population, in contrast to genetic heterogeneity which describes

changes at the level of the genotype that lead to the expression of different phenotypes (S. Huang 2009).

One of the best studied examples of drug resistance caused by non-genetic heterogeneity is bacterial persistence. Through bacterial persistence, a subset of the population will spontaneously express a persister phenotype, allowing it to tolerate drug treatment by assuming a dormant state (K. Lewis 2010). This survival strategy is usually termed bet-hedging, and is characterized by the ability to dynamically switch between phenotypic states, combined with a fitness differential between the phenotypes (Veening, Smits, and Kuipers 2008). Through bet-hedging, some cells in the population adopt a resistance phenotype that allows them to survive drug treatment, but makes them less fit when growing in normal conditions, as would be the case for cells entering dormancy. By 'hedging its bets' in this way, there is a tradeoff between a population's absolute fitness in optimal conditions and the ability to persist through unexpected stresses.

The phenomenon of bacterial persistence highlights the importance of understanding not only how distinct phenotypic states can be expressed from the same genome but also how cells are able to switch between these states. The first notable description of this was put forth by Conrad Waddington in his proposed explanation of how development is regulated (Waddington 2014). Waddington uses the metaphor of a pebble rolling down a landscape filled with hills and valleys, where the pebble will eventually settle within one of the valleys. This metaphor of an 'epigenetic' landscape is used to represent all potential gene expression states of a cell where some (the hills) are unstable and others (the valleys) are stable. The landscape is defined by the genome, but the fate of each individual cell has a random component. As new valleys emerge, a cell may be directed towards one valley or another, and the fate of otherwise identical cells can begin to diverge at these bifurcation points. In other words, as different cells traverse the same landscape, each takes an independent path, drawn towards valleys and away from hills. Each bifurcation point will lead to a more restricted set of potential fates until the cell eventually settles in a stable steady state.

Huang *et al.* used Waddington's metaphor to develop a framework where the landscape represents a surface in a higher-dimensional phase space. In this framework, the shape of the surface

is defined by genetically-encoded parameters and distinct phenotypic states are defined by stable states of gene expression that are attractors of a dynamic system (S. Huang, Eichler, et al. 2005). While the epigenetic landscape serves as an intuitive explanation for cell fate determination, it presents problems for interpretation (Moris, Pina, and Arias 2016). First, it is very difficult to calculate a position in a phase space of thousands of variables, as would be required to represent a cell state dependent on the expression of thousands of different genes. Second, the reality of state switching is likely not as smooth and continuous as the rolling pebble metaphor presents, as it is currently believed that genes switch between states in a more discrete manner, having to actively overcome barriers between steady states.

The biochemical mechanisms underlying transitions between distinct phenotypic states are not very well understood. However, from dynamical systems theory, two general mechanisms can be considered to underlie the ability of a cell to switch between states: transitions caused by a random perturbation of the system, or a transient alteration of the epigenetic landscape by extrinsic factors that reduce or remove barriers between states (S. Huang, Guo, et al. 2007). Both mechanisms have been demonstrated experimentally using modified or synthetic transcriptional regulatory networks (M. B. Elowitz, Levine, et al. 2002; Gardner, Cantor, and Collins 2000; Kashiwagi et al. 2006). In these networks, the relevant epigenetic landscape is defined by a relatively small number of transcription factors that regulate the transcription of each other as well as reporter proteins. Due to their small size, it is possible to analyze analogues of natural fate-determination transitions and gain detailed insight into regulatory processes at a single-cell level. This is important because the large number of genes contributing to a cell's state makes quantitative studies mostly intractable. The problem is made more manageable by using synthetic networks, which may be viewed as simplified embodiments of the hierarchical and modular characteristics used to understand the properties of larger networks (MacNeil and Walhout 2011).

1.2.1 Gene Expression Noise

It has been argued that the accumulation of mutations cannot explain many instances of drug resistance, including resistance of some early-stage infections and tumors to antibiotic or chemotherapeutic treatment (Sharma et al. 2010; Deris et al. 2013; Niepel, Spencer, and Sorger 2009). These diseases typically begin with populations of genetically identical cells, and it is unlikely that a sufficient amount of time would have elapsed since the onset of the disease for mutations conferring resistance to accumulate given the relatively slow rate of mutation in general (Drake et al. 1998). As an alternative, it has been proposed that gene expression noise may play important roles in producing phenotypic diversity and increased resistance of a subset of cells to drug treatment. Gene expression noise can in general be defined as variability in gene expression levels between cells in an isogenic population. The sources of this noise can be classified as extrinsic or intrinsic (Swain, M. B. Elowitz, and Siggia 2002; M. B. Elowitz, Levine, et al. 2002; Pedraza and Oudenaarden 2005). Extrinsic noise globally effects all processes within a single cell, but may vary between cells, and results from variabilities in cell-wide factors such as microenvironments, metabolic or cell cycle states, organelle distribution and inheritance, and cell age, that might cause changes to the epigenetic landscape. However, extrinsic noise may also be caused by cells occupying distinct stable gene expression states and does not require variability in the epigenetic landscape to be present.

Intrinsic noise results from the inherent stochasticity of the molecular interactions involved in the process of gene expression. It is an inevitable source of cell-to-cell variability that arises from the biochemical processes governing the synthesis and turn-over of mRNA and protein molecules. Even in the extremely unlikely scenario that all cells are at identical states in an identical environment, cells will still exhibit variability in gene expression. This was demonstrated by Elowitz *et al.* by placing different fluorescent reporter genes under the regulation of identical promoters in the same cell (M. B. Elowitz, Levine, et al. 2002). By measuring fluorescence of both reporters in a population of cells, it was shown that individual cells differed from one another in their expression of each reporter, owing to extrinsic noise, and that within each individual cell there were observ-

able differences between reporter expression, owing to intrinsic noise. This variability results from the reality that all molecular interactions occurring in the cell are biochemical reactions and thus follow the same thermodynamic principals that govern all chemical reactions (Coulon et al. 2013). In this way, molecules do not interact deterministically, but rather probabilistically with an element of randomness. Different cellular factors affect the likelihood that a molecular interaction will occur, such as molecule concentrations and cell volume, as well as environmental factors like temperature. Ideal interaction conditions, such as high molecule concentrations, small cell volume, and optimal temperature, do not guarantee that the interaction will take place, but rather increase the likelihood that a successful interaction will occur. Similarly, non-ideal conditions such as low molecule concentrations, large cell volume or sub-optimal temperatures make it very unlikely that an interaction will occur, although not impossible. Whereas systems may be approximated as deterministic if they are sufficiently large with sufficiently high concentrations of reaction species, stochastic differences are especially pronounced when considering the small volume and low number of molecules interacting in many cellular processes, including gene expression (Spudich and Koshland Jr 1976; McAdams and A. Arkin 1999).

At the level of genes, randomness in molecular-level interactions manifests as stochastic bursts in protein synthesis over time. These bursts may be explained by two mechanisms: translational bursting and transcriptional bursting (Kærn et al. 2005; McAdams and A. Arkin 1997). Translational bursting occurs when translation efficiency is high and mRNA abundance is low. In this scenario, when mRNA templates are present they will be highly translated and lead to bursts of protein expression (Ozbudak et al. 2002). This is in contrast to the case where mRNA template is highly abundant and translation is relatively inefficient, which will result in a relatively consistent rate of protein expression. Transcriptional bursting is described by slow reaction kinetics at the gene's promoter, resulting in slow transitions between active and inactive states. A gene will thus switch between states of high and low transcriptional activity. If the transcribed mRNA is unstable and translation is relatively efficient, these states of transcriptional activity will manifest as bursts of protein expression. This is supported by work focused on the recruitment of RNA polymerase

II and other transcription factors to gene promoters, leading to transcription initiation and successive reinitiation events when certain pieces of the transcriptional machinery remain bound to the promoter (Zawel, Kumar, and Reinberg 1995; Yudkovsky, Ranish, and Hahn 2000). As the initial binding and assembly events are thought to be rate limiting steps in transcription (Chatterjee and Struhl 1995; Klages and Strubin 1995), the ability to reinitiate transcription after an initial binding event would lead to transcriptional bursting. Although transcriptional and translation bursting are separate processes resulting from distinct cellular processes, both can contribute to noisy gene expression.

The randomness inherent to gene expression challenges the attractive but simplistic view of the cell as a fine-tuned deterministic machine where one process routinely and predictably leads to another. For example, intrinsic noise in the expression of transcription factors that cause epigenetic marks to be added or removed from specific regions of the genome may cause some genes to be actively transcribed in one cell but not in another cell that is otherwise completely identical. The situation is further complicated by the fact that intrinsic and extrinsic noise are not independent of one another, as intrinsically noisy expression of genes involved in cellular metabolism and growth can lead to variability in cell size, cell cycle stage, and metabolic state, factors which will globally affect the entire cell as sources of extrinsic noise (Kærn et al. 2005; Kiviet et al. 2014).

Gene expression noise is generally viewed as having potentially detrimental effects, and it is expected that mechanisms would have evolved to attenuate cellular noise so that signals may become more precise and reliable. Cellular strategies do exist for maintaining robust gene expression (Rao, Wolf, and A. P. Arkin 2002) such as negative feedback loops (Becskei and Serrano 2000), ultrasensitive cascades (Thattai and Oudenaarden 2002), gene dosage (Cook, Gerber, and Tapscott 1998), and cellular checkpoints (Hartwell and Weinert 1989), which can all function to diminish noise and maintain order in cellular processes. However, noise can actually have beneficial properties as well, and can be exploited by the cell. Cellular noise can underlie the development of diversity and cellular heterogeneity (Eldar and M. B. Elowitz 2010), which are beneficial for population survival (Fraser and Kaern 2009) and division of labour (Ispolatov, Ackermann, and Doebeli 2012) in

single cell communities, and lineage determination and differentiation in multicellular organisms (Balázsi, Oudenaarden, and Collins 2011; H. H. Chang et al. 2008).

1.2.2 Gene Regulatory Networks

Gene regulatory networks play an essential part in mediating gene expression levels and patterns and are described by the interactions of genes, typically transcription factors, that regulate each other's expression. These networks are essential for understanding non-genetic heterogeneity because they define the shape of the epigenetic landscape. Gene regualtory networks can be depicted as graphs of nodes, representing the genes, and edges, representing the interactions between them (Alon 2006). While gene regulatory networks can involve tens to hundreds to thousands of genes, specific connection patterns, or motifs, formed between small numbers of genes are observed in these networks at a greater frequency than would be predicted by chance, indicating that they play functional roles that are selected for through evolution (Alon 2007; Milo et al. 2002). Indeed many of the motifs observed in gene regulatory networks are common to other biological networks, such as those formed between neurons, as well as non-biological information processing networks such as electrical circuits and those found in the internet. This interesting fact indicates that information processing in biological systems behaves much the same as in engineered systems. The most prominent motifs found in gene regulatory networks include simple direct regulation, autoregulation, feedforward regulation, single-input regulation of multiple genes, and multiple input regulation of a single gene (Alon 2007). These networks can produce a plethora of different functional outputs as they can be composed of either positive or negative interactions with varying degrees of strength, and can act on downstream genes whose promoters can act as logic gates with a variety of input requirements (Hunziker et al. 2010). Many functional properties are associated with specific gene network motifs, some of which could serve as mechanisms for cells to switch to, and remain in, new gene expression states.

Positive Feedback Loop

Certain regulatory network motifs are able to mediate gene expression noise by either attenuating or amplifying it, where diminished noise may be desirable for maintaining gene expression levels within a defined range while amplified noise can be used to generate heterogeneity (Chalancon et al. 2012). Feedback loops are common network motifs where a gene product regulates its own expression (Figure 1.1a). Negative feedback loops are formed when a gene product negatively regulates its own expression, and can function to attenuate noisy signals or activating events (Alon 2007; Hinczewski and Thirumalai 2016). As the amount of gene product increases, so too does the level of negative regulation it exerts on its own expression. This type of feedback effectively prevents the expression level from exceeding a certain threshold, maintaining it fairly constant within a specified range. Conversely, a positive feedback loop (PFL) functions to amplify stochastic variability in gene expression where a gene product positively regulate its own expression (Alon 2007). As a gene is expressed, even at a relatively low level, the product promotes more expression in a self-perpetuating cycle. This results in a switch-like amplification of small increases in gene expression, which can contribute to non-genetic heterogeneity where cells are expressing the gene at either a low level, if no activation has occurred, or a high level, where any activation is amplified to a produce a greater gene expression response (Ferrell 2002). While negative feedback loops are capable of reducing noise (Becskei and Serrano 2000), this is often associated with a decrease in sensitivity as well as increased noise in other parts of the network (Bruggeman, Blüthgen, and Westerhoff 2009; Hornung and Barkai 2008). Alternatively, PFLs can retain sensitivity while somewhat ironically reducing noise in their gene expression output. Due to their slow dynamics, where cells can exist for long periods of time in one state or the other, PFLs can improve time averaging of gene expression levels, thus reducing noise (Hornung and Barkai 2008). A PFL can also produce non-genetic memory by maintaining gene expression after the initial signal is removed, due to the self-perpetuation of its own expression (Ferrell 2002). A PFL can thus underlie stochastic switching between gene expression states and also provide a mechanism to maintain memory of these expression states that could be passed on to future generations of cells. Both of these properties are

likely mechanisms underlying the ability of cells to switch between, and then remain in, different stable states of gene expression, implicating this network motif in the development of non-genetic heterogeneity.

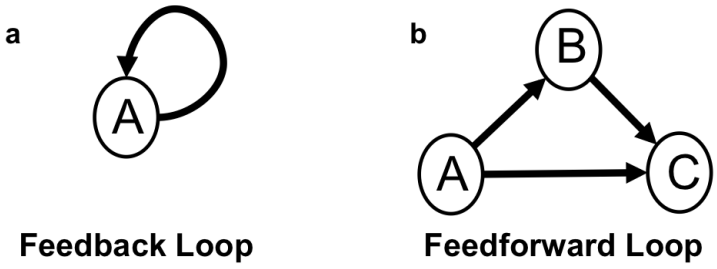


Figure 1.1: Feedback loop and feedforward loop network motifs. **a)** A feedback loop, where the product of gene A autoregulates its own expression. The autoregulation can be inhibitory or excitatory, forming a negative or positive feedback loop respectively. Although shown to directly regulate its own expression, a gene product may form a feedback loop by influencing any gene upstream of itself in the network as well. **b)** A feedforward loop, where an upstream gene A regulates a downstream gene C both directly and indirectly through the intermediate gene B. Any connection in the network may be either inhibitory or excitatory.

Feedforward Loop

The feedforward loop is another gene regulatory network motif that can function to mediate gene expression noise (Figure 1.1b). Feedforwrad loops occur when one transcription factor (A) regulates a downstream gene (C) both directly and indirectly through an intermediate transcription factor (B), which itself regulates gene C (Alon 2007). Feedforward loops can be classified as one of eight possible types, as each interaction can be either positive or negative (three connections in the network that have two possible types of regulation = $2^3 = 8$). These eight types of feedforward loops can be generally classified as either coherent, where the regulation from both arms is the same (both positive or both negative), or incoherent, where the regulation from each arm is opposite (one positive and one negative). The eight possible feedforward loops are shown in Figure 1.2. On top of this, C can be regulated by A and B through either an 'OR gate', where activity of either transcription factor is sufficient to regulate gene C, or an 'AND gate' where activity of both transcription factors is required. Regulation through an 'OR gate' can have an added layer

of complexity where activities of the transcription factors A and B may be additive, synergistic, or antagonistic, depending on the nature of their individual methods of regulation. Feedforward loops can both process noisy inputs and regulate noisy outputs. The role of feedforward loops in the latter was demonstrated by Kittisopikul and Süel, who showed that the type of regulation that gene A exerts on gene B can group all possible feedforward loop variations into two distinct categories (Kittisopikul and Süel 2010). If A activates B (Fig. 1.2 coherent and incoherent types 1 and 3), expression of C will be more noisy when the network is in the OFF state. Alternatively, if A represses B (Fig. 1.2 coherent and incoherent types 2 and 4), expression of C will be equally noisy in both the ON and OFF states. These characteristics were correlated with naturally occurring feedforward loops in *E. coli*, where it was observed that the former category (A activating B, high noise in the OFF state) is more common in the regulation of genes involved in stress response phenotypes which could benefit from noisy gene expression.

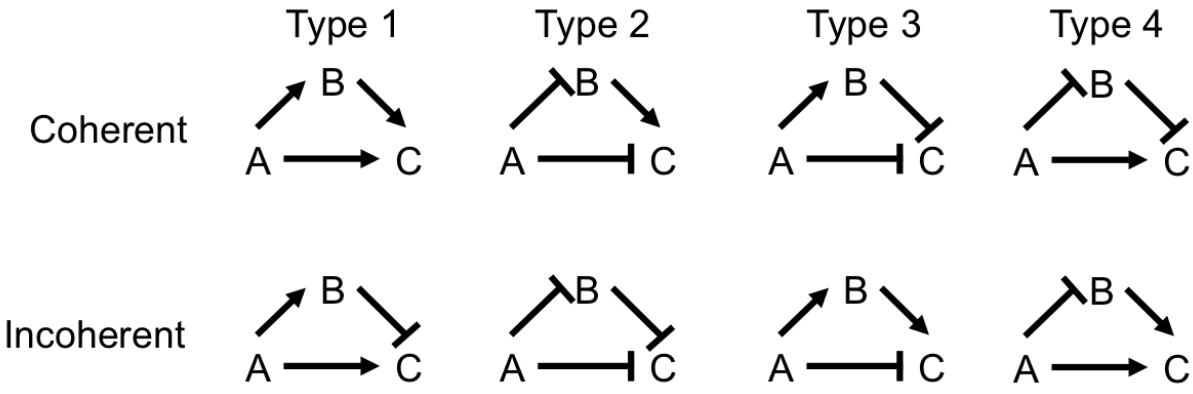


Figure 1.2: Feedforward loops. All eight possible feedforward loop motifs, classified as either coherent or incoherent, types one through four. In coherent feedforward loops, both the direct and indirect branches regulate the downstream gene in the same direction. In incoherent feedforward loops, each branch regulates the downstream gene in opposite directions.

Feedforward loops also show interesting and variable behaviour in their response to signals (Alon 2007). The type I incoherent feedforward loop (I1FFL) for example, where A activates both B and C, and B represses C (Fig. 1.2, incoherent, type 1), produces a rapid and pulsatile response to a signal that activates A. Activation of C by A can occur rapidly through the direct connection, quickly reaching a maximum expression level, but as A activates B, C will eventually become

repressed, resulting in a pulse of C expression. The type I coherent feedforward loop (C1FFL), which involves all positive interactions (Fig. 1.2, coherent, type 1), can act as a delay element in C expression, and can produce a delay in either the ON or OFF state, depending on the logic of C regulation. If A and B regulate C through an AND gate, then there will be a delay in the ON state, where A activity is not sufficient to activate C, and time must pass for A to first activate B and then for B to also activate C. This architecture and logic combination can also act as a filter for persistent activating signals, where spurious activation of the network will not allow both transcription factors to reach sufficient levels to activate C, and only signals persisting long enough to allow A to produce and adequate amount of B will result in expression of C. Alternatively, if C is regulated through an OR gate, there will be no delay in turning C on, as the direct connection between A and C will result in rapid activation, but persisting activity of B after A is turned off will result in the continued expression of C even after removal of A, and thus a delay in the OFF state. Considering an added layer of complexity, where C is regulated by an OR gate and the activities of A and B are additive or synergistic, then the end result of C activation by both A and B will be amplified expression relative to activation by either A or B alone.

The functional properties associated with this last case of C1FFL architecture and logic, where C is regulated by an OR gate and A and B can act synergistically, are similar to those associated with a PFL. Both network motifs can amplify an activating signal, resulting in increased gene expression output, and can cause slow dynamics in the OFF state, although both of these properties are more pronounced in the PFL. One property of the C1FFL that is not shared with the PFL, however, is the ability to generate a rapid response to an activating signal (again when the promoter of the downstream gene functions as an OR gate). When considering a C1FFL, and comparing it to a cascade network where gene A activates gene C only indirectly through gene B, it can be seen that the direct activation between gene A and gene C allows activation of C immediately after gene A is expressed. In the cascade network, the immediate response of expressing gene A would only be the activation of gene B. By the time gene B is expressed and activates gene C in the cascade network, the same occurs in the C1FFL. Now, with two potentially additive activating signals, the C1FFL

will express gene C at a higher level than the cascade. If a PFL were regulating a downstream gene, there could similarly be a delay due to an activity threshold that must be surpassed before the PFL is activated (Savageau 1974; Maeda and Sano 2006).

These common gene regulatory network motifs, the PFL and C1FFL, have functional properties that implicate them in the two processes required for the development of non-genetic heterogeneity: the availability of more than one gene expression state, and the ability to maintain lasting expression of each individual state. However, each network motif is not equal in its ability to perform these functions. The PFL is assumed to be more efficient at generating stochastic heterogeneity through its ability to amplify noisy activating signals, fitting the theoretical model of phenotypic state switching due to random perturbations. The C1FFL, on the other hand, is predicted to be more apt at producing heterogeneity by more effectively responding to external signals. This would fit into the theoretical model for state switching through alteration of the cell-state landscape by external factors. Although both the PFL and C1FFL are predicted to produce lasting gene-expression states, that resulting from the C1FFL would be much shorter lived, as only a slight delay would be expected before activity of the intermediate transcription factor eventually falls below a threshold required to activate the downstream gene. The PFL would alternatively allow a transcription factor to perpetuate its own expression, and thus the expression of a downstream gene, indefinitely. The non-genetic heterogeneity that these network motifs could underlie can have many important biological consequences, as mentioned above, one of which is drug resistance and survival in single cell populations.

1.2.3 Non-genetic Heterogeneity and Drug Resistance

The inability of a pharmacological intervention to completely eliminate an entire population of genetically identical disease cells is commonly observed in the treatment of both bacterial infections (Harms, Maisonneuve, and Gerdes 2016; K. Lewis 2010) and tumors (Roux et al. 2015; Brock, H. Chang, and S. Huang 2009; Pisco et al. 2013; Sun and Yu 2015). Drug treatments often wipe out a majority of the population while a portion of it survives, seeding a new population. This

phenomenon typically manifests as persistence in bacteria, as described above, and may occur randomly or through changes in bacterial gene expression that are induced by the presence of a stress such as drug treatment (Fisher, Gollan, and Helaine 2017). In chemotherapeutic treatment of tumors, the phenomenon is also referred to as fractional killing (Skipper et al. 1970) and describes the observation that identical doses of a chemotherapeutic agent applied for identical lengths of time will always kill a constant portion of the tumor cells, and not a specific number. In both of these cases, the development of resistance in only a portion of the population requires that it exhibit heterogeneity in the expression of a drug resistance phenotype. Further, as persisters are able to resume normal growth when the selection pressure is removed, and as constant fractions of tumor cells, regardless of the popoulation size, are effected by chemotherapy, this heterogeneity must be dynamic so that cells can switch in to or out of resistant states. This precludes genetic mutation as a mechanism underlying the required heterogeneity as genetically coded resistance would not be this dynamic. While the exact mechanisms underlying this non-heterogeneity are unclear, some have been suggested including cell cycle stage-dependent tolerance, rapid adaptation to the stress, and general dormancy (Balaban et al. 2004).

Two mechanisms for the development of resistance-related non-genetic heterogeneity receiving lots of recent attention are the stochasticity in gene expression associated with cellular noise (Niepel, Spencer, and Sorger 2009; Daniel A Charlebois, Abdennur, and Kaern 2011; El Meouche, Siu, and Dunlop 2016; Pisco et al. 2013) and the induced response of cells to an environmental stress (Selvarajoo 2013; Pisco et al. 2013; Chaudhary and Roninson 1993). While either of these means can plausibly generate heterogeneity in an isogenic population, the resistance phenotype must be able to persist for long enough to confer a survival advantage to the population. Through the heterogeneous expression of a resistance gene, cells with high levels of expression will be able to tolerate the stress of a drug treatment while cells with low expression will likely be killed off. The surviving cells can then seed a new population and re-establish the initial distribution of resistance levels through dynamic state switching. If this high expression is merely transient, and only persists for a period of time shorter than the length of the drug treatment, then even the initially resistant cells

will eventually be wiped out. If the high expression state can be maintained, however, it may be passed on between generations of cells. If the drug treatment is either recurrent or persistent, these temporarily-resistant cells will become enriched in the population. Increased resistance, even temporarily, provides more time for these cells to accumulate mutations, which may even be induced by the stress of drug treatment (Kohanski, DePristo, and Collins 2010), and eventually acquire permanent resistance (Brock, H. Chang, and S. Huang 2009; Pisco et al. 2013). Interestingly, it has also been suggested that increased heterogeneity may allow a broader set of adaptive mutations to confer drug resistance (Bódi et al. 2017), shortening the time required for permanent resistance to develop. Both the requirement for non-genetic heterogeneity and for its inheritance in the development of drug resistance have been investigated. Blake *et al.* demonstrated that both cell-to-cell heterogeneity and the ability to respond rapidly are beneficial for a population encountering an acute stress (Blake et al. 2006). Noisy gene expression can underly both cell-to-cell variability and rapid responses, and it was shown that cells in which a stress response gene was expressed from promoters with decreased noise (achieved through mutating the TATA box) were less able to survive the presence of an environmental stress. It was also demonstrated theoretically that both gene expression noise and sufficient memory produce fitness advantages for cell populations encountering unexpected stresses such as drug treatment (Daniel A Charlebois, Abdennur, and Kaern 2011). In this study, gene expression noise was important mainly for generating heterogeneity and producing highly resistant cells. The memory was required for the high-resistance phenotype to be passed on between generations of cells, as populations in which memory was shorter than the generation time were eventually wiped out by the drug treatment. Alternatively, all populations in which memory lasted longer than the generation time were able to survive, where greater fitness was associated with longer memory.

Various cellular mechanisms could potentially be responsible for the development of non-genetic heterogeneity and for its stable maintenance, including the functional properties associated with gene regulatory network motifs. As outlined above, two common network motifs, the coherent feedforward loop and the positive feedback loop, have properties that implicate them in

heterogeneous gene expression and slow dynamics for switching between gene expression states. The regulation of a drug resistance gene by these network motifs may thus underlie its heterogeneous and heritable expression which could implicate it in non-genetic drug resistance. Despite the apparent consensus and fairly large bodies of work both on the functional properties of these network motifs and on the benefit from non-genetic heterogeneity for surviving in unpredictably stressful environments, little work has been done on linking the two, or on the role of gene regulatory networks in drug resistance in general. It has been demonstrated that positive feedback can indeed produce distinct heterogeneity in an isogenic population, and that an ideal balance between the PFL-mediated heterogeneity for a drug resistance gene produces a 'sweet spot' for drug resistance (Nevozhay et al. 2012). In this work it was shown that induction of the PFL at too low a level did not produce enough of the drug resistance gene to survive treatment, while induction at too high a level resulted in cellular toxicity and a fitness defect in drug-free conditions. Bódi *et al.* have also demonstrated that gene circuits generating more noise, in particular those involving a PFL, produce increased phenotypic heterogeneity, and that this heterogeneity facilitated survival in an adverse environment when treated with a drug (Bódi et al. 2017). It was also shown that phenotypic heterogeneity even evolves in response to stressful conditions and facilitates the evolution of other adaptive mutations leading to permanent drug resistance. This was in agreement with previous work showing that a PFL network module regulating a drug resistance gene can be eliminated or fine-tuned through evolution, depending on the balance between the survival benefits and fitness costs that it confers (González et al. 2015). Lu *et al.* have also demonstrated that interfering with gene networks can increase the effectiveness of an antibiotic treatment (Lu and Collins 2009), supporting the potential benefit of targeting gene regulatory networks in combatting drug resistance. Aside from these limited studies, focused mainly on the PFL and its ability to generate beneficial heterogeneity, little else has been done to investigate how gene regulatory networks, and their functional properties, might underlie non-genetic drug resistance. On top of this, most of the work on non-genetic resistance in general has focused specifically on antibiotic resistance and bet-hedging in bacteria, with very little work investigating other survival strategies, or even resistance

in general, in eukaryotic cells.

1.3 The Yeast PDR Network

In the bakers yeast *Saccharomyces cerevisiae*, a model eukaryote, the pleiotropic drug resistance (PDR) network is the main pathway implicated in multidrug resistance. The PDR network consists of many genes with various functions including transmembrane transport, DNA damage repair, and metabolism, at least 15 of which appear to be regulated directly by the master regulators of the network, the transcription factors Pdr1p and Pdr3p, through both positive and negative regulation (DeRisi et al. 2000). As a multidrug resistance network, many of the effector genes code for transmembrane efflux pumps that actively detoxify the cell. One of the major effector proteins, and one of the best studied, is Pdr5p, a transmembrane active transporter that works to export a broad range of structurally unrelated drugs.

1.3.1 The Multidrug Efflux Pump *PDR5*

The implication of many ABC transporters, such as MDR1, in cancer multidrug resistance make them a prime candidate to study in order to develop new therapies to combat chemotherapeutic resistance. However, similar insights may be gained from studying more manageable model organisms such as *S. cerevisiae* and its MDR1 homologue *PDR5*, the founding member of a subfamily of ABC transporters found only in fungi (Taglicht and Michaelis 1998). This Pdr subfamily of ABC transporters, on top of their relative ease to study compared to human transporters, is also clinically significant in that the human pathogen *Candida albicans* can develop multidrug resistance through the expression of its Pdr5p homologue Cdr1p (Kontoyiannis and R. E. Lewis 2002). Pdr5p is responsible for exporting hundreds of different, chemically unrelated compounds (Rogers et al. 2001), with some overlap in substrates between two other ABC transporters Yor1p and Snq2p. It has been shown that Pdr5p specificity is based mainly on substrate size (Golin et al. 2003), a characteristic that is shared with MDR1 (Loo and Clarke 2001). Pdr5p's established role in multidrug

resistance has also supported its recent use as a model effector protein to study eukaryotic multidrug resistance and fitness (Bódi et al. 2017). *PDR5* expression is directly regulated by the two master regulators of the PDR network, Pdr1p and Pdr3p.

1.3.2 The Master Regulators *PDR1* and *PDR3*

Pdr1p and Pdr3p control the PDR response by regulating the expression of genes for transmembrane efflux, DNA damage repair, cellular metabolism, and other regulators. These master regulators are homologous zinc finger transcription factors sharing 36% identity and a conserved domain organization, binding to DNA through the N-terminal cysteine-rich zinc finger domain and activating transcription through a C-terminal acidic activation domain (Mamnun, Pandjaitan, et al. 2002). Both Pdr1p and Pdr3p bind to PDREs (pleiotropic drug resistance elements) with the consensus sequence 5'-TCCG/aC/tGG/cA-3' found in the promoters of their target genes (Katzmann, Hallstrom, et al. 1996) and have been shown to both activate and repress gene expression. Pdr1p and Pdr3p are both phosphoproteins, binding as either homo- or heterodimers to their PDREs (Mamnun, Pandjaitan, et al. 2002), and at least one of either Pdr1p or Pdr3p is required for expression of *PDR5* (Katzmann, Burnett, et al. 1994). While the roles of *PDR1* and *PDR3* in *PDR5* regulation, and in the PDR response in general, are fairly well understood, there remain some discrepancies regarding the precise regulation of these transcription factors. It has been suggested that Pdr1p is constitutively bound to the PDREs in the promoter for *PDR5* as well as other target genes (Fardeau et al. 2007). However, it has also been demonstrated that Pdr1p may bind certain compounds that are exported by Pdr5p, inducing Pdr1p binding and thus *PDR5* expression (Thakur et al. 2008). Interestingly, *PDR3* has been shown to be up-regulated by defective mitochondria, or their complete absence (Hallstrom and Moye-Rowley 2000), activating the PDR response and *PDR5* expression in a *PDR3*, but not *PDR1*, dependent manner. While some finer details and certain intricacies of *PDR1* and *PDR3* regulation remain unclear, the genes that they target are generally agreed upon.

Pdr1p and Pdr3p both regulate *PDR5* by binding to the PDREs in its promoter, of which there are four (Katzmann, Hallstrom, et al. 1996). There are also two PDREs in the *PDR3* promoter

(Delahodde, Delaveau, and Jacq 1995) suggesting that *PDR3* is regulated by *PDR1* and also auto-regulates its own expression. This pattern of regulation forms a transcriptional regulatory network consisting of a CFFL, where *PDR1* regulates *PDR5* directly and indirectly through *PDR3*, and a PFL, where *PDR3* positively auto-regulates its own expression (Figure 1.3). These network motifs, and their associated functional properties, may themselves play an important role in regulating *PDR5* expression to facilitate non-genetic multidrug resistance and population survival. In modelling the *PDR5* regulatory network, it was observed that a feedforward loop with positive feedback produced greater mean gene expression that was more robust to fluctuations than just a feedforward loop or direct activation when subject to fluctuating activating signals (Daniel A. Charlebois, Balázsi, and Kaern 2014). It was further shown that this elevated level of stable expression provided a fitness benefit when the population was treated with a drug, although the same fitness benefit was conferred by the feedforward loop alone. There is strong evidence for the regulation of *PDR5* by a CFFL+PFL, although there are likely other, and possibly unknown, genetic interactions that play potentially important roles in regulating Pdr5p expression. As is the case when studying any cellular process, it is nearly impossible to say for certain that the observed occurrences are dependent only on the small number of components being studied.

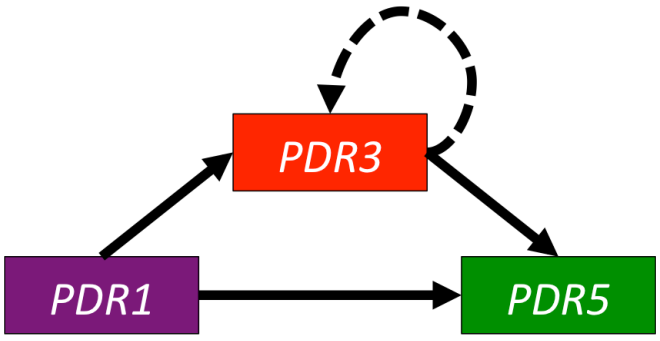


Figure 1.3: PDR5 regulatory network. A graphical depiction of the regulation of PDR5 by the transcription factors PDR1 and PDR3. A coherent feedforward loop is formed between PDR1, PDR3, and PDR5 (solid lines). A positive feedback loop is formed by PDR3's positive autoregulation (dashed line).

1.4 Synthetic Biology

The field of synthetic biology has been steadily gaining interest and popularity over the past couple of decades, as has its uses in both creating new biological systems and in studying existing ones (Oldham, Hall, and Burton 2012; Mukherji and Van Oudenaarden 2009). One useful application of synthetic biology for studying existing biological systems is the ability to create new systems that mimic natural ones, but that are composed of well defined and characterized parts (M. Elowitz and Lim 2010; Liu, Fu, and J.-D. Huang 2013). In this way, researchers can build a synthetic system based on their current knowledge of a natural one and investigate whether, and under what conditions, it behaves similarly. This allows them to refine or deepen their understanding of how the natural system works and address what might be lacking from their current knowledge. One area of biological research in which this approach has proved very useful is the study of biological circuits and networks (Mukherji and Van Oudenaarden 2009).

1.4.1 Synthetic Gene Regulation

An important aspect of synthetic biology is the ability to create systems that regulate gene expression in a well-defined way (Ding, Wu, and Tan 2014). This regulation can be built in using a variety of genetic and cellular components, including gene coding regions, promoters, and terminators, and regulatory proteins such as transcriptional activators and repressors. These parts may be naturally occurring, with their natural functions either exploited or repurposed, or they may be completely synthetic and foreign to the cellular systems in which they are implemented (Sprinzak and M. B. Elowitz 2005; Mukherji and Van Oudenaarden 2009). The use of completely synthetic machinery in regulating gene expression allows greater control over the typically well-characterized parts as well as the ability to isolate the synthetic system from the rest of the cell in order to avoid complicating, and possibly poorly understood, interactions with other cellular machinery (Nandagopal and M. B. Elowitz 2011). However, without creating completely synthetic cells it would be impossible to isolate these synthetic systems from all influencing factors in the cell as, for example, a

synthetic gene network will still rely on the natural transcription and translation machinery present in the cell. These systems can still be isolated from other genetic interactions that may affect their activity and can still be largely buffered from global sources of variation such as cell cycle stage, metabolic state, and cell age, all of which are likely to influence the activity of natural systems. Early and notable examples of isolated synthetic systems in action include the design and implementation of a genetic oscillator (M. B. Elowitz and Leibler 2000) and a genetic toggle switch (Gardner, Cantor, and Collins 2000) in bacteria, using transcription factors and promoters that are not naturally involved in these processes. The design and implementation of these synthetic circuits highlights the ability to engineer genetic networks with both desirable and novel functions, and with approximated natural functions to help understand a natural biological system. These systems, although relatively simple in comparison to those that are found in nature, can nonetheless provide insight into more complex biological processes that involve these simpler patterns (Mukherji and Van Oudenaarden 2009). This has been demonstrated in studying basic processes such as transcription, both in terms of transcription factor activity (Rosenfeld et al. 2005) and promoter architecture (Hammer, Mijakovic, and Jensen 2006), signalling pathway connectivity(Dueber et al. 2009), and network topology and architecture (Alon 2007).

1.4.2 Synthetic Gene Regulatory Networks

Synthetic gene networks are built using synthetic transcription factors, allowing the expression of a gene of interest to be controllably regulated without interfering with, or being subject to interference from, the expression of other genes (Yaghmai and Cutting 2002; Perez-Pinera et al. 2013). These transcription factors are typically fusion proteins possessing an activation or repression domain, a ligand-binding site for regulation by a small molecule inducer, and a DNA binding domain. These domains may originate from a variety of different proteins, different organisms, and even different domains of life. The modulation domains, which can have activating or repressing functions on the regulated gene, can act in a variety of ways, but usually work to recruit other cellular machinery to the gene. This machinery may be other transcription factors and members of the pre-initiation

complex for activating gene expression, or chromatin silencers for repressing gene expression (in eukaryotic cells). The ligand binding domain, if present, allows the activity of the transcription factor to be precisely modulated due to the requirement for a chemical inducer to become active. The level of transcription factor activity will be proportional to the concentration of inducer in the system, allowing gene expression to be desirably increased or decreased over a potentially very broad and dynamic range. The DNA binding domain is responsible for binding to specific and well-defined DNA sequences upon induction. These transcription factors can thus be recruited to specific genes by engineering their promoters to include a binding site recognized by the DNA binding domain of a particular transcription factor. Promoters can be engineered to respond to multiple transcription factors, synthetic and natural, by including particular binding sites.

Above the level of synthetic gene regulation using synthetic transcription factors and engineered promoters, more complex systems of regulation can be built by creating synthetic gene networks, such as those early ones created by Elowitz (M. B. Elowitz and Leibler 2000) and Gardner (Gardner, Cantor, and Collins 2000), and more advanced ones to regulate more complex processes such as metabolism (Oyarzún and Stan 2013). These networks can be composed of multiple synthetic transcription factors that regulate each other's expression as well as the expression of a gene of interest. Synthetic gene networks can be built using the coding sequences of synthetic transcription factors and engineering their promoters to recruit other transcription factors in the network. These synthetic networks can be built with a variety of transcription factors and connections between them, allowing different network patterns to be created and studied. In this way, specific network topologies that are studied in natural systems can be built in order to investigate, in isolation, the functional roles that these networks might play in regulating gene expression and their potential phenotypic consequences (Mukherji and Van Oudenaarden 2009).

1.5 Rationale

Heritable non-genetic heterogeneity is believed to be important in the development of drug resistance in an isogenic population without mutation. Two motifs commonly found in gene regulatory networks, the coherent feedforward loop and the positive feedback loop, have functional properties that implicate them in the development of non-genetic heterogeneity as well as gene expression memory. Despite general support for these two ideas from previous work, they have not been explicitly linked. It is predicted that regulation of a drug resistance gene by these network motifs would result in expression dynamics that confer drug resistance and aid in population survival in adverse conditions.

1.6 Objectives

1. *PDR5* is regulated by *PDR1* and *PDR3* forming a coherent feedforward loop with positive feedback. These network motifs have predicted functional properties that are predicted to underlie the development of heritable non-genetic heterogeneity, aiding in drug resistance and population survival. The first objective is thus to investigate *PDR5* expression patterns from the natural PDR regulatory network, and variants, and the associated population fitness. This will be done by tracking *PDR5* expression by tagging it with the yeast enhanced green fluorescent protein (yEGFP), and assessing the fitness of different levels of expression by growing these tagged yeast in the presence and absence of cycloheximide, an antifungal and Pdr5p substrate.

2. The second objective is to support the predicted functions of the coherent feedforward loop and positive feedback loop in producing heritable non-genetic heterogeneity through the creation and study of synthetic gene networks. In doing this, expression patterns and associated resistance phenotypes can be attributed to the network specifically, and in comparing network variants, the functional role of each connection in the network can be determined.

Gene regulatory networks will be constructed from synthetic transcription factors and their associated promoter response elements, effectively isolating the networks from other genetic interactions. Expression patterns from the network variants can be tracked through expression of the yEGFP reporter, and fitness in the presence and absence of cycloheximide can be assessed by synthetic regulation of *PDR5* expression.

Chapter 2

Materials and Methods

2.1 Genetic Manipulation and Strain Construction

2.1.1 DNA Fragment Cloning and Construct Assembly

Yeast strains were created through a combination of polymerase chain reaction (PCR), DNA splicing by overlap extension PCR, and transformation followed by homologous recombination. Desired DNA sequences were cloned from the genomes of bacteria or yeast, or from plasmids, by PCR using primers that would produce overhangs. These overhangs of approximately 40-50 base pairs were designed to have homology with either another DNA fragment to allow assembly of the two DNA monomers, or with a region in the yeast genome to allow insertion by homologous recombination. PCR cloning reactions were prepared using 10 μM custom designed primers (Invitrogen), 200 μM deoxynucleotide triphosphate 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 was used when cloning from a genomic template and approximately 50 ng of plasmid DNA when cloning from a plasmid template. The PCR reactions were performed as follows: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 50°C - 60°C (depending on the melting temperature of the primers) for 30 seconds, elongation at 72°C for 15 seconds per kilobase of

DNA in the final product, and completed with five minutes of elongation at 72°C. DNA fragments were combined into larger constructs through either PCR or transformation followed by homologous recombination. PCR-based assembly was performed as above, using 50 ng/kilobase of each DNA part to be assembled, but was preceded by 10 pre-cycles without primers using an annealing temperature based on the melting temperature of the homologous sequence in the added overhang. These pre-cycles function to attach the separate DNA parts without amplification. After the 10 pre-cycles, primers were added to exponentially amplify the entire assembled piece and the PCR reaction was carried out according to the conditions outlined above. PCR reactions (cloning and assembly) were performed in either a BioRad C1000 Touch or BioRad T100 Thermal Cycler.

2.1.2 Yeast Transformation

Yeast homologous recombination can be exploited to both insert exogenous DNA (flanked by sequences homologous to yeast genomic sequences) and to assemble multiple DNA fragments while inserting them into the genome. DNA pieces, either cloned fragments to be assembled by homologous recombination or assembled constructs to be inserted into the genome, were introduced to the yeast using a standard lithium acetate (LiAc) transformation protocol based on the Gietz and Schiestl method (Gietz and Schiestl 2007). Briefly, cells in mid log-phase growth were spun down at 4000 rpm for five minutes, washed once with ddH_2O and then with 100 mM LiAc. Cells were aliquoted into tubes containing approximately 10^8 cells each and resuspended in a 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 heat shocking for 45 minutes at 42°C, cells were either plated on selection plates immediately, if transformed with an auxotrophic selection marker, or pre-incubated in 1 mL YPD for one hour at 30°C before plating on drug plates, if transformed with a drug resistance selection marker. Selection plates were composed of either YP media supplemented with 2% glucose and the appropriate drug for drug selection or synthetic dropout media supplemented with 2% glucose. Positive transformants were confirmed by PCR using one primer internal to the transformed DNA part and one primer for the flanking genomic region.

2.2 Media and Growth conditions

Cells were grown in liquid YP media (2% w/v yeast extract, 4% w/v bacteriological peptone) supplemented with 2% w/v glucose (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 (E8875, Sigma) was dissolved in ddH_2O for a stock concentration of 5 mM, both stock solutions were aliquoted and stored at -20°C. To induce the synthetic networks, cells in mid-log phase growth were diluted in YPD supplemented with the required concentration of either or both small molecule inducers β-estradiol or doxycycline. Unless otherwise indicated, cells were induced for six hours. 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 then diluted in liquid or pre-solidified solid media to achieve the desired concentration. Cultures were maintained in mid-log phase growth during experiments and diluted every 12 hours in fresh media for multi-day experiments. All incubations were at 30°C, liquid cultures were agitated using a tissue culture rotator.

2.3 Flow Cytometry

For flow cytometry experiments cultures were grown to reach mid-log phase growth (OD_{600} of around 0.4) at the time of the experiment. Cultures were diluted 1:10 in 50 mM sodium citrate buffer. For flow cytometry experiments investigating the PDR5 network, a CyAn ADP 9 Analyzer (Beckman Coulter, Inc.) was used and for synthetic network experiments, a BD FACSCelesta (BD Biosciences) was used. On both cytometers yEGFP was excited by a 488-nm laser and fluorescence was detected with a 530/40 filter. For experiments with MitoTracker Deep Red, an LSR Fortessa Analyzer (BD Biosciences) was used, with MitoTracker fluorescence excited by a 640-nm laster and detected with a 670/30 filter. Flow Cytometry Standard (.fcs) files were analyzed with Matlab® R2015a using a custom written script to extract data and analyze data. Fluorescence

data is presented as arbitrary units and cell counts are normalized by dividing the number of cells in each binned population by the total number of cells in the sample.

2.4 Spot Assay

For spot assay experiments 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 used to prepare four 1:10 serial dilutions in a shallow 96-well plate. The diluted cultures were spotted on agar plates with different growth conditions using an 48-prong hand pinner. The plates were incubated until at least one spot reached full growth.

Chapter 3

The Yeast PDR5 Regulatory Network

3.1 Rationale

Previous work (Azizi 2014) tagged Pdr5p with the yeast enhanced green fluorescent protein (yEGFP) to track its expression in different growth and drug treatment conditions. Briefly, this work found that under certain growth conditions (using galactose as a carbon source, which is not fermented by the BY4741 yeast strain that was used) there exists a subpopulation of cells with high Pdr5p expression. These high-Pdr5p cells grow better in the presence of the drug (and Pdr5p substrate) cycloheximide, but worse in rich media, than low-Pdr5p cells, and the high-Pdr5p phenotype can be maintained for a prolonged period of time. It was also shown that *PDR1* is required for a rapid response and *PDR3* is required for a full response to drug treatment. While these results appear consistent with the predicted functions of the network motifs in question, endogenous *PDR5* expression is linked closely to cellular growth phase (Mamnun, Schüller, and Kuchler 2004), which was not properly controlled for in these experiments. The first goal of the project was thus to replicate these results while properly controlling for cellular growth phase, maintaining cultures in exponential log phase growth consistently.

3.2 Hypothesis

Regulation of *PDR5* by a combined coherent feedforward loop and positive feedback loop (formed between *PDR1* and *PDR3*) produces heritable non-genetic heterogeneity in Pdr5p expression. This expression pattern confers non-genetic drug resistance, improving survival when treated with a drug that is a substrate of Pdr5p.

3.3 Specific Aims

1. Assess *PDR5* expression patterns to determine whether it is heterogeneous and if it is inherited between generations of cells.

2. Assess fitness associated with different expression levels of *PDR5* in normal and drug treatment growth conditions.

3. Alter the *PDR5* regulatory network by deleting either or both of *PDR1* and *PDR3* to determine the role that each transcription factor plays in *PDR5* expression.

3.4 Results

In order to obtain consistent results, it is best to perform experiments on yeast growing at the same growth phase. Mid-log phase, corresponding to an OD_{600} (optical density measured at a wavelength of 600 nm) of around 0.3-0.8, is typically most desirable as this is when the cells are actively metabolizing, growing, and reproducing with a consistent doubling time. To reliably attain mid-log phase cultures, without ever allowing them to reach stationary phase, doubling times were calculated for each strain used in each different media condition. Using these doubling times, along with the desired OD_{600} at the time of the experiment, and the approximate time elapsing between inoculation and the start of the experiment, the starting density of the cultures could be calculated and the freshly inoculated cultures were simply diluted to this density.

3.4.1 Heterogeneous Pdr5p expression

Using this method to consistently obtain cells in mid-log phase growth, the first objective was to replicate the previous observation that yeast cultures growing in YPgal (yeast extract peptone media with galactose as the carbon source) have a small subpopulation of high-Pdr5p cells. Using the previously made strain in which Pdr5p was tagged with yEGFP (Table 3.1), a culture was grown to mid-log phase in YPgal and *Pdr5-yEGFP* expression was analyzed by flow cytometry (Figure 3.1). Compared to autofluorescence in wild type cells not tagged with yEGFP, there is an elevated basal level of fluorescence in the *PDR5-yEGFP* cells indicating low but constitutive *PDR5* expression. There is also a distinct small subpopulation of cells, accounting for approximately 6% of the population, with even higher fluorescence, indicating elevated levels of *PDR5* expression. While these results agree with the previous observation that there is a small subpopulation of high-Pdr5p cells when grown in YPgal, the subpopulation observed here is much larger and more distinct, accounting for approximately 6% of the population and clearly separated from the low-Pdr5p subpopulation, as opposed to the approximately 1% that was previously observed, which appeared as more of a tail on the low-Pdr5p population.

Table 3.1: Yeast strains used to study the PDR network.

Strain	Genotype
BY4741	S288C[a]: *MAT*a; *his3*Δ*1*; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*
WT	BY4741: *pdr5*Δ::*PDR5-yEGFP-KanMX*
*pdr1*Δ	WT: *pdr1*Δ::*URA3*
*pdr3*Δ	WT: *pdr3*Δ::*URA3*
*pdr1*Δ*pdr3*Δ	*pdr3*Δ: *pdr1*Δ::*HIS3*
*pdr5*Δ*::yEGFP*	WT: *pdr5*Δ::*yEGFP-KanMX*

[a]The parental strain is indicated before the colon.

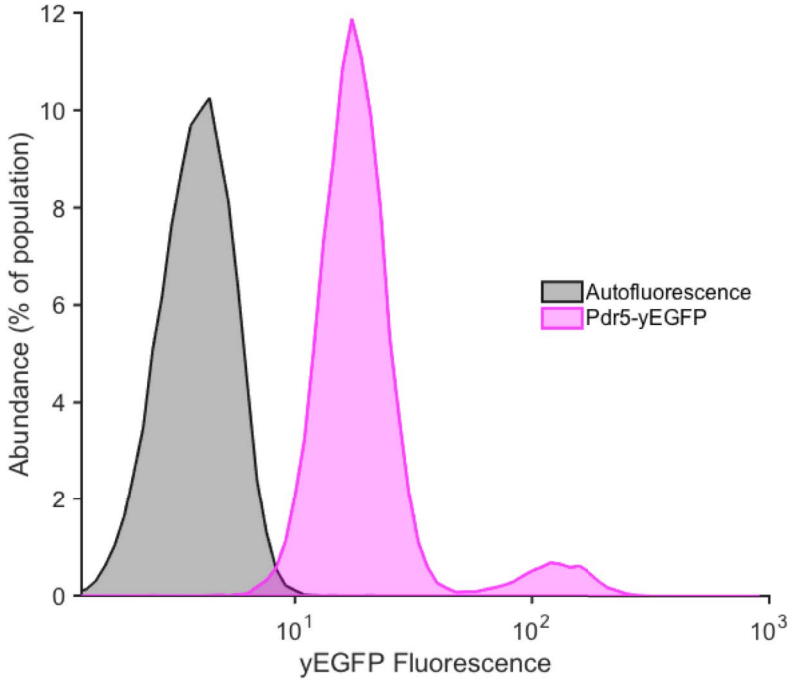


Figure 3.1: Characterization of *PDR5-yEGFP* expression by flow cytometry. The yEGFP fluorescence levels are shown for a wild type strain (BY4741, autofluorescence) and for the strain in which Pdr5p was tagged with yEGFP.

3.4.2 Fitness differences between high- and low-*PDR5* expressing cells

The next objective was to replicate the observation that high-Pdr5p cells are more fit when grown in the presence of cycloheximide and less fit relative to the low-Pdr5p cells when grown in rich media. The two populations were previously separated using fluorescence-activated cell sorting (FACS), which proved not to not be completely efficient, resulting in a sorted low population that was about 80% enriched for low-Pdr5p cells and a sorted high population that was about 60% enriched for high-Pdr5p cells. The sorting efficiency was improved using the new growth phase-controlling protocol, likely due to the more distinct separation of the subpopulations that was attained, however sorting in this way proved to be very time and cost consuming. Serendipitously, it was discovered that when plating cultures growing at mid-log phase in YPgal in order to obtain single clonal colonies, both small and large colonies could be observed (Figure 3.2a). *PDR5* expression was assessed in these different sized colonies by flow cytometry and it was observed that

the small colonies exhibited much greater yEGFP fluorescence than the large colonies, indicating much higher expression of *PDR5* (Figure 3.2b). The *PDR5* expression level in the large colonies was very comparable to that of the low-expressing subpopulation that was previously observed and the expression level in the small colonies was similarly comparable to that of the high-expressing subpopulation. This indicates that upon plating a culture of cells grown in YPgal, with a mixed population of low- and high-Pdr5p cells, the low expressers seed large colonies and the high expressers seed small colonies. Already this supports the fitness disparity between low and high *PDR5* expression that was previously observed. The observation of different sized colonies also provides a new potential way to sort low and high *PDR5*-expressing populations. This method appears much more efficient than the previously used FACS method as the small colonies appear to be composed exclusively of high-Pdr5p cells and the large colonies of low-Pdr5p cells (Figure 3.3a).

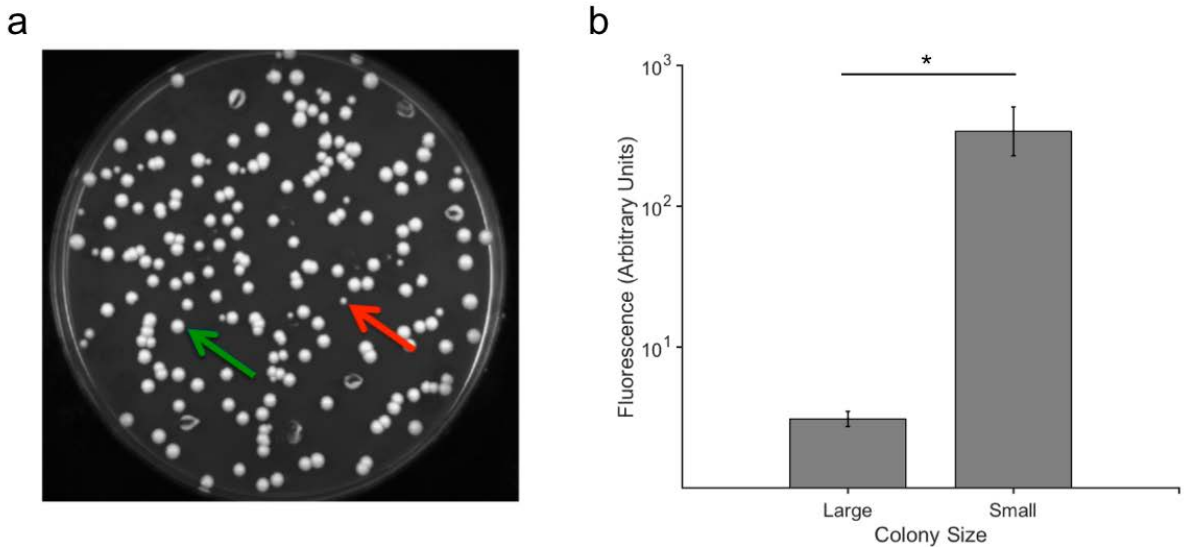


Figure 3.2: Different levels of *PDR5* expression are associated with different colony sizes. **a)** Yeast grown to mid-log phase in YPgal were plated on YPgal agar plates at a density to obtain approximately 200 colonies. The green arrow indicates an example large colony and the red arrow indicates an example small colony. **b)** yEGFP fluorescence from large and small colonies. Colonies were picked from the plate and inoculated in YPgal before assessing fluorescence by flow cytometry. Bars show the mean +/- standard deviation for five biological replicates of each colony size.
* Unpaired t-test, $p < 0.01$.

By sorting low- and high-Pdr5p cells based on colony size after plating, the fitnesses of these subpopulations could be compared. The sorted populations resulting from this method were much

more enriched for their respective *PDR5* expression state than had previously been achieved by FACS (Azizi 2014). First, three each of large and small colonies were inoculated in YPgal and their *PDR5* expression was assessed by flow cytometry (Figure 3.3a), showing that all three large colonies had low *PDR5* expression and all three small colonies had high *PDR5* expression, where each subpopulation was almost entirely exclusive of the other. These six cultures were then used to seed new cultures in either rich media (YPgal) or drug media (YPgal + 0.1 μg/mL cycloheximide) and their growth was tracked over 10 hours (Figure 3.3b). It can be seen that low-Pdr5p cells grow very well, and have the highest fitness out of all four conditions tested, when grown in rich media without cycloheximide. However, the low expressers exhibited virtually no growth when grown in the presence of cyclohximide. The high-Pdr5p cells, alternatively, were able to grow in the presence of cycloheximide, and were only slightly less fit in this condition than when grown in drug-free media. High expressers grown in rich media, however, showed a markedly lower fitness than low expressers grown in the same condition. These liquid culture growth results were supported by spot assays comparing the fitness of high-Pdr5p cells, low-Pdr5p cells, and an unsorted culture, all grown in the presence and absence of 0.1 μg/mL cycloheximide (Figure 3.3c,d). The spot assays also showed that high-*PDR5* expressers ate relatively more fit when grown in the presence of cycloheximide, but relatively less fit when grown on rich media, compared to low-*PDR5* expressers and an unsorted population.

3.4.3 Sustained high-Pdr5p expression over time

The previous observations of a small subpopulation of high-Pdr5p cells that are more fit than low-Pdr5p cells when grown in the presence of cycloheximide were replicated with a greater sorting efficiency. The next result to replicate was the maintenance of the high-PDR5 phenotype in this subpopulation. This expression memory is also the second requirement, besides the heterogeneous expression of the drug resistance gene that was just demonstrated, for non-genetic drug resistance to improve population survival. It was previously shown that the sorted high-PDR5 population shifts back to low expression after 42 hours growing in rich media. To replicate this, a small colony

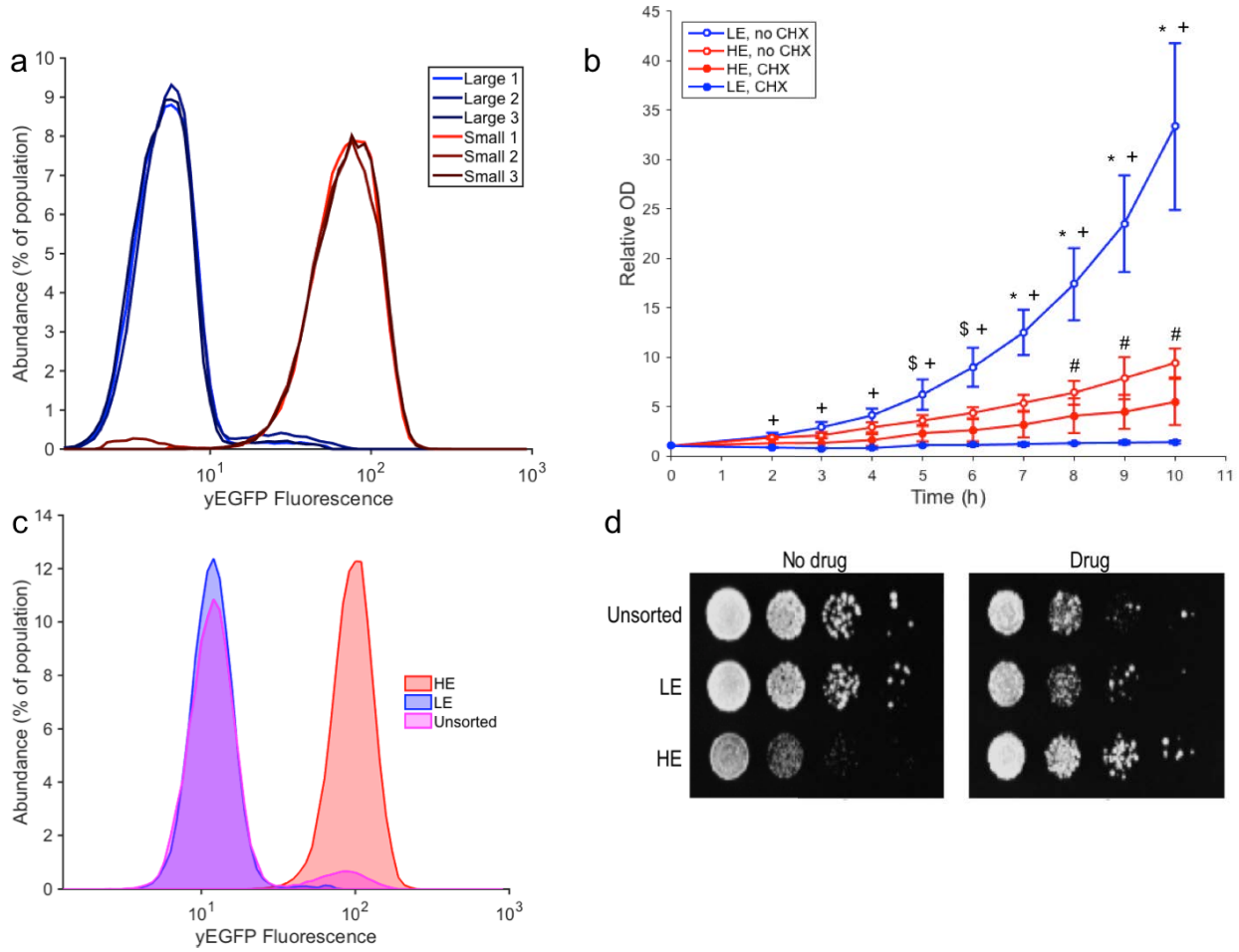


Figure 3.3: High-*PDR5* expressers are more fit in the presence of cycloheximide but less fit in rich media relative to low-*PDR5* expressers. **a)** yEGFP fluorescence of colonies picked for liquid culture growth analysis. Three each of large and small colonies were inoculated in YPgal before assessing fluorescence by flow cytometry. Large colonies are shown in different shades of blue and small colonies are shown in different shades of red. **b)** Liquid culture growth analysis for the six colonies picked and shown in (**a**). Red plots show small colonies and blue plots show large colonies. Closed circles indicate treatment with cyclohximide (CHX) while open circles indicate the absence of cycloheximde (no CHX). OD_{600} was measured every hour and normalized to the initial OD_{600} for each respective culture to obtain relative OD. * LE vs HE, no CHX $p < 0.01$; $ LE vs HE, no CHX $p < 0.05$; + LE, no CHX vs CHX $p < 0.01$; # HE vs LE, CHX $p < 0.05$ **c)** yEGFP fluorescence of colonies picked for spot assay. One large and one small colony were picked and inoculated in YPgal before assessing fluorescence by flow cytometry, along with an unsorted population. Small colony is shown in red, large colony is shown in blue, and unsorted culture is shown in purple. **d)** Spot assays of unsorted cultures and those seeded by small and large colonies. After inoculating colonies in YPgal, cultures were prepared for spot assays as described in methods. Cultures were spotted on YPgal without cycloheximide (no drug) and with 0.1μg/mL cycloheximide (drug). Pictures were taken after two days for the no drug condition and after four days for the drug condition. HE - High Expressers (seeded by small colonies), LE - Low Expressers (seeded by large colonies).

was used to seed a population of high-Pdr5p cells in rich media and *PDR5-yEGFP* expression was tracked over multiple days (Figure 3.4). It was observed that after maintaining a population of exclusively high-*PDR5* expressers for seven days, the low-expressing subpopulation begins to emerge at the eighth day and gradually increases, becoming almost completely enriched in the population at day 11. During this time, the population distribution remains bimodal, with the low-expressing subpopulation gradually increasing and the high-expressing subpopulation gradually decreasing.

3.4.4 Roles of *PDR1* and *PDR3* in *PDR5* expression

Having successfully replicated the previous results showing that *PDR5* expression is heterogeneous in a population of cells growing in YPgal media, that the high-PDR5 phenotype can be stably maintained over time, and that it is associated with increased resistance to cycloheximide, the next objective was to replicate the results of altering the *PDR5* regulatory network and the effects on *PDR5* expression. Strains had previously been made in which either *PDR1*, *PDR3*, or both were deleted (Table 3.1). These strains were similarly grown in YPgal media and *PDR5-yEGFP* expression was assessed by flow cytometry (Figure 3.5). It can be seen that there is not much difference in *PDR5* expression between the wildtype network and the *pdr1*Δ network, except a slight decrease in fluorescence of the low-expressing subpopulation. In the *pdr3*Δ network however, the high-expressing subpopulation is completely abolished, indicating that *PDR3*, the site of the positive feedback loop, is required for development of the high-expressing subpopulation. In the *pdr1*Δ*pdr3*Δ network there is essentially no *PDR5* expression and the population exhibits only autofluorescence. These results support the requirement for one of either *PDR1* or *PDR3* for the expression of *PDR5*, and that *PDR3* is required for development of the high-expression subpopulation.

Upon characterizing Pdr5p expression from network variants, replicating the previously observed results, the next objective was to controllably over-express *PDR1* and *PDR3* to observe the effects on *PDR5* expression. A gene cassette was designed to allow the controlled over-expression of a target gene using the synthetic transcription factor rtTA (reverse tetracycline-controlled trans-

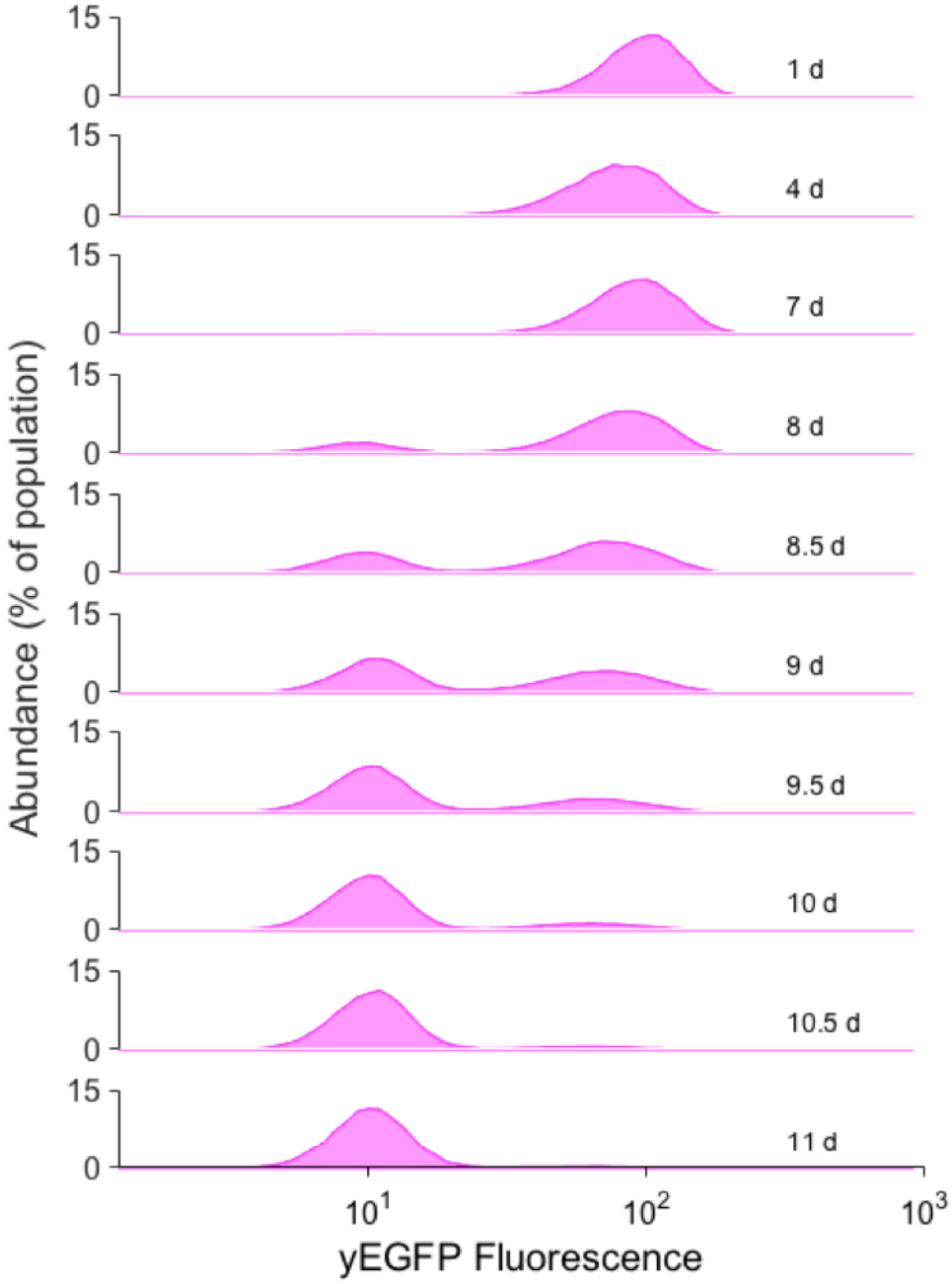


Figure 3.4: High *PDR5* expression is maintained for a prolonged period of time. A small colony was inoculated in YPgal to seed a culture of high-*PDR5* expressing cells. yEGFP fluorescence was assessed by flow cytometry every 12 hours (not every reading reading is shown). Cultures were diluted every 12 hours to maintain log phase growth.

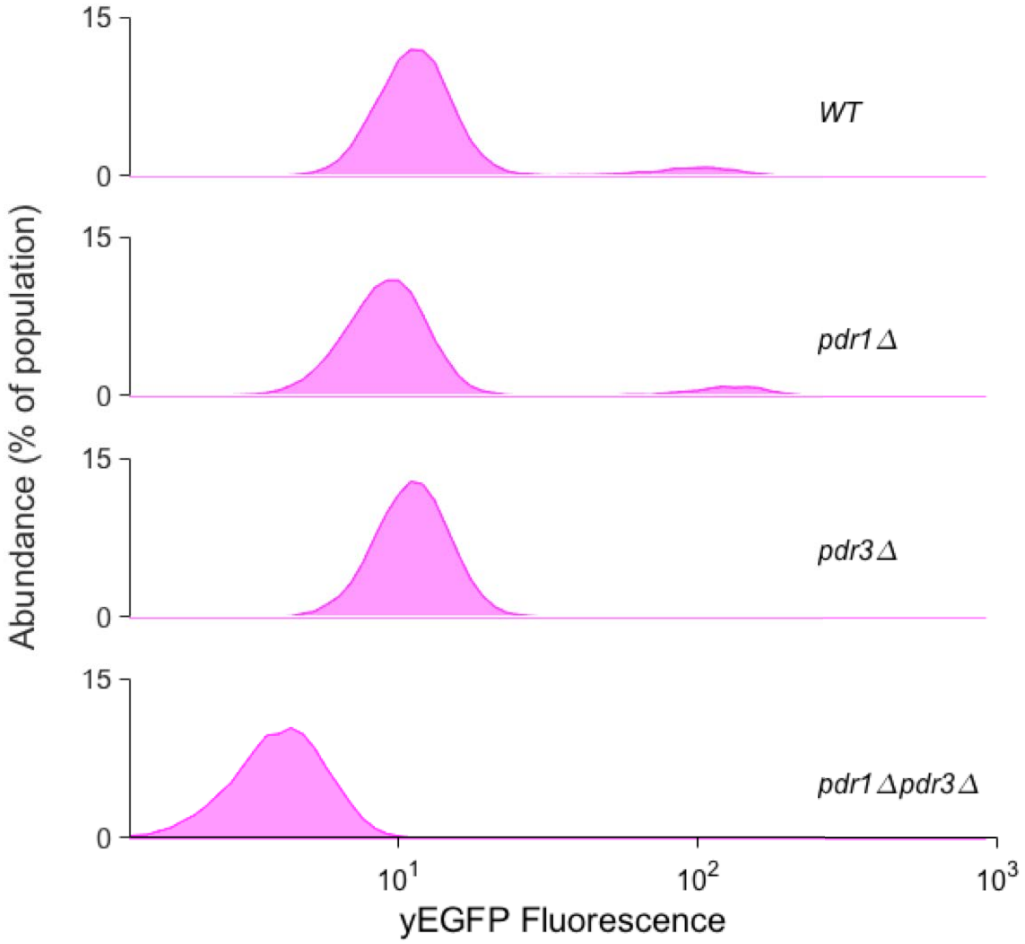


Figure 3.5: yEGFP fluorescence in *PDR5* regulatory network variant strains. Cells were grown to mid-log phase in YPgal and fluorescence was assessed by flow cytometry.

activator), which can be induced to activate gene expression at a desired level using the small molecule doxycycline (Figure 3.6). The entire cassette can be inserted upstream of a gene of interest, replacing the endogenous promoter in order to controllably regulate the expression of the gene, in this case either *PDR1* or *PDR3*. Tetracycline and its derivatives (including doxycycline), the inducers of rtTA, are substrates of Pdr5p and will thus be exported (Diao et al. 2016), altering their effective concentration inside the cell and creating a complicating negative feedback loop between *PDR5* expression and its induction by doxycycline. In order to avoid this, *PDR5-yEGFP* was replaced by just *yEGFP* at the *PDR5* locus (*pdr5Δ::yEGFP*). In this way, expression from the *PDR5* locus can be tracked without the complicating negative feedback imparted by Pdr5p-mediated ex-

port of doxycycline.

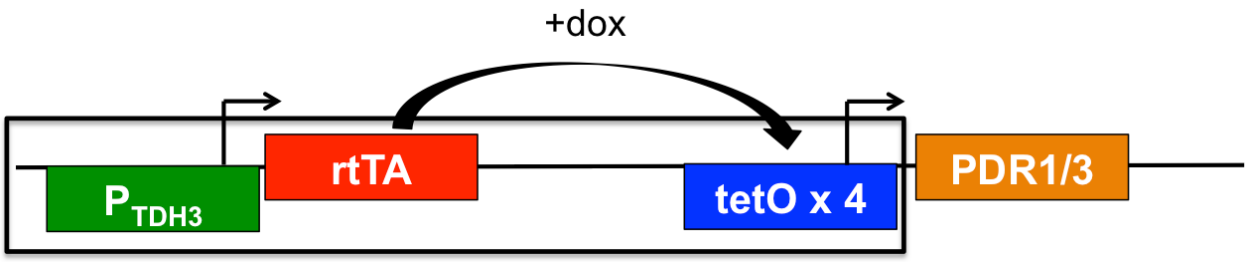


Figure 3.6: Over-expexpression cassette. A DNA cassette (boxed) was designed to include the rtTA coding sequence (red) under the regulation of the constitutive TDH3 promoter (green) upstream of a rtTA-responsive promoter with four tetO sites for rtTA binding (blue). rtTA binding is induced by doxycycline as indicated by the arrow.

3.4.5 *PDR5* expression and mitochondrial activity

An interesting observation was made when plating *pdr5*Δ*::yEGFP* cells in order to obtain single colonies for experimentation, in that both large and small colonies had formed (Figure 3.7b). This was unexpected as it had been assumed that the size difference in colonies was a result of differential *PDR5* expression, as they were essentially perfectly correlated. Investigating this further, it was observed that the *PDR5* network variant strains, in which the *pdr3*Δ strain had no high-PDR5 subpopulation and the *pdr1*Δ*pdr3*Δ strain had no *PDR5* expression at all, nonetheless gave rise to both large and small colonies (Figure 3.7c-e). These results strongly indicate that Pdr5p is not responsible for the fitness differences observed between high- and low-expressers, but instead is over-expressed as a result of some other phenotype resulting in a fitness defect.

Small colony formation is a hallmark of the petite phenotype commonly observed in yeast which cannot perform cellular respiration (Day 2013). This may result from a loss of mitochondrial DNA (as in ρ^0 yeast), mutations to mitochondrial DNA, or mutations to nuclear genes that are involved in respiration. It has also been demonstrated that *PDR5* expression is up-regulated by mitochondrial defects through a signalling pathway involving *PDR3* (Hallstrom and Moye-Rowley 2000). It appears that the small colonies that are observed in all strains are the result of mitochondrial defects which also lead, in strains possessing *PDR3*, to an over-expression of *PDR5*. This was investigated

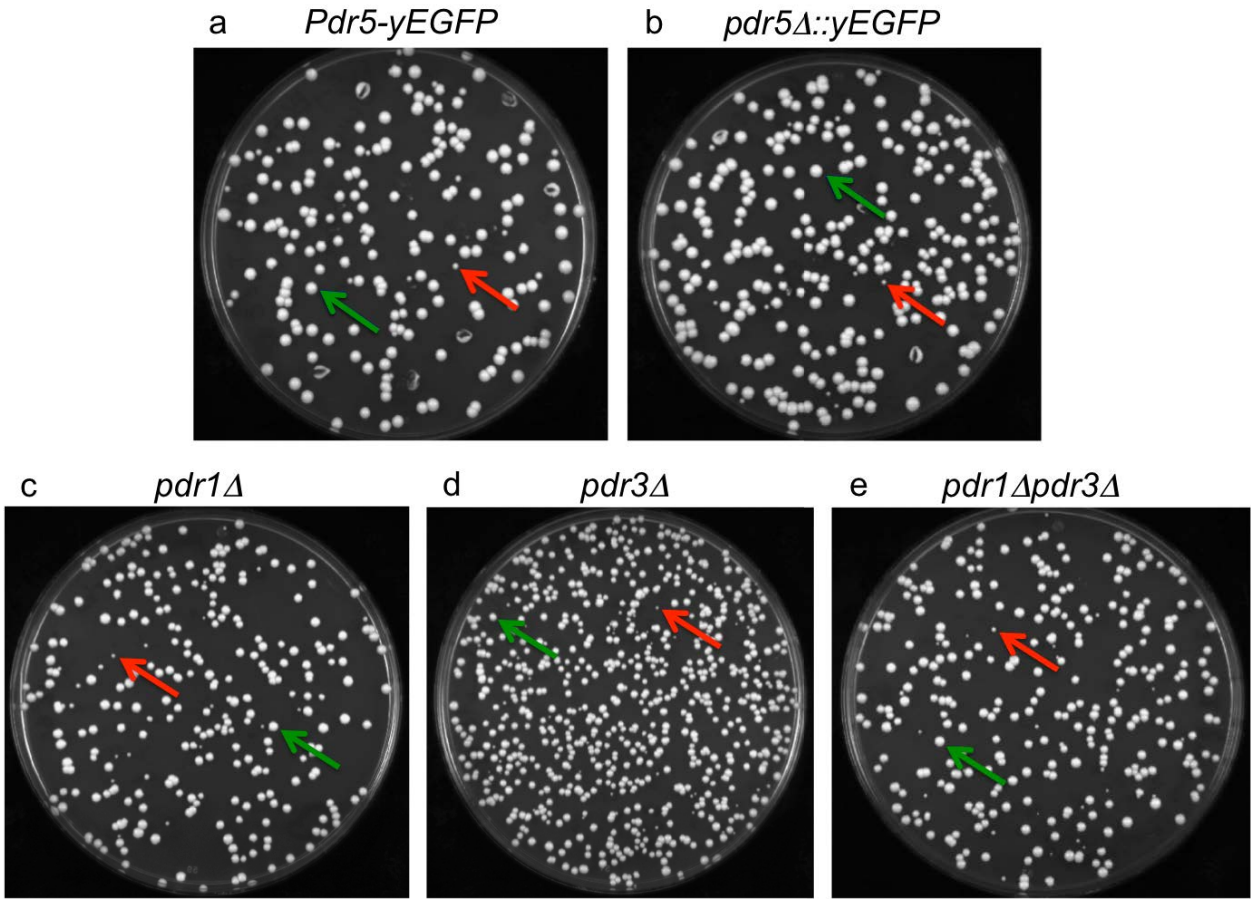


Figure 3.7: Different sized colonies are produced by all PDR network mutant strains. Cells were grown to mid-log phase in YPgal and plated on YPgal agar plates at a density to obtain approximately 200 colonies. Green arrows indicate example large colonies and red arrows indicate example small colonies.

by correlating *PDR5-yEGFP* expression with mitochondrial activity using the MitoTracker Deep Red fluorescent dye to report on mitochondrial activity in both large and small colonies (Figure 3.8). It was observed that small colonies with high *PDR5* expression had relatively lower mitochondrial activity than large colonies with low *PDR5* expression.

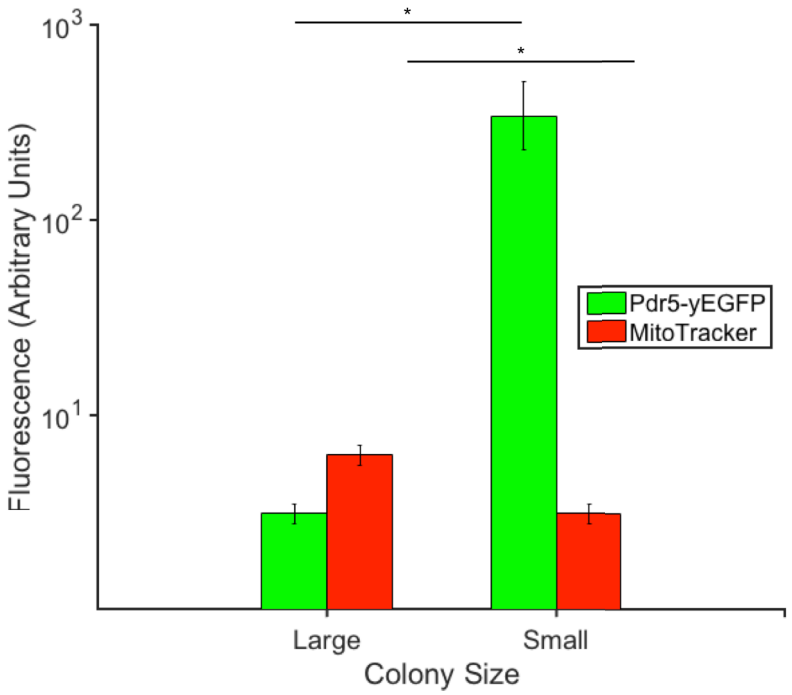


Figure 3.8: Small colonies have higher *PDR5* expression and lower mitochondrial activity than large colonies. Single colonies were picked and inoculated in YPgal before staining with Mito-Tracker Deep Red and assessing fluorescence by flow cytometry. Bars show the mean +/- standard deviation for five biological replicates of each colony size. * Unpaired t-test $p < 0.01$.

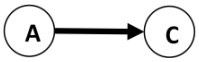
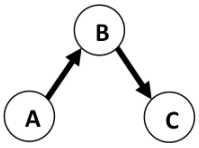
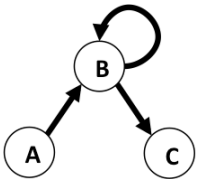
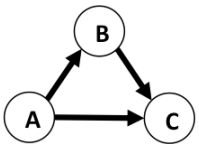
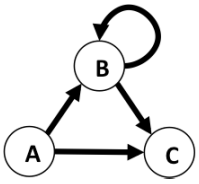
Chapter 4

Drug Resistance in Synthetic Gene Networks

4.1 Rationale

The observed heterogeneous expression of *PDR5*, which was predicted to be a result of the regulatory network formed between itself, *PDR1*, and *PDR3*, was apparently caused by mitochondrial defects in a subset of the population. Thus, the functional roles that the motifs present in the *PDR5* regulatory network play in producing heritable non-genetic heterogeneity could not be reliably investigated. To study these network motifs specifically, synthetic networks would be designed and built to mimic the natural *PDR5* regulatory network. These networks would involve a combined coherent feedforward loop and positive feedback loop, as well as variations on this architecture. The synthetic network variants, their predicted responses, and potential roles in drug resistance are presented in Table 4.1. In creating completely synthetic networks they can be isolated from the rest of the cell and other complicating interactions, allowing the observed expression dynamics and associated phenotypes to be associated to the specific network topologies with much more confidence.

Table 4.1: Gene regulatory network variants of the feedforward loop with positive feedback loop and their predicted responses, associated heterogeneity, and implications in drug resistance.

Network Topology	Response Rate	Response Duration	Degree of Heterogeneity	Potential Drug Resistance
 Direct activation	Fast	Short	Low	Induced
 Cascade	Slow	Medium	Low	Predisposed (short-lived)
 Cascade with positive feedback	Slow	Long	High	Predisposed
 Feedforward loop	Fast	Medium	Low	Predisposed (short-lived), induced
 Feedforward loop with positive feedback	Fast	Long	High	Predisposed, induced

4.2 Hypothesis

A coherent feedforward loop with a positive feedback loop on the intermediate gene can produce heritable non-genetic heterogeneity. Specifically, the coherent feedforward loop can underlie induced heterogeneity due to its ability to rapidly respond to an activating signal, and the positive feedback loop can underlie the stochastic development of heterogeneity due to its ability to amplify noisy activating signals. The positive feedback loop can also confer memory of gene expression which can be inherited by new generations of cells, while the feedforward loop also has the potential to confer memory through slow expression-switching dynamics.

4.3 Specific Aims

1. Design and construct synthetic network variations of the combined coherent feedforward loop and positive feedback loop. These should include a direct activation only network, a coherent feedforward loop network with and without a positive feedback loop on the intermediate gene, and a cascade network (a feedforward loop lacking the direct activation connection) with and without a positive feedback loop on the intermediate gene.

2. Characterize reporter expression from the synthetic network variants at varying concentrations of both inducers and assess expression memory after fully inducing networks and removing the first inducer.

3. Modify networks to regulate the expression of *PDR5-yEGFP*. Use these modified networks to relate observed expression patterns with drug resistance phenotypes.

4.4 Results

4.4.1 Synthetic network design and construction

Synthetic networks were designed and built using the common synthetic transcription factors rtTA (reverse tetracycline-controlled transactivator) and GEV (Gal4 DNA binding domain, Estradiol receptor, VP16 activation domain), and promoters containing their respective DNA binding sites. Promoters were engineered to contain different combinations of DNA binding sites to respond to one or both of the synthetic transcription factors. These promoters were used to regulate the expression of the other transcription factor and a fluorescent reporter in order to form a gene regulatory network controlling expression of the reporter gene.

Reverse tetracycline transactivator (rtTA) is a commonly used synthetic transcription factor and a derivative of tetracycline transactivator (tTA). tTA is a fusion protein composed of the TetR repressor, from the Tc resistance operon in *E. coli* (Tn10), fused to the VP16 transactivation domain from Herpes Simplex Virus, a strong transactivator (Gossen and Bujard 1992). TetR binds to the 19 base pair tet operator sequence (tetO) as a dimer, exerting its function in activating transcription of the downstream gene. While the naturally occurring TetR binds to the tetO site in the absence of tetracycline and tetracycline derivatives, the reverse TetR protein has been mutated so that it conversely binds to the tetO site only in the presence of tetracycline or a derivative (Gossen, Bender, et al. 1995). This allows the use of an inducer, typically doxycycline due to its increased stability over tetracycline, to induce binding and gene expression. Promoters can thus be engineered to contain one or more tetO sequences upstream of the minimal promoter in order to controllably activate expression of a downstream gene.

Another commonly used synthetic transcription factor in yeast is the GEV fusion protein, consisting of a Gal4 DNA binding domain (Gal4dbd) from yeast, an estrogen receptor hormone binding domain from humans, and a VP16 transactivation domain (Louvion, Havaux-Copf, and Picard 1993). GEV is normally inactive in the cell's cytoplasm, sequestered by the Hsp90 chaperone complex and prevented from entering the nucleus. Upon binding of β-estradiol to the estrogen receptor

binding domain, GEV is released from the Hsp90 chaperone complex and diffuses freely into the nucleus where it can activate transcription of a target gene. The Gal4dbd recognizes and binds to the 17-mer galactose upstream activating sequence (UAS_{gal}) as a dimer to activate expression of a downstream gene.

All synthetic networks were built using these synthetic transcription factors, where GEV acts as the first gene in the network, rtTA as the intermediate gene, and yEGFP as the downstream gene to report on expression patterns and dynamics (Figure 4.1). The *PDR5-yEGFP* gene can alternatively be placed downstream of the network to associate expression patterns with drug resistance and fitness phenotypes. GEV is placed under control of the constitutive MYO2 promoter, to mimic the predicted constitutive expression of *PDR1* in the *PDR5* regulatory network, and can be induced with β-estradiol to bind the Gal4 binding site and activate expression of a downstream gene. rtTA, the intermediate gene in the network mimicking *PDR3*, is placed under the control of an engineered promoter which can possess either Gal4 sites, tetO sites, or both, in order to respond to GEV (mimicking *PDR3* regulation by *PDR1*), and to itself (mimicking *PDR3* autoregulation). rtTA will bind to tetO sites when induced with doxycycline, activating expression of a downstream gene. yEGFP, as the downstream reporter, is similarly placed under the regulation of an engineered promoter that can possess Gal4 sites, tetO sites, or both, to respond to GEV (mimicking *PDR1*'s regulation of *PDR5*) and rtTA (mimicking *PDR3*'s regulation of *PDR5*). The promoters for rtTA and yEGFP can be designed with different combinations of synthetic transcription factor binding sites to effectively add or remove specific network connections. Using this method, synthetic networks mimicking the *PDR5* regulatory network of a combined coherent feedforward loop and positive feedback loop, and variations on this network, were built into the BY4742 background with GEV inserted at the *GAL4* locus, rtTA at the *ADE4* locus, and yEGFP at the *ADE2* locus.

Using the synthetic transcription factors GEV and rtTA, and the yEGFP reporter, promoters for rtTA and yEGFP were engineered to contain different combinations of GEV and rtTA binding sites (Gal4 and tetO sequences respectively). The direct activation network (Figure 4.2a) was the first to be built, with P_{Myo2} constitutively expressing GEV at the *GAL4* locus, and yEGFP at the

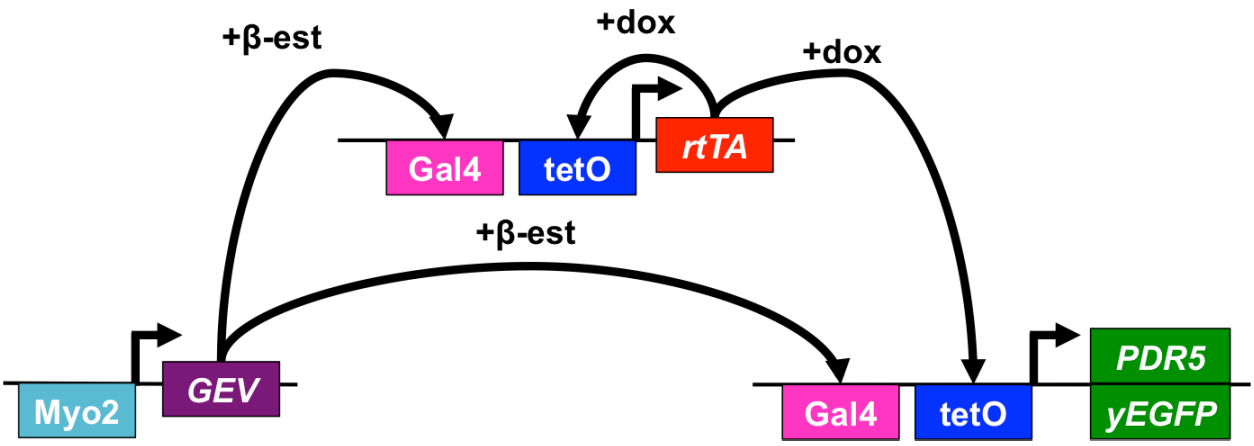


Figure 4.1: General synthetic network design. Arrows indicate activating interactions. Myo2 = MYO2 promoter, GEV = GEV coding sequence, Gal4 = Gal4 binding site (for GEV), rtTA = rtTA coding sequence, tetO = tet operator sequence (for rtTA), PDR5 = PDR5 coding sequence, yEGFP = yEGFP coding sequence, β-est = β-estradiol, dox = doxycycline.

ADE2 locus was regulated by a promoter possessing three Gal4 binding sites located 300 base pairs upstream of the transcriptional start site and two tetO sites located 100 base pairs upstream of the transcriptional start site. The presence of the Gal4 sites allows yEGFP to be regulated by GEV in this direct activation network. Building on this, the rtTA gene, regulated by a promoter possessing only three Gal4 sites, was inserted at the *ADE4* locus. The presence of this rtTA gene and promoter effectively produced a coherent feedforward loop (FFL) (Figure 4.2b), where GEV can activate rtTA and yEGFP expression, and rtTA can also activate yEGFP expression by binding to the tetO sites already in its promoter. By replacing the rtTA promoter in the FFL with a promoter possessing the same three Gal4 sites as well as two tetO sites (identical to that regulating yEGFP), a coherent feedforward loop with positive feedback (FFL+PFL) is produced (Figure 4.2c). In both the FFL and FFL+PFL networks, replacing the yEGFP promoter with one that contains only the two tetO sites, and no Gal4 sites, the direct activation connection can be removed and cascade networks, without and with positive feedback, are produced (Figure 4.2d,e). The genotypes of all of the synthetic networks can be seen in Table 4.2.

Using these synthetic networks the expression patterns and associated fitness phenotypes produced by the FFL+PFL network, and variations on this architecture, can be investigated. By com-

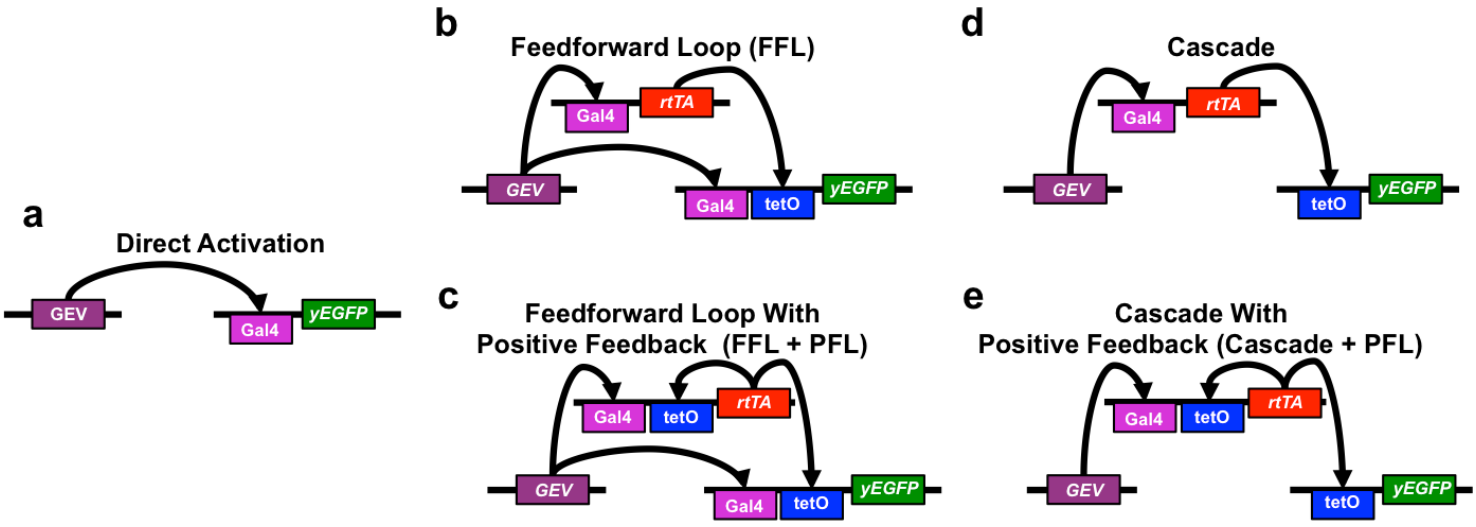


Figure 4.2: Synthetic network variants. The five synthetic networks that were designed and built to study the functional properties of the combined coherent feedforward loop and positive feedback loop. The networks include the coherent feedforward loop with positive feedback itself, and four other variants lacking certain connections. Arrows indicated activating interactions. GEV = GEV coding sequence, Gal4 = Gal4 binding site, rtTA = rtTA coding sequence, tetO = tet operator binding site, yEGFP = yEGFP coding sequence.

paring similar networks that differ by only one connection, the functional roles that specific connections play in expressing the downstream gene can be determined, as well as the effects that they have on drug resistance. The networks were first characterized by assessing the expression patterns of the downstream reporter gene, *yEGFP*, at various concentrations of both inducers. Expression memory was then assessed by tracking expression after removing the initial inducing signal. Finally, resistance imparted by Pdr5p was investigated by growing cells, with synthetically regulated *PDR5-yEGFP*, in the presence of cycloheximide under different inducing conditions.

4.4.2 Synthetic network expression characterization

The synthetic network variants were first characterized by assessing their steady state yEGFP expression at various concentrations of β-estradiol and doxycycline (Figure 4.3). Strains in log phase growth were induced for six hours to reach approximate steady state as the expression level of yEGFP did not change much before or after this time. Looking only at the average expression lev-

Table 4.2: Yeast strains with synthetic gene regulatory networks.

Strain	Genotype
BY4742	S288C[a]: *MAT*α; *his3*Δ*1*; *leu2*Δ*0*; *lys2*Δ*0*; *ura3*Δ*0*
Direct Activation (DA)	BY4742: *gal4*Δ::*HIS3-*P_{MYO2}*-GEV-*T_{PGK1}; *ade2*Δ::*KanMX-*P_{GT}[b]*-yEGFP*
Feedforward Loop (FFL)	DA: *ade4*Δ::*NrsR-*P_G[c]*-rtTA-*T_{PGK1}
FFL + Positive Feedback Loop (FFL+PFL)	DA: *ade4*Δ::*NrsR-*P_{GT}*-rtTA-*T_{PGK1}
Cascade	FFL: *ade2*Δ::*URA3-*P_T[d]*-yEGFP*
Cascade + PFL	FFL + PFL: *ade2*Δ::*URA3-*P_T*-yEGFP*

[a]The parental strain is indicated before the colon.

[b]P_{GT} promoter has Gal4 sites and tetO sites.

[c]P_G promoter has only Gal4 sites.

[d]P_T promoter has only tetO sites.

els from each network in the 2-dimensional dose response surface plots it can be seen that in the FFL networks β-estradiol can activate reporter expression even in the absence of doxycycline (Figure 4.3a,b), in contrast to the cascade networks in which there is no reporter expression in the absence of doxycycline for any concentration of β-estradiol (Figure 4.3c,d). This indicates that the direct activation connection had effectively been eliminated with the engineered promoter used to regulate yEGFP expression in the cascade networks. It can also be seen that there is more of a switch-like response in the PFL networks (Figure 4.3b,d), where there is no yEGFP expression in the absence of β-estradiol, for any concentration of doxycycline, and a steeper increase in expression within the low–moderate concentrations of β-estradiol compared to the corresponding networks lacking the PFL (Figure 4.3a,c). This ultra-sensitivity, observed as a steep, switch-like response, is a predicted characteristic of the PFL, indicating the engineered promoters for the intermediate gene in the networks can effectively respond to autoregulation.

The data presented here represent only one trial of inducing yEGFP expression from the syn-

thetic networks. This initial experiment was performed in order to asses the response of the synthetic networks to different concentrations of inducers and determine a working range for the next experiments. Similar levels of induction were routinely observed in subsequent experiments performed under the same conditions.

Expression distributions from the synthetic networks

One major benefit of using flow cytometry to assess gene expression is the ability to discern the expression in single cells, allowing the detection of heterogeneity within the population. The population distribution of yEGFP expression was thus also analyzed in the same samples for which average expression is shown in Figure 4.3. The most interesting differences in expression distribution between networks was observed at 10 nM β-estradiol, a relatively low concentration. At this level of induction, with increasing concentrations of doxycycline, yEGFP expression showed a graded increase from the FFL network (Figure 4.4a). From the FFL+PFL network however, a bimodal distribution of yEGFP expression was observed, where cells existed in either a low- or high-expressing state, with only a small portion of the population expressing yEGFP at an intermediate level (Figure 4.4b). From this network, as the concentration of doxycycline increased, so too did the size of high-expressing subpopulation, while the low-expressing subpopulation decreased. A similar trend was observed between the cascade and the cascade+PFL networks, where the presence of the PFL resulted in a bimodal population in which increasing doxycycline concentrations increased the size of high-expressing population while decreasing the size of the low-expression population (Figure 4.4c,d). It is interesting to note that expression from the cascade network (without a PFL) is slightly bimodal at intermediate concentrations of doxycycline, where cells with increasingly higher levels of expression are distinct from a subset of cells that continue to show no expression. It can also be seen that expression from the FFL networks can be induced at lower concentrations of doxycycline compared to their cascade counterparts. Expression from the FFL network is noticeable at 0.5 μg/mL doxycycline while noticeable expression is not seen from the cascade network until 1 μg/mL doxycycline, which induces expression from the FFL almost an order of magnitude higher.

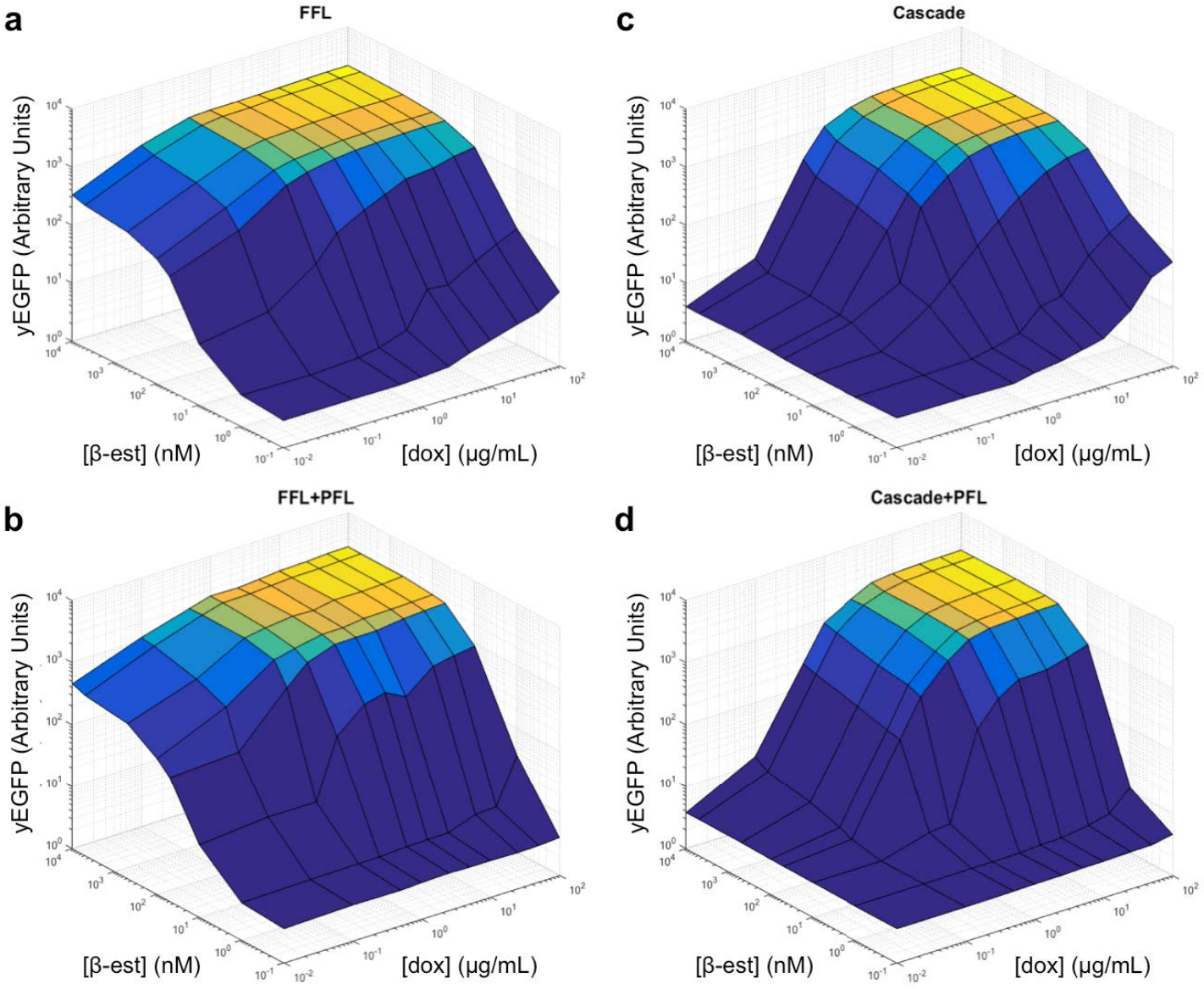


Figure 4.3: Average yEGFP expression from synthetic network variants at various levels of induction. 2-dimensional dose response surface plots for β-estradiol and doxycycline induction of yEGFP expression. Cells were grown to mid-log phase then induced for six hours with the indicated concentrations of both inducers before measuring fluorescence by flow cytometry. yEGFP expression is presented in arbitrary fluorescence units and averaged over the entire population for the feedforward loop network (FFL, **a**), the feedforward loop network with positive feedback loop (FFL+PFL, **b**), the cascade network (**c**), and the cascade network with positive feedback (cascade+PFL, **d**).

Similarly, the high-expressing population can be observed from the FFL+PFL network when induced with 1 μg/mL doxycycline whereas the high-expression population does not arise from the cascade network until induced with 2.5–5 μg/mL. Response to lower concentrations of doxycycline is also observable in comparing similar networks with and without the PFL, where the FFL begins to express yEGFP above basal levels before the FFL+PFL (at 0.5 μg/mL doxycycline as opposed to 1 μg/mL doxycycline), and the same trend is seen in comparing the cascade to the cascade+PFL (1 μg/mL doxycycline as opposed to 2.5–5 μg/mL doxycycline). Finally, at the lowest concentrations of doxycycline, expression from the cascade networks is lower than from the FFL networks, and in comparing the FFL+PFL to the cascade+PFL, the low-expressing population remains lower in the cascade+PFL network, which also appears to produce less cells with intermediate expression (Figure 4.4b,d). From these observations, it appears that the PFL is responsible for producing a bimodal population of low- and high-expressers, while the direct activation edge present only in the FFL networks is responsible for increased basal expression levels.

Rapid response to induction form the FFL

Basic characterization of expression patterns, by assessing steady-state expression from the network variants, shows that the presence of a PFL can underlie the development of stochastic heterogeneity with the presence of two distinct subpopulations, one low-expressing and one high-expressing. Stochastic heterogeneity mediated by positive feedback was one way by which heterogeneity was predicted to develop from a combined FFL+PFL. The second method is induced heterogeneity produced by the rapid response facilitated by the direct activation connection of the FFL. To assess the ability of the feedforward loop to respond rapidly to an inducing signal, each synthetic network was treated with both inducers and yEGFP expression was tracked over the time immediately after induction (Figure 4.5). From the FFL networks, yEGFP expression increases immediately within the first hour and reaches near-maximum expression level by 4–6 hours (Figure 4.5a-c). Conversely, there is no expression at all from the cascade networks until 4 hours after induction (Figure 4.5a,d,e). This indicates that the direct activation connection, the only difference between

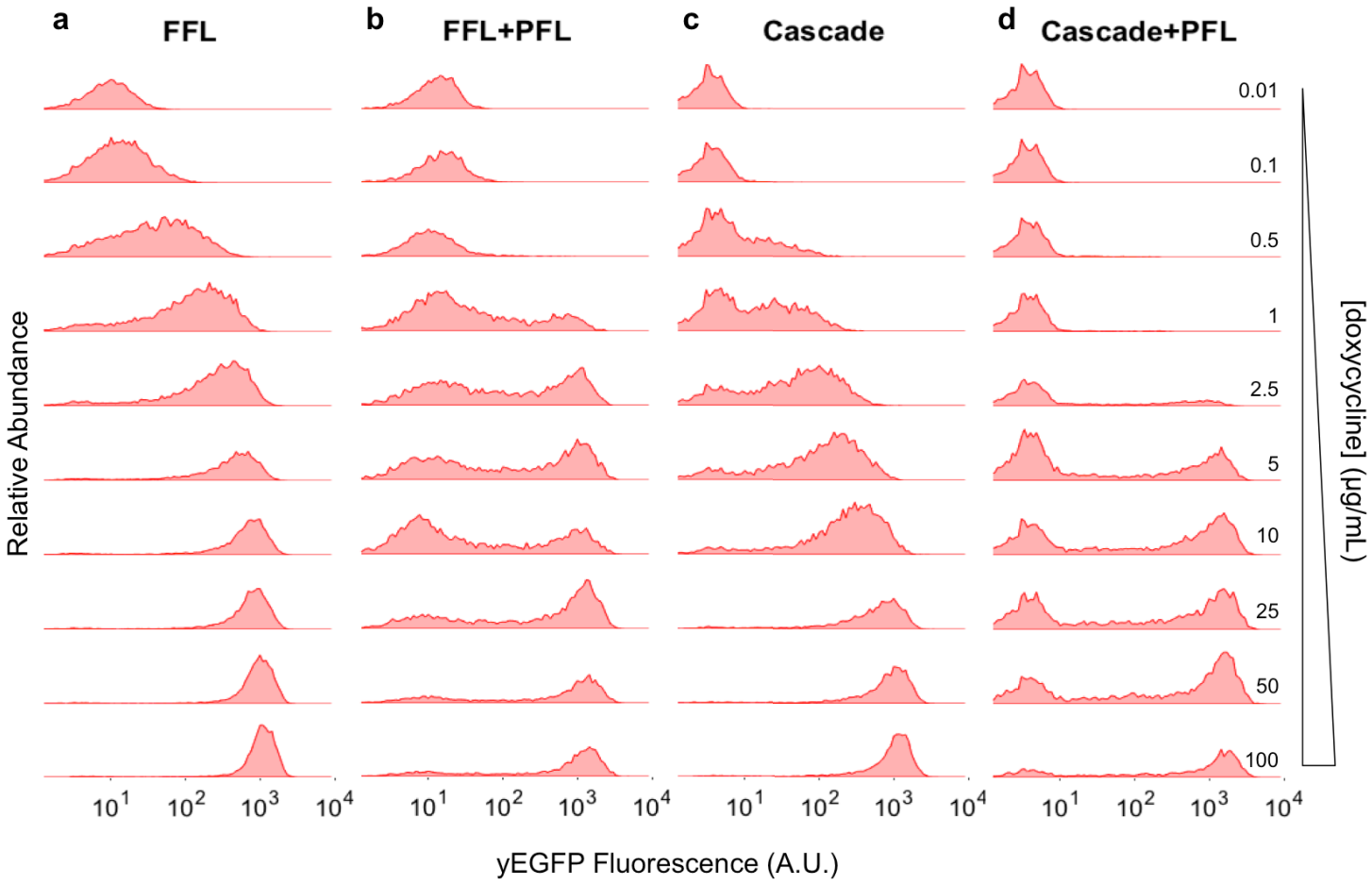


Figure 4.4: Steady state expression distributions from synthetic networks. Population distributions of yEGFP expression from synthetic network variants at 10 nM β-estradiol and increasing concentrations of doxycycline. Cells were grown to mid-log phase then induced for six hours with both inducers before measuring fluorescence by flow cytometry. Histograms show yEGFP expression for samples induced with 10 nM β-estradiol from Figure 4.3, displaying relative number of cells at each expression level. yEGFP expression is presented in arbitrary fluorescence units (A.U.) for the feedforward loop (FFL, **a**), the feedforward loop with a positive feedback loop (FFL+PFL, **b**), the cascade (**c**), and the cascade with a positive feedback loop (Cascade+PFL, **d**).

the FFL and cascade networks, can allow a rapid response to an activating signal. Further, the maximum expression level reached by the FFL networks is slightly higher than that reached by the cascade networks. It can also be seen that yEGFP expression is induced a bit faster from the FFL network than from the FFL+PFL, indicating that the presence of a PFL causes a slight delay in response speed.

Maintenance of high-expression state

It was demonstrated that the FFL+PFL network can produce non-genetic heterogeneity, through induction mediated by the direct activation connection, or stochastically, mediated by the PFL. This non-genetic heterogeneity must also be stably maintained for a biologically relevant amount of time, at least longer than one generation, in order to effectively contribute to drug resistance and population survival. It is predicted that the PFL can maintain memory of an activating signal by self-perpetuation of its own expression, and that the FFL has slow switching dynamics resulting in a delayed decrease in gene expression after removal of a signal. To investigate whether this occurs in the synthetic networks, yEGFP expression was induced maximally with both inducers before removing β-estradiol, but not doxycycline, to maintain the activity of rtTA but abolish the activity of GEV. After fully inducing the networks and removing β-estradiol, yEGFP expression was tracked over 36 hours (Figure 4.6). It can be seen that while expression from the direct activation network immediately begins to decrease (Figure 4.6a,b), the high-expression state is maintained from the FFL and cascade briefly (Figure 4.6a,c,e), remaining high for about 6 hours before beginning to decrease. The almost identical decreases in expression from the FFL and cascade networks indicate that the indirect branch of the network is responsible for this delay as this is the only similarity between the two networks. Expression is maintained for much longer from the FFL+PFL (Figure 4.6a,d), which maintains expression near maximum for at least the 36 hours shown. Both the FFL and FFL+PFL-mediated expression memory persist for longer than the time of one generation, which is approximately 2 hours. This indicates that the presence of either a FFL or a PFL can effectively maintain memory of a high-expression state for a biologically relevant time scale, where

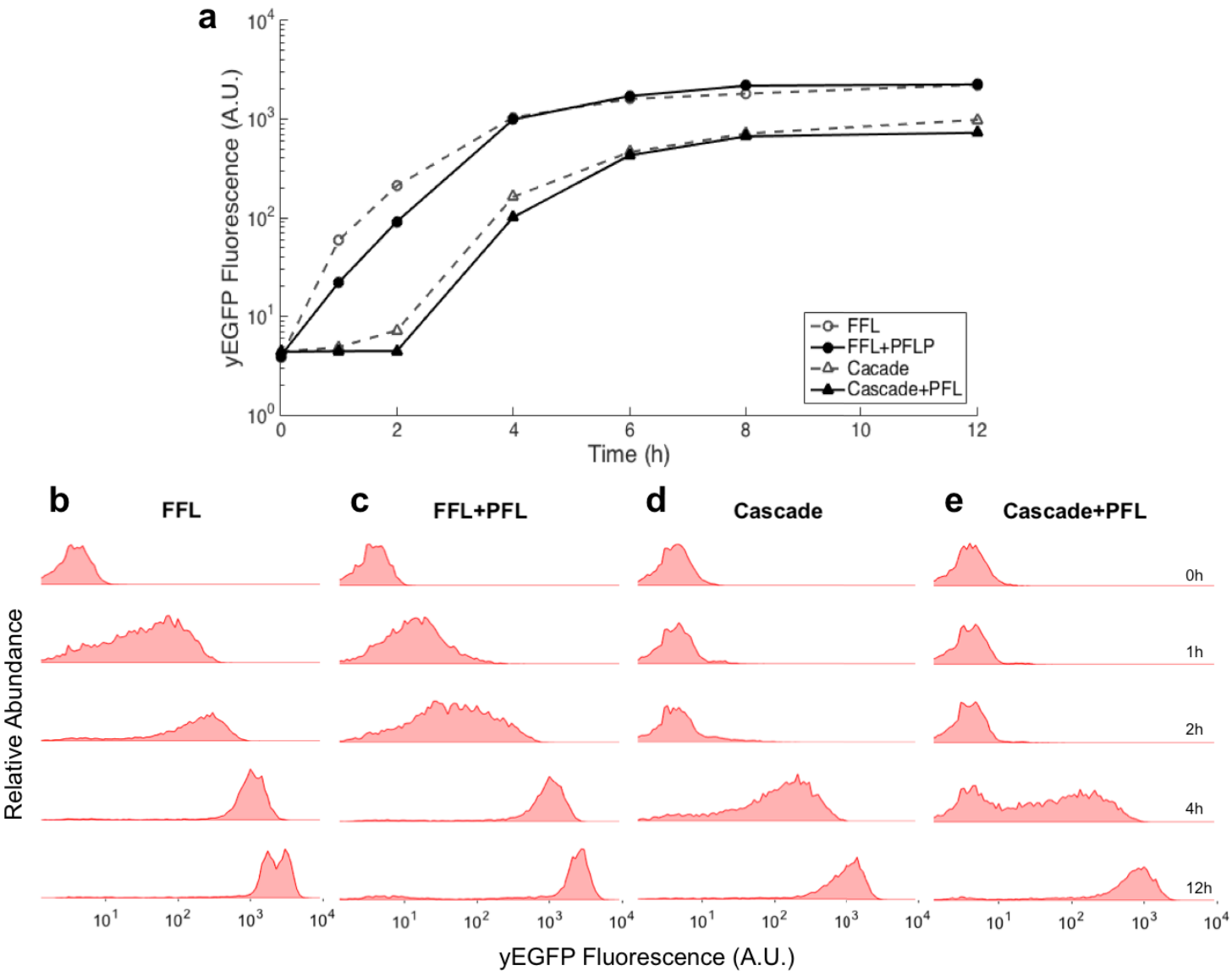


Figure 4.5: FFL networks induce expression faster than cascade networks. Cells were grown to mid-log phase then induced with 5 μM β-estradiol and 5 μg/mL doxycycline. yEGFP expression was tracked for 12 hours by assessing fluorescence by flow cytometry every two hours after the first two. **a)** Mean yEGFP expression from the synthetic strains over the 12 hours of induction. yEGFP expression is presented as arbitrary units (A.U.) of fluorescence. **b-e)** Histograms showing relative abundance of cells at each yEGFP fluorescence level, presented as arbitrary fluorescence units for select time points. Histograms are shown for the feedforward loop (FFL, **b**), the feedforward loop with a positive feedback loop (FFL+PFL, **c**), the cascade (**d**), and the cascade with a positive feedback loop (Cascade+PFL, **e**).

the PFL can maintain this state for much longer as was predicted.

4.4.3 Drug resistance from synthetic networks

Characterization of reporter expression from the synthetic network variants has shown that the FFL, specifically the direct activation connection, can underlie induced heterogeneity by responding rapidly to an inducing signal, and that the PFL can produce stochastic non-genetic heterogeneity by amplifying noisy activating signals, and can also maintain memory of high-expression states. To associate these expression patterns and dynamics with drug resistance phenotypes that contribute to population survival, the synthetic networks were modified to regulate the expression of the PDR5-yEGFP fusion protein (Figure 4.1). In this way, Pdr5p-mediated drug resistance can be induced through different dynamics using the synthetic networks, where expression can still be tracked by yEGFP fluorescence. Survival advantages conferred by different synthetic network variants were assessed by the classic spot assay using different combinations of inducers and the drug cycloheximide to mimic scenarios where different network connections are predicted to be beneficial. Although doxycycline and β-estradiol are exported by Pdr5p, the protein whose expression they induce, Pdr5p expression and associated fitness is assessed after expression reaches a steady state, when there is an equilibrium between the amounts of inducers entering the cells and being exported by Pdr5p. This particular system, where an efflux pump exports the same molecules that induce its expression, may more accurately depict the regulation of *PDR5* expression, which is predicted to be induced by some of its substrates (Thakur et al. 2008). To provide confidence that any fitness advantages observed in the presence of cycloheximide are due to the expression patterns of Pdr5p, it was first demonstrated that resistance to cycloheximide can be rescued in a *pdr5*Δ strain by inserting the *PDR5-yEGFP* gene downstream of the direct activation synthetic network and inducing expression sufficiently with β-estradiol (Figure 4.7). While naturally-expressed Pdr5p provides resistance in the wild type strain, its deletion results in sensitivity to cycloheximide and growth inhibition at the concentration used. Expressing Pdr5-yEGFP from the synthetic direct activation network, however, rescued Pdr5p-mediated resistance to cycloheximide.

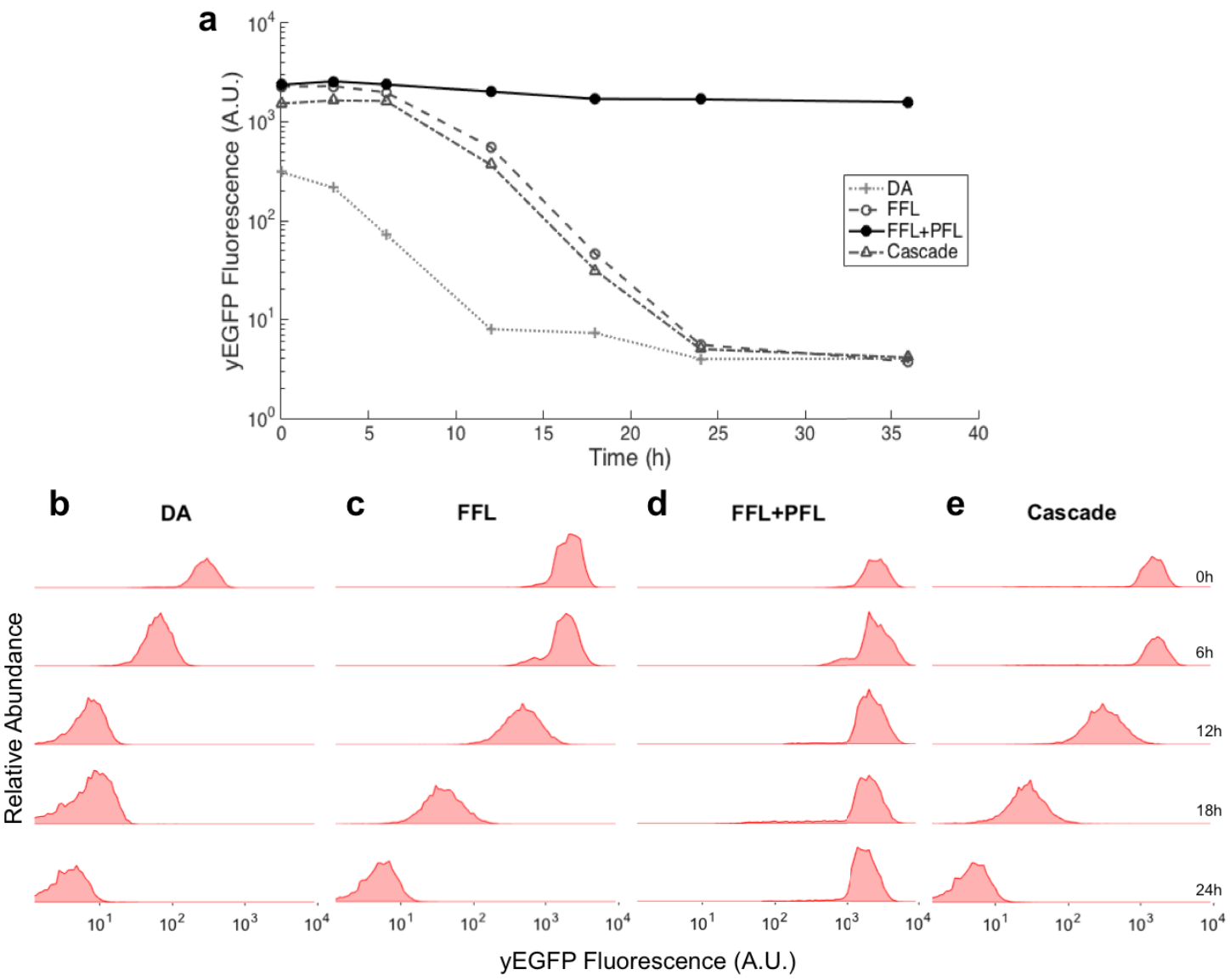


Figure 4.6: FFL maintains expression for a short period while PFL maintains memory for a prolonged period of time. Cells were grown to mid-log phase then induced with 5 μM β-estradiol and 5 μg/mL doxycycline for six hours. The cells were then washed and resuspended in fresh media with 5 μg/mL doxycycline but no β-estradiol. After removing β-estradiol, yEGFP expression was tracked over 36 hours by measuring fluorescence by flow cytometry. **a)** Mean yEGFP expression from the synthetic strains over 36 hours. yEGFP expression is presented as arbitrary units (A.U.) of fluorescence. **b-e)** Histograms showing relative abundance of cells at each yEGFP fluorescence level, presented as arbitrary fluorescence units, for select time points during the experiment. DA = direct activation, FFL = feedforward loop, FFL+PFL = feedforward loop and positive feedback loop.

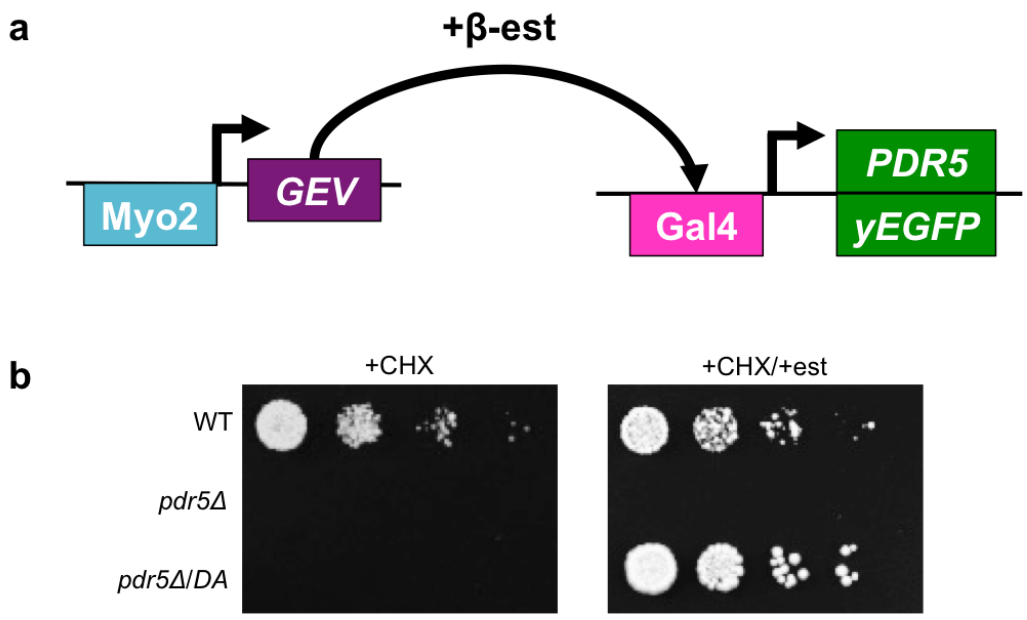


Figure 4.7: Cycloheximide resistance, lost in *pdr5*Δ, can be rescued by expressing Pdr5-yEGFP from the direct activation synthetic network. **a)** Diagram of Pdr5-yEGFP induction in the direct activation network. GEV is constitutively expressed from the MYO2 promoter and induced in the presence of β-estradiol to bind to the Gal4 binding site in the Pdr5-yEGFP promoter, inducing expression. **b)** *pdr5*Δ cells without and with the direct activation synthetic network (*pdr5*Δ*/DA*) were grown to mid-log phase and spot tests were performed on plates containing only cycloheximide (0.1 μg/mL, +CHX), or cycloheximide and 5 μM β-estradiol (+CHX/+est). WT = BY4742.

4.4.4 Induced resistance conferred by the FFL

The first predicted fitness advantage to be tested was the ability of the FFL to induce heterogeneity and thus drug resistance when the resistance phenotype is induced by the presence of a drug. To simulate this, the synthetic networks were activated at the same time as drug treatment by spotting un-induced cells on plates containing both inducers, β-estradiol and doxycycline, as well as the drug cycloheximide (Figure 4.8a,b). Pdr5-yEGFP expression was assessed prior to performing the spot assay and it was seen that all samples began with no expression (Figure 4.8a). Starting from this state of no Pdr5-yEGFP expression, only the cells with synthetic networks containing a direct activation connection, the direct activation network itself, the FFL, and the FFL+PFL, were able to survive and grow when induced at the same time as drug treatment (Figure 4.8b). This indicates that the direct activation connection in the FFL network can underly the development of induced heterogeneity, which can in turn provide a fitness advantage when rapid response to a stress signal is required.

4.4.5 Predisposed and heritable resistance conferred by the PFL

The second predicted fitness advantage from the FFL+PFL network is the predisposed high-expression of a resistance phenotype from the PFL, which would also maintain this high-expression level. This was simulated by inducing Pdr5-yEGFP expression from the networks with both inducers and then spotting the cells on a plate containing only doxycycline, to maintain the positive feedback, as well as cycloheximide (Figure 4.8c,d). Assessing Pdr5-yEGFP expression prior to performing the spot test showed that all samples had been induced to high-expression levels. Although not all samples had identical expression levels, it was most significant that the networks that differed by only the presence of the PFL began with the same level of Pdr5-yEGFP expression. It can indeed be seen that the FFL and the FFL+PFL both had the same initial expression level, as did the cascade and the cascade+PFL (Figure 4.8c). Spotting these samples on plates containing only doxycycline and cycloheximide, removing GEV activity which acted as the initial activating signal, it can be seen that only cells possessing a network with a PFL were able to survive and grow (Figure 4.8d). This

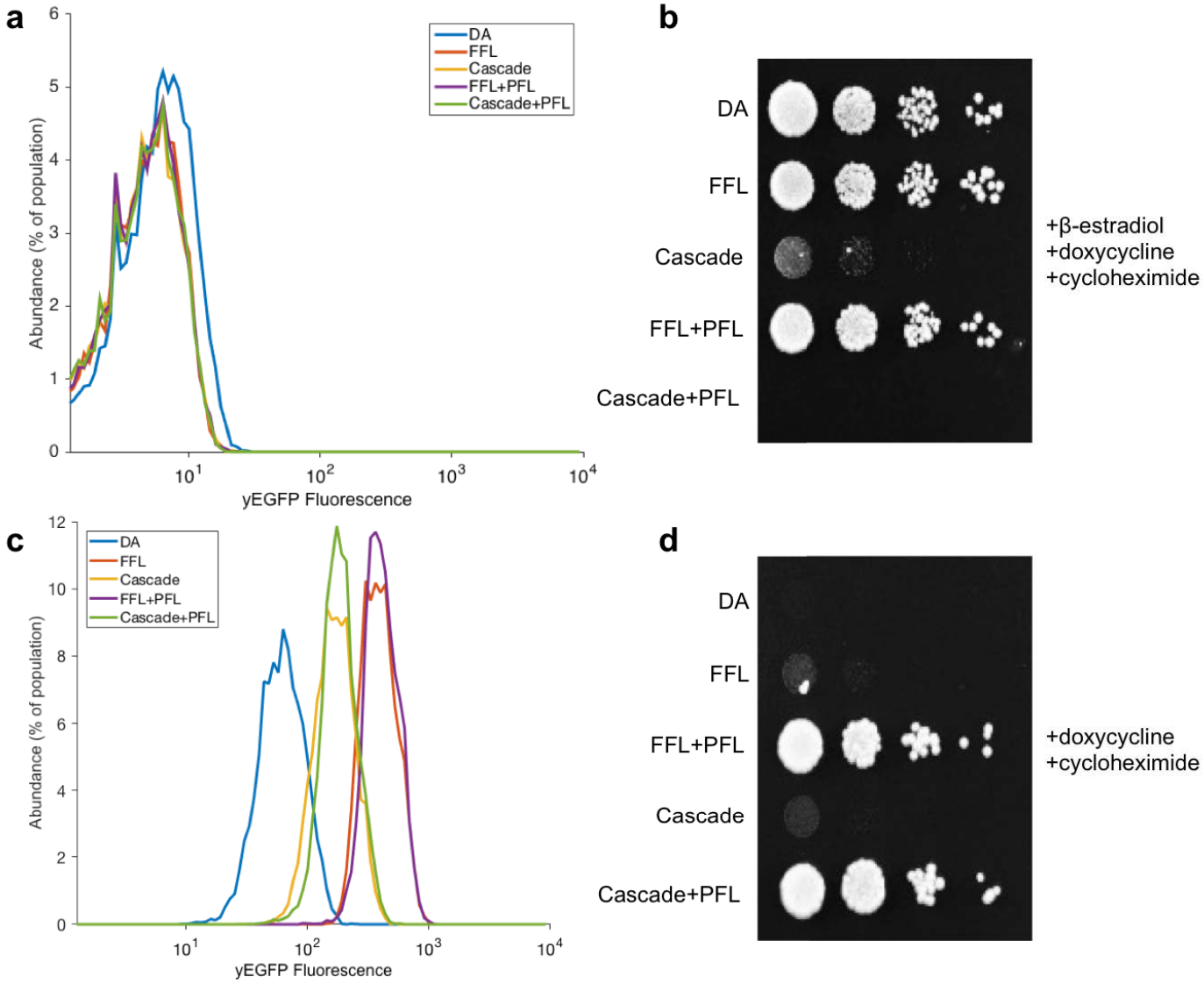


Figure 4.8: FFL networks provide induced resistance and PFL networks provide predisposed and heritable resistance. **a,b)** Cells were grown to mid-log phase and yEGFP fluorescence was measured by flow cytometry **(a)** before performing spot assays on plates containing 5 μM β-estradiol, 5 μg/mL doxycycline, and 0.1 μg/mL cycloheximide **(b)**. Plates were incubated for 5 days before taking pictures. **(c,d)** Cells were grown to mid-log phase growth before inducing for six hours with 5 μM β-estradiol and 5 μg/mL doxycycline. After 5 hours of induction, yEGFP fluorescence was read by flow cytometry **(c)** before performing spot assays on plates containing 5 μg/mL doxycycline and 0.05 μg/mL cycloheximide **(d)**. Plates were incubated for 3 days before taking pictures. Both spot assays were performed on 5 replica plates and the picture shown is representative of the 5 technical replicates. DA = direct activation, FFL = feedforward loop, FFL+PFL = feedforward loop and positive feedback loop, Cascade+PFL = cascade and positive feedback loop.

indicates that the positive feedback, which was kept active by the presence of doxycycline, is able to maintain the high-expression state for a biologically relevant length of time. The maintained high-expression state can be passed on between generations of cells and confer drug resistance to each new generation, facilitating population survival.

Chapter 5

Discussion

The development of mutation-independent heterogeneity in clonal populations has been generally overlooked despite its potential importance as a mechanism underlying drug resistance. For this non-genetic drug resistance to develop, there must exist a mechanism for the heterogeneous expression of a drug resistance phenotype within the isogenic population, which must also be heritable between generations. In this way, the population can become enriched with highly-resistant cells which can effectively buy more time for genetic mutations to accumulate and confer permanent resistance. It is known that certain gene regulatory network motifs have functional properties that may implicate them in either the development of heterogeneity or in maintaining a long-lasting and heritable expression state. It was thus predicted that the combined coherent feedforward loop and positive feedback loop (FFL+PFL) that regulate the expression of Pdr5p, a major drug resistance protein in *S. cerevisiae*, function to produce heritable and heterogeneous expression, and that this facilitated drug resistance and population survival.

5.1 *PDR5* expression

It was initially observed that *PDR5* is expressed heterogeneously in an actively growing yeast culture, with a small subpopulation of high-Pdr5p cells that was very distinct from the subpopulation of low-expressing cells. This distinctly bimodal expression pattern seemed to agree with the pre-

dicted role of the FFL+PFL in generating non-genetic heterogeneity. The switch-like characteristic of the PFL, combined with a general lack of expression activity in drug free conditions, would result in the majority of the population expressing low levels of Pdr5p while a small subset expresses high Pdr5p due to PFL-mediated amplification of stochastic activating signals. The low-expressing subpopulation still exhibited fluorescence above the level of yeast autofluorescence, indicating a possible role for the direct activation of *PDR5* by *PDR1* in maintaining a basal level of *PDR5* expression. This would also agree with previous studies that found that Pdr1p is constitutively bound to the *PDR5* promoter (Fardeau et al. 2007).

5.1.1 *PDR5* expression could underlie a bet-hedging mechanism

The seemingly fortunate observation that the high- and low-Pdr5p subpopulations could be easily sorted based on the size of the colonies they formed, where low-expressing cells seeded large colonies and high-expressing cells seeded small colonies, pointed towards a fitness disparity between the different states of *PDR5* expression. It would be reasonable to assume, and was indeed observed, that high-Pdr5p cells have increased fitness in the presence of a drug that is a substrate for Pdr5p. It was also observed that these cells were less fit, relative to the low-Pdr5p cells, when grown in normal, drug-free conditions. It appeared that high-Pdr5p cells had similar levels of fitness when grown with or without drug treatment, while low-Pdr5p cells grew either very well without drug treatment, or did not grow at all when treated with cycloheximide. This indicates that high Pdr5p expression allows cells to maintain a constant level of fitness, despite the environmental conditions, enabling their continued growth in the adverse conditions of drug treatment. This is in contrast to low-expressing cells in which fitness fluctuates drastically with the environmental conditions. The observed survival strategy, in which some cells in the population assume a phenotype that lowers their fitness in ideal conditions but provides them with a fitness advantage in adverse conditions, resembles the bet-hedging risk-spreading strategy. Bet-hedging has been proposed as a heterogeneity-based survival strategy for single-cell populations inhabiting unpredictably fluctuating environments (Veening, Smits, and Kuipers 2008). The requirements for bet-hedging are that

phenotypic heterogeneity exists in the population, that different levels of fitness are associated with each phenotypic state, and that the heterogeneity is not genetically-encoded, allowing individuals in the population to dynamically switch between states (de Jong, Haccou, and Kuipers 2011). Through bet-hedging, cells can essentially sample different phenotypes by dynamically switching between states without becoming fixed in a state that is not favourable. This stochasticity-based dynamic state-switching should result in a consistent ratio of cells in each possible phenotypic state, some of which may not provide optimal fitness in the current conditions. However, if the environment should change unexpectedly to one in which one of these phenotypes provides a fitness benefit, the subset of cells that assumed this state will be able to at least survive, while the rest of population may not. In this way, the population as a whole sacrifices some amount of absolute fitness in optimal conditions in exchange for the possible ability to survive if unfavourable conditions should be encountered. As a risk-spreading strategy, this will provide the most advantage when the loss of absolute fitness in optimal conditions is outweighed by the potential benefit gained in adverse conditions. If the benefit gained is survival of the population as opposed to complete extinction, this would likely outweigh any potential loss of absolute fitness in optimal conditions.

It was also observed that the high-Pdr5p phenotype could be stably maintained over an extended period time, days to weeks, which would allow it to be passed between generations in order to maintain population survival. In the previous work the high-expression state was only observed to persist for a couple of days at most, however this observation was made in populations that still contained a relevant fraction of low-expressing cells. As the low-Pdr5p phenotype was shown to be relatively more fit in optimal conditions, it is likely that the faster decrease in the size of the high-Pdr5p population was a result of the low-Pdr5p cells merely enriching the population due to a faster growth rate, and not to high-expressing cells reverting back to a low-expression state. When the subpopulations were more effectively sorted by picking different sized colonies from the plates, those seeding high-Pdr5p cultures were essentially free of any low-Pdr5p cells and the high-expression phenotype persisted for much longer. Once a low-expressing population began to arise, likely due to the spontaneous state switching of one or a few previously high-expressing cells, this

population of low-Pdr5p, high fitness cells could again overtake the rest of the population simply through faster growth.

While bet-hedging is not the only survival strategy associated with non-genetic heterogeneity, it appeared that it could be a likely mechanism occurring in yeast through *PDR5* expression, regulated by a FFL+PFL network. There was a fitness disparity between the low-Pdr5p and high-Pdr5p subpopulations, and the high-Pdr5p population was abolished when *PDR3*, the site of the PFL which would be responsible for the switch-like state switching, was deleted. It was also shown that either phenotypic state could persist for a long enough period of time to be passed between generations and would thus be biologically relevant in protecting the population against drug treatment.

5.1.2 *PDR5* expression is linked to mitochondrial activity

The observation that all strains, including PDR network variants in which *PDR1*, *PDR3*, both *PDR1* and *PDR3*, or *PDR5* had been deleted, produced both large and small colonies did not agree with the proposed bet-hedging mechanism. It appeared that *PDR5* expression was not responsible for the fitness defect, but merely associated with it. It has been shown that *PDR5* can become over-expressed in response to mitochondrial defects through signals that are mediated by *PDR3* (Hallstrom and Moye-Rowley 2000). It is likely that the small colonies are produced by cells expressing the petite phenotype due to loss or mutation of their mitochondrial DNA (Bernardi 1979). These defective mitochondria would independently result in a decrease in fitness when grown in optimal conditions and in an over-expression of *PDR5* through Pdr3p signalling. This would explain why small colonies were still observed in *pdr3*Δ cells, but the high-Pdr5p subpopulation was not.

The commonly used strain BY4741, which was used as a background for all strains used in this part of the project, is derived from the S288C strain (Brachmann et al. 1998). S288C and thus its derivatives posses a mutation in the *MIP1* allele (Young and Court 2008), which codes for a mitochondrial DNA polymerase, resulting in an increased frequency of the petite phenotype. The *mip1* mutation results in an increased incidence of replication errors in the mitochondrial DNA and thus an increase in the amount of mutations in mitochondrial DNA, resulting in an elevated chance

of disrupting mitochondrial activity. It was indeed observed that small colonies, with high *PDR5* expression, also had lower mitochondrial activity than large colonies with low *PDR5* expression. The appearance of small colonies, now assumed to be of the petite phenotype, is actually quite puzzling as these cells, without the ability to perform oxidative phosphorylation, should only be able to grow in the presence of a fermentable carbon source. One hallmark of the S288C strain, and its derivatives, is the deletion of the *GAL2* gene, preventing it from utilizing galactose anaerobically (Mortimer and Johnston 1986). This should mean that petite cells, which can only survive on anaerobic respiration, should not be able to grow in the presence of galactose, which was the carbon source used for experiments investigating the PDR network. One possible explanation is that the yeast that are observed to form small colonies when grown on galactose are not true ρ^0 petites that lack the entire mitochondrial genome. The high-Pdr5p cells did still exhibit mitochondrial activity, supporting this theory that they are not completely deficient in mitochondria, but may harbour mutations in specific genes that both cause a defect in respiration and are also linked to Pdr5p over-expression. It has been demonstrated previously that certain mitochondrial genes, such as *FZO1*, *TIM17*, and *OXA1*, are specifically responsible for up-regulating *PDR3* and thus *PDR5* (Hallstrom and Moye-Rowley 2000). Mutations in these genes specifically could cause a decrease in, but not complete loss of, mitochondrial activity. These respiratory-deficient mitochondria would then signal through Pdr3p to the PDR network, up-regulating the expression of *PDR5* along with other PDR genes. This means of over-expressing PDR genes may still underlie a bet-hedging strategy for population survival, where the increased resistance resulting from mitochondrial deficiency does provide a fitness advantage when treated with a drug and a fitness defect otherwise. The low-fitness, high-Pdr5p state did not appear to be permanent, as populations of exclusively high-Pdr5p cells eventually reverted back to low-expression states, allowing cells to avoid becoming stuck in this non-optimal phenotypic state. The reversion that was observed, however, was likely not due to stochastic state switching as was predicted. It is possible that these reversions occurred due to the accumulation of further mutations that rescue the petite phenotype, as it has been shown that increasing nucleotide pools, possibly by over-expression of *RNR1*, can negate the defects caused

by *mip1* mutation (Lecrenier and Foury 1995; Baruffini et al. 2006). It is also known that in wild type populations, mating between petite and non-petite yeast results in non-petite offspring (Clark-Walker and Miklos 1975), which may be another potential way for high-Pdr5p cells to revert back to a low-expression state. This mechanism, however, could not underlie the reversions observed here as mating type is homogeneous within each strain. Another possible explanation for the observed reversion is that some fraction of mitochondria in the high-Pdr5p cells became dysfunctional while others remained fully functional. Expansion of the pool of functional mitochondria, overtaking the dysfunctional pool, could thus potentially underlie the reversion from high to low *PDR5* expression.

Non-genetic heterogeneity in *PDR5* expression resulting from defective mitochondria in petite yeast could underlie a potential risk-spreading survival strategy. However, the heterogeneity evidently does not result from the functional properties of the FFL+PFL network that regulates *PDR5* expression. Although this may be an interesting mechanism contributing to drug resistance and population survival, the initial hypothesis was that the combined functional properties of the FFL and PFL result in more dynamic non-genetic heterogeneity, which could underly drug resistance and survival. As evidenced by the unexpected complicating interactions between the PDR network and mitochondria, endogenous networks are difficult to study specifically. Isolated, synthetic networks present one option for investigating the properties of the networks themselves.

5.2 Synthetic gene networks

Synthetic networks were successfully designed and built to mimic the FFL+PFL network, as well as variations on this network that differ by only one or two network connections. This enabled the investigation of each network connection, the expression patterns it mediates, and the associated drug resistance phenotypes. The major functional properties predicted to be associated with the FFL+PFL network, and that would contribute to drug resistance and population survival, are the development of non-genetic heterogeneity and its stable maintenance and heritability. Non-genetic heterogeneity may develop either stochastically due to the noisy nature of gene expression, or it

may be induced by some activating signal, each of which may result from one of the network motifs in the FFL+PFL. The heritable maintenance of a phenotypic state is predicted to result from slow dynamics in gene expression state switching, which may also be produced by either the FFL or PFL motifs, although through different mechanisms.

5.2.1 Synthetic networks and non-genetic heterogeneity

The stochastic development of non-genetic heterogeneity is predicted to occur when noisy activating signals are amplified into a distinct difference in gene expression, which can be mediated by a PFL. This heterogeneity manifests as the co-existence of more than one gene expression state in a population exposed to the same environmental conditions. This was observed in the synthetic networks when induced with a relatively low level of β-estradiol, simulating a low-level activating signal, and various levels of doxycycline, simulating various positive feedback strengths. In networks lacking the PFL, there was a unimodal distribution of yEGFP expression among the population, with the entire population showing an increase in expression in response to increasing concentrations of the second inducer doxycycline. Alternatively, networks which possessed a PFL exhibited a bimodal distribution of yEGFP expression within the population, where the subpopulation of high-expressing cells grew in response to increasing concentrations of doxycycline, or increased positive feedback strength. This demonstrates the ability of the PFL to underlie the development of stochastic non-genetic heterogeneity as two different expression states were observed to coexist in the same environmental conditions. Heterogeneity was only observed at a low–intermediate level of activation by β-estradiol. This represents a potential sweet spot where there is enough GEV activity to express rtTA and initiate the PFL, but not too much activity that all cells in the population express rtTA maximally. This agrees with previous work on noise in eukaryotic gene expression which demonstrated that noise is greatest at intermediate levels of expression (Blake et al. 2006). Within this sweet spot of network activation, the portion of the population existing in each expression state is probabilistic and depends on the strength of the positive feedback. With relatively weak positive feedback, the noisy activation will be amplified in only a small portion

of the population in which the initial level of rtTA activity surpasses the threshold to activate the PFL. With strong positive feedback, however, any activation of rtTA expression will be sufficiently amplified to surpass the threshold, activating the PFL and resulting in the entire population existing in the high-expressing state. In a naturally-occurring FFL+PFL network, both the basal level of activity and the strength of the PFL could be tuned through evolution to produce a population distribution in which an optimal proportion of cells are high-expressers. This optimal proportion may be relatively low, if there is a fitness penalty associated with the high-expressing state as in the bet-hedging strategy, or relatively high if the high-expressing state does not confer as much of a fitness defect. Regardless of the size of the high-expressing population, through this mechanism there will exist some subset of cells that are high-expressers. If the gene in question is related to drug resistance, this subset of cells will be predisposed with a high-resistance phenotype and will thus be amply prepared to survive a drug treatment should it be encountered.

The second means by which non-genetic heterogeneity may develop is through induction by some environmental signal that alters the gene expression landscape (S. Huang, Guo, et al. 2007). In this way, cells can switch from one phenotypic state to another without genetic alteration. This new phenotype may develop in all or in a subset of the population in response to the environmental cue, depending on the mechanism that exists to respond to it. To achieve a widespread response of expressing a significantly different phenotype, it would be beneficial for individual cells to respond rapidly to the inducing signal and reach a state of significantly high expression, both of which could result from regulation by a FFL. Studying the synthetic networks demonstrated that activation is induced rapidly from a FFL as compared to a cascade network, where the main difference between the two is the direct activation connection. This difference is intuitive as the ability to directly activate the downstream gene, as opposed to having to activate an intermediate gene first, is expected to result in a faster response. It was also demonstrated that regulation by a FFL network ultimately results in a higher level of expression than regulation by a cascade, which is likely due to the dual input from both branches of the FFL, where the transcription factors likely activate expression in an additive manner. Finally, it was demonstrated that response is slightly faster in the PFL-free

networks than in those possessing a PFL, indicating a potential benefit of having the PFL in a separate branch of the network, so that the direct activation can act independently. Rapid activation that culminates in an increased expression level would be beneficial when regulating the expression of a drug resistance gene. This was demonstrated by spot assays showing that only networks with the direct activation connection, like the FFL networks, can respond fast enough to survive when exposed to a drug at the same time as inducing expression of the drug resistance gene.

5.2.2 Synthetic networks and drug resistance

Heterogeneous expression of a drug resistance gene, produced either stochastically through the PFL or induced through the FFL, would only be relevant for population survival if it persisted long enough to be inherited by future generations of cells. The PFL was shown to effectively maintain states of high-gene expression, even after the initial activating signal was removed, for long enough that the population could survive drug treatment. Networks lacking a PFL, however, could not survive these conditions after removal of the initial activating signal, despite displaying essentially identical levels of expression when first encountering the drug. It was demonstrated previously that the presence of a FFL confers sufficient memory of a beneficial high-expression state for a population to survive drug treatment, and that the presence of a PFL merely enhances this effect, but was not required for survival (Daniel A. Charlebois, Balázsi, and Kaern 2014). Although it was observed that a FFL can delay turning gene expression off, this short expression memory did not appear sufficient to confer drug resistance. The discrepancy between this *in vivo* observation and the previous *in silico* observation may be explained by the choice of parameter values used in the simulations, as it was also demonstrated that at certain levels of induction the FFL+PFL network will respond faster than the direct activation network, which is contrary to what was previously reported (Maeda and Sano 2006). It is likely, then, that certain parameters used in the simulations are significantly different from those dictating the actual biological systems, at least in these experiments. Heterogeneous expression of a drug-resistance gene in a population is thus not sufficient for survival, but this heterogeneity, in particular the high-resistance phenotype,

must be maintained for a biologically relevant period of time in order for new generations of cells to benefit as well.

While the properties of the FFL and PFL were studied separately in general, the observations can be combined into a more complete picture of how a FFL+PFL regulatory network can produce heritable non-genetic heterogeneity to facilitate drug resistance and population survival. In a population of isogenic cells, there may exist a subpopulation predisposed with high resistance due to noisy activation of the regulatory network that is amplified by the positive feedback loop. If the population encounters a drug, these cells will be primed to resist and will continue to survive. At the same time, the presence of the drug may induce expression of the drug resistance phenotype in the rest of the population, which would occur rapidly through the direct activation connection of the FFL. This would help ensure that a larger portion of the population survives the drug treatment as opposed to only the small subset that had been predisposed with high resistance. Reaching a high-expression state through either of these mechanisms will allow the cells to survive the initial drug treatment, but if treatment is persistent or recurrent the cells may dynamically revert back to states of low-expression. These cells would lose the resistance phenotype, becoming susceptible once again to drug treatment. Through expression memory conferred by the PFL, the high-expressing cells will be able to maintain this high-resistance phenotype for a prolonged period of time, passing it on to new generations of cells, and the population in general will be able to either continue resisting the drug treatment, or will be predisposed to resist it should it be encountered in the future.

A couple of issues may be realized when considering the above scenario of drug resistance facilitated by a FFL+PFL network. These issues mainly pertain to potential redundancies between the two motifs in the network. First, the stochastic development of predisposed high resistance, mediated by the PFL, may appear unnecessary if the resistance phenotype can also be effectively induced by the presence of the drug through the FFL. One potential benefit of having the PFL combined with the FFL is that the cells predisposed with high resistance may be better able to initially survive drug treatments at higher doses. These doses could negatively affect non-resistant cells before they have a chance to induce expression of the resistance gene to a high enough level. Pre-

disposed resistance would thus mainly be beneficial for surviving sudden treatments of extremely high doses, whereas it may play less of a role in surviving lower doses that allow the resistance phenotype to be sufficiently induced in the rest of the population. Alternatively, the stochastic development of heterogeneity may merely be a side effect of the presence of the PFL in the network. The major role of the PFL might only be to maintain memory of the high-expression state, while the main mechanism of reaching this state may be through induction. Relying mainly on induced heterogeneity for resistance is a potential mechanism to avoid a fitness penalty associated with the expression of a drug resistance phenotype in the absence of the drug. One aspect of the bet-hedging survival strategy is that the different phenotypes are associated with different fitnesses, where resistant cells are less fit than non-resistant cells in the absence of a drug, resulting in an overall decrease in fitness for a mixed population. If the drug resistance phenotype must be induced, and is not expressed in the absence of the drug, then the population can avoid this fitness penalty when in optimal, drug free, conditions. The PFL, then, would mainly be required for maintaining the resistance phenotype after it has been induced. It was indeed shown that without memory imparted by the PFL, cells that encounter a drug even while expressing a resistance gene at a high level are unable to survive as this high-expression level is eventually lost.

The second issue is related to the role of the PFL in maintaining memory of the high-resistance state, which might be considered redundant if the population can respond to the presence of a drug sufficiently fast through the FFL. First, it may be that the resistance phenotype is only induced immediately upon exposure to the drug, and that induction subsides during prolonged treatment. In this case memory imparted by the PFL would benefit the population by maintaining the high-resistance state after the induction subsides. Second, it has been theoretically demonstrated that the ability to respond to environmental changes, or phenotypic plasticity, is most beneficial when the environmental fluctuations have some aspect of consistency or predictability (Botero et al. 2015). Drug treatment would not be described by either of these characteristics, and so the survival advantage of being able to induce a resistance phenotype in the presence of a cytotoxic drug may be limited. Much of the issue with a redundant mechanism for memory may also stem from the

experimental methods used, in that the resistance phenotype was represented as the expression of a single efflux pump that, according to the predictions inspired by the *PDR5* regulatory network in yeast, confers resistance to the same drugs that induce its expression. Many drug resistance phenotypes, however, are much broader than the expression of a single gene. A benefit of maintaining a resistance phenotype induced by one stress or a single drug might then be that the population will be predisposed to resist different drugs or stresses in the future, stresses that might not themselves induce expression of the resistance phenotype. This may be gleaned from the experiment showing that networks with a PFL can survive drug treatment in the absence of an activating signal, where the drug treatment does not itself induce expression of the resistance phenotype.

These issues should still be addressed experimentally, where the relative fitness benefits of each network motif can be compared as opposed to merely elucidated individually as was done here. While the classic spot assay is useful for qualitatively assessing fitness in different conditions, it does not allow observation of the finer dynamics leading to this steady state. It would be interesting to perform similar experiments in liquid culture using microscopy where both the expression of the resistance gene and cell fitness can be assessed simultaneously and over shorter time scales. Using this technique, it would be possible to assess the immediate effects of exposure to a drug and how each of the different network variants responds. This would allow the observation of whether the predisposed resistance resulting from the PFL does in fact confer a fitness advantage immediately upon encountering a potentially high dose of drug. Similarly, if different strains are tagged with different reporters, network variants can be competed against each other to determine relative advantages imparted by different network connections in different scenarios. For example, a FFL strain could be competed against a FFL+PFL strain to determine which is better at responding initially to a drug treatment, and which is better at continuing to survive through a prolonged treatment. Different pre-conditions of network induction may also be used to determine possible advantages associated with varying proportions of cells with predisposed resistance.

Long-term experiments may also be performed in liquid culture, exposing the network variants to different regimens of induction and drug treatment and assessing the evolution of the popula-

tion. At different levels of positive feedback strength, cultures can be exposed to both induction and drug treatment either persistently or periodically at various levels and frequencies. The development and possible fixation of drug resistance can then be monitored. It is theoretically predicted that non-genetic drug resistance can act as a sort of pre-selection, allowing the population to become enriched with resistant cells that can eventually become permanently resistant. It would be interesting to use these long term experiments to determine whether heritable non-genetic drug resistance imparted by the FFL+PFL does eventually facilitate the accumulation of genetic mutations conferring permanent resistance. Previous studies investigating the relative benefits of high-noise gene regulatory modules and the resulting stochastic heterogeneity have demonstrated that higher noise is favoured and may even evolve in populations growing in stressful conditions (Bódi et al. 2017) but also that this stochasticity is only favoured in conditions of variable and fluctuating stress, whereas a more stringent signal-response mechanism is favoured in more stable environments (New et al. 2014). The results here indicate that a FFL+PFL network can underlie both of these mechanisms, where the PFL can generate stochastic heterogeneity and the FFL can respond rapidly to a stress signal. As it has been demonstrated that network modules can evolve differently depending on the environmental conditions (González et al. 2015), it would be interesting to see if either of these different functionalities are favoured when strains are evolved in environments that are either more variable or more stable. It may be predicted that variable environments will maintain or strengthen the role of the PFL, relaxing constraints on the FFL. Alternatively, more stable environments might favour the maintenance of the FFL and may weaken or even eliminate the PFL if its associated predisposed heterogeneity proves too costly.

Chapter 6

Conclusion

It appears that the *PDR5* regulatory network that initially inspired the investigation into the role of a combined FFL+PFL in facilitating drug resistance and population survival does not play as important of a role as was predicted. The use of synthetic networks, however, did allow the functional properties of these networks to be studied more specifically. It was observed that *PDR5* is naturally expressed bimodally, but that this heterogeneous expression is likely due to a relatively high frequency of petite yeast which up-regulate *PDR5* expression in a *PDR3*-dependent manner. Since the *PDR5* regulatory network did not appear to play as much of a role in mediating a useful drug resistance phenotype as was predicted, synthetic networks mimicking the *PDR5* regulatory network, and variants, were constructed to study instead. Through studying these synthetic networks, it was observed that non-genetic heterogeneity can develop either stochastically through noise amplification by the PFL or by induction through the FFL direct activation connection. It was also shown that the PFL can act to provide memory of a high-expression state after the initial activating signal is removed. The indirect activation present in the FFL and cascade only temporarily delays a decrease in expression. Both the development of non-genetic heterogeneity and its heritability are theoretically required for drug resistance to develop in an isogenic population and confer a survival advantage. This was indeed seen as the FFL was responsible for inducing drug resistance and the PFL played a major role in maintaining memory of a high-resistance phenotype long enough for

the population to survive.

Issues concerning potential redundancies between the FFL and PFL in facilitating non-genetic drug resistance remain to be addressed experimentally, as do the combined properties of the FFL+PFL network in responding to drug treatment both immediately and over a prolonged period of exposure. While these properties were studied individually, it will be interesting to further study their combined effects on development of non-genetic heterogeneity and associated implications for drug resistance and survival. Upon fully characterizing the functions and benefits of regulating a drug resistance phenotype using a FFL+PFL network, or perhaps a variation, it would also be interesting to investigate potential strategies to combat resistance by targeting the regulatory network along with, or instead of, the effector gene or protein itself. For example, the first transcription factor in the network could be targeted so that the drug resistance response cannot be induced as rapidly, where the delay in response may allow sufficient time for the drug to exert its effects (as was demonstrated through the delayed response from the cascade networks). Targeting the positive feedback loop could also be useful as a pre-treatment to abolish a potential subpopulation of cells predisposed with high resistance, or to prevent the prolonged maintenance of a high-expression state. In doing so, even if resistance is induced by drug treatment it will eventually subside, and a prolonged exposure to the drug could eventually wipe out the population.

The role of the FFL+PFL in producing heritable non-genetic heterogeneity was investigated in relation to development of drug resistance in a non-resistant population and the associated implications on population survival. This non-genetic drug resistance in clonal populations will continue be an exciting area to study in society's constant battle against drug resistance in infections and cancers. The underlying mechanisms can also shed light on the development and maintenance of non-genetic heterogeneity in general, an important biological phenomenon that has implications in other areas such as development of optimally fit single-cell populations, and development and differentiation of multicellular organisms.

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