Construction and Optimization of Tetracycline-Responsive Gene Expression Systems

by

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Abstract

Conditional gene expression systems that enable inducible and reversible transcriptional control are essential research tools and have broad applications in biomedicine and biotechnology. The reverse tetracycline transcriptional activator is a canonical system for engineered gene expression control that enables graded and gratuitous modulation of target gene transcription in eukaryotes from yeast to human cell lines and transgenic animals. However, the system has a tendency to activate transcription even in the absence of tetracycline and this leaky target gene expression impedes its use. Here, we identify single amino-acid substitutions that greatly enhance the dynamic range of the system by reducing leaky transcription to undetectable levels while retaining high expression capacity in the presence of inducer. Furthermore, we show that these improved DNA binding domains can be fused to repression domains to create synthetic transcriptional repressors. The function of these transcriptional repressors is dependent on the location of their recruitment and their mechanisms of action.

Much of the data presented in this thesis was re-adapted for a paper that was submitted and has recently been published in Scientific Reports [77] under a CC BY license (Creative Commons Attribution 4.0 International License) and as such I retained all copyright of the published work. I am the lead author of the manuscript and the sole trainee contributing to the study. I conducted all the experiments, wrote the manuscript with assistance from my advisor, Dr. Kærn, designed the study with Dr. Kærn and Dr. Couture, and received assistance in the lab from Dr. Rudner.

This work has lead to the filing of a provisional patent on new rtTA variants, which is currently being completed in partnership with the University‘s Technology Transfer Office.

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Introduction

What I cannot create, I do not understand - Richard Feynman

1.1 Transcriptional regulation

1.1.1 Basic principles of transcriptional initiation in Eukaryotes

Gene expression is largely regulated at the level of transcriptional initiation. Transcriptional initiation occurs in the promoter region of the gene to be transcribed and is dependent on the proper assembly of the pre-initiation complex (PIC) [2, 62], illustrated in Figure 1.1. For most protein coding genes, the PIC includes the general transcription factors (TFIIA, B, D, E, F, H) and RNA polymerase II (RNA pol II). The assembly of the PIC is initiated by the binding of TFIID to the promoter followed by TFIIA and TFIIB; the remaining general transcription factors and RNA polymerase II are then recruited to the promoter. Upon successful assembly of the PIC, the RNA polymerase can then transcribe the gene from the transcriptional start site (TSS), while the majority of the PIC remains at the promoter, ready to initiate another transcriptional event [15].

The assembly of the PIC is directed by trans and cis-regulatory elements. Cis-regulatory

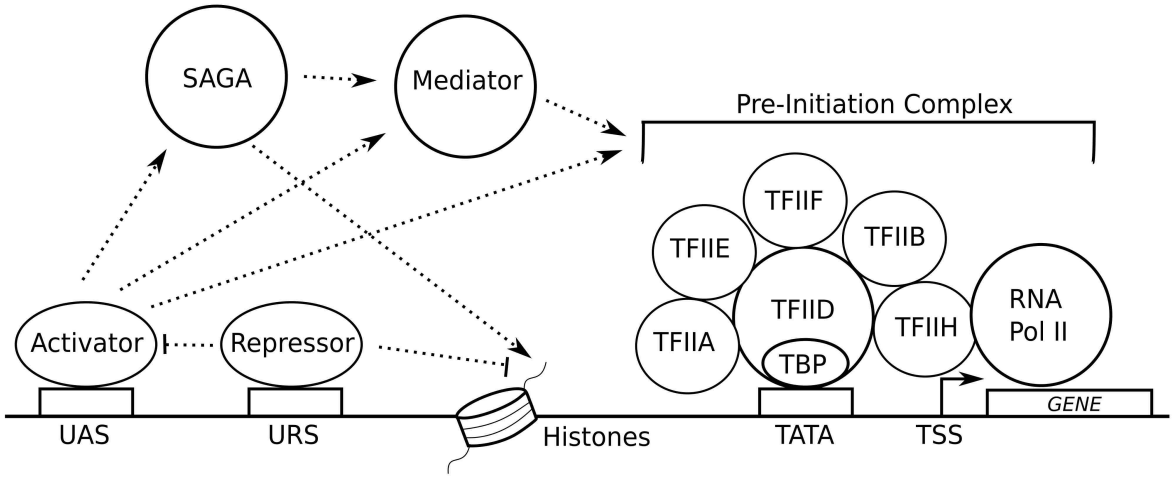

Figure 1.1: Schematic of transcriptional regulators and the pre-initiation complex at a TATA-containing promoter. The arrows represent stimulatory interactions that can occur. The blunt ended arrows represent inhibitory interactions that can occur. Adapted from [62]

elements (CREs) are DNA sequences that influence transcription "on this side of" the gene. These DNA sequences, originally called "operators" by Monod and Jacob [51], are found upstream of the gene they regulate and serve as binding sites for proteins. Trans-regulatory elements are DNA sequences that influence transcription from "across" the gene. These DNA sequences are often genes themselves, which are transcribed into mRNA and translated into diffusible transcription factors that can bind cis-regulatory elements [51].

Cis-regulatory elements can be further divided into two classes, promoter proximal elements and distal elements [62]. Promoter proximal elements include the TATA box and initiator element, which are mutually exclusive in eukaryotic promoters [62], and serve as the DNA scaffold for the assembly of the PIC. In yeast, the initiator element or TATA box is generally found within 25 to 50 nucleotides of the TSS [62]. Approximately 20 % of yeast promoters contain TATA boxes and these promoters are typically actively regulated and associated with stress responses, whereas TATA-less promoters display more constitutive

behaviour and drive the expression of house-keeping genes [50, 8, 15].

Distal cis-regulatory elements are found in upstream activating sequences (UAS) and upstream repressing sequences (URS) and serve as binding sites for transcription factors which can up-regulate or down-regulate the basal transcription rate. In yeast, UASs and URSs are typically found within 1500 nucleotides of the transcriptional start site [95] and have a local effect on transcription [76]. Although less common, longer-range transcriptional silencing mechanisms guided by cis and trans-regulatory elements do exist [76].

1.1.2 Transcriptional activation

Transcriptional activators are transcription factors that up-regulate the transcription rate and can be largely classified into two categories, activation by recruitment or activation by directed enzymatic activity.

The activation by recruitment model suggests that DNA bound transcriptional activators have contacts with components of the transcriptional machinery and recruit these components to the promoter region [74]. This results in a higher effective concentration of essential transcriptional machinery at the promoter and increases the probability of successful PIC assembly and subsequent transcriptional events. These interactions can occur directly between the transcriptional activator and PIC, as has been shown in artificial recruitment studies [33, 34], or indirectly through interactions with the mediator complex [13, 22], as illustrated in Figure 1.1.

On the other hand, the activation by enzymatic activity model, suggests that DNA bound transcriptional activators recruit enzyme complexes to the promoter region which up-regulate transcription. Enzyme complexes, such as SAGA and SWI/SNF, increase the transcription rate primarily by altering local chromatin structure, by acetylating histones [6] or repositioning nucleosomes [86]. This has been shown to increase the accessibility of the core promoter to the transcriptional machinery [87] and therefore increases transcrip-

tional output.

Transcriptional activators have been found to be highly modular, with distinct domains responsible for DNA binding or activation [82]. This modularity has allowed for the rational engineering of synthetic activators by mixing and matching DNA binding domains (DBDs) and transcriptional activation domains (TADs). The ability to mix-and-match protein domains is instrumental in synthetic biology because it allows for the creation of novel activators that can conditionally regulate natural [68] or synthetic promoters [67, 66]. Furthermore, separating the DNA binding and activation domains of activators lead to the development of the powerful yeast two hybrid technology for identifying protein-protein interactions that can occur *in vivo*.

Transcriptional activation domains (TADs) are highly conserved among different transcriptional activators. The most widely studied transcriptional activators in yeast; Gal4, Pho4 and Gcn4 all have regions abundant in acidic amino acids and these regions are essential for their activation capacity [87]. Acidic activation domains have been shown to work in every eukaryotic system they have been tested in, including yeast, drosophila and mammals [82]. The herpes simplex virus that infects mammalian cell lines also uses an acidic activation domain, VP16, to activate the expression of its immediate-early genes. The VP16 activation domain is very potent and is one of the most widely used transcriptional activators in synthetic biology [47].

Acidic activation domains have no apparent secondary structure as individual proteins, but form alpha helixes when they interact with their target proteins [37, 75]. Acidic activating domains have been found to interact with components of the SAGA complex, mediator, and PIC [41]. It is believed that these acidic regions act as an adhesive [75] that can stick to many components of the transcriptional machinery and recruit them to the promoter. With substantial contacts between acidic activation domains and the PIC and the SAGA complex, it is likely that acidic activation domains function by both recruiting the transcriptional machinery, and by directing enzymatic activity to the promoter. This

mechanism would explain how these activation domains are so potent, as a single transcriptional activator at a promoter could recruit multiple components of the transcriptional machinery and alter local chromatin structure [61]. Furthermore, it may allow for synergistic increases in transcription rates when multiple activators are recruited to the same promoter, as each activator can make contacts with different parts of the transcriptional machinery [21].

Since transcriptional initiation is the first step in gene expression it is often desirable from a synthetic biology point of view to put regulation at this step, preventing un-necessary transcription, which may be a burden on the cellular metabolism. Strong, acidic, activation domains such as VP16 are important tools for constructing synthetic transcriptional activators as their redundant mechanisms of activation allow them to be recruited to various regions near a promoter to induce transcription [20, 54, 76]. Whereas, activators that depend on the recruitment of specific pieces of the transcriptional machinery are very location specific [33, 34, 22]

1.1.3 Transcriptional repression

The foundational work of Monod and Jacob on the lac regulatory operon of *E. coli* [51] was the first study of transcriptional regulation. While studying *E. coli* growth in different carbon sources, namely glucose and lactose, they observed a differential growth rate when switching between the two sugars. They hypothesized that the bacteria did not waste resources producing the enzymes required for lactose metabolism when glucose was present and the lactose metabolic pathway was negatively regulated by the presence of glucose. By culturing *E. coli* in various levels of lactose and measuring the production of the key lactase enzyme, β-Galactosidase, they confirmed their hypothesis. This work uncovered the role of cis and trans regulatory elements in regulating gene expression and initiated the investigation of transcriptional repressors in controlling prokaryotic transcription. The lac operon

has a single promoter that controls the expression of genes essential for the metabolism of the sugar lactose. In the absence of lactose the key component of the system, LacR, represses the promoter. LacR is a DNA binding protein that recognizes a palindromic operator site. There are three lac operators, which are located in the core region of the lac promoter, with one operator positioned between the transcriptional start site and the first gene of the operon[72]. By inhibiting the passage of the RNA polymerase to the gene, transcription of the operon is repressed[51, 24]. Continued work on the lac operon has uncovered that the two additional operator sites in the promoter contribute to the repression by enabling DNA looping through LacR-LacR interactions and further inhibiting the RNA polymerases ability to access the promoter or transverse the gene[72]. The lac operon has served as a model system of transcriptional repression and experimental and theoretical investigations of it continue to this day [84]. Similar mechanisms of transcriptional repression mediated by DNA binding proteins were found in many other bacterial systems, such as the TetR repressor of the bacterial tetracycline resistance transposon[43] and the LexA repressor of the *E. coli* SOS DNA damage response[30]. It is now widely accepted that prokaryotic transcriptional repression is primarily achieved through promoter occlusion, mediated by DNA binding proteins[87].

As the modular nature of eukaryotic transcriptional regulators was being uncovered [82], researchers attempted to use bacterial repressors in yeast[17]. By inserting lexA operators into the core *GAL1* promoter and expressing a yeast codon optimized lexA protein, they were able to repress transcription from the promoter[17]. Repressors based on the bacterial LacR and TetR were subsequently found to function in a similar way [39, 5] and because they were inducible with lactose/IPTG or tetracycline derivatives, DNA binding could be controlled by the addition of small molecules.

The finding that bacterial repressors could function in eukaryotes was a key step in establishing the field of synthetic biology, which aimed to create synthetic gene regulatory networks and characterize the biophysical properties of gene expression[32, 29, 10]. The

field of synthetic biology continued to popularize and characterize bacterial mechanisms of transcriptional repression in eukaryotes by creating libraries of eukaryotic promoters that could be repressed by TetR or LacR [70, 28]. These promoter libraries have been used extensively in yeast[15, 65, 16, 71], yielding insight into the role of noise in gene expression[15] and have allowed for the predictable construction of synthetic gene regulatory networks [28].

Despite the success of using bacterial transcriptional repression mechanisms in eukaryotes, there is little evidence that these mechanisms are naturally used. Eukaryotes and prokaryotes use fundamentally different mechanisms to regulate transcription[87], most notably, prokaryotes lack histones. Without chromatin, bacterial promoters are considered 'open' in the ground state and are fully accessible to the transcriptional machinery[87]. Repressors are therefore needed to inhibit strong promoters. In eukaryotes, chromatin is able to maintain a closed ground state where core promoters that have high affinity to the transcriptional machinery *in vitro*, are inaccessible to the transcriptional machinery *in vivo*, in the absence of transcriptional activators [58, 87]. Enzymes that regulate chromatin structure play a large role in eukaryotic transcriptional regulation and many transcriptional regulators have been found to recruit chromatin-modifying enzymes.

The two most studied transcriptional repressors in yeast are the Sin3-Rpd3 complex and the Ssn6-Tup1 complex. DNA binding transcription factors recruit these complexes to promoter regions where they function as gene-specific repressors, rather than long-range silencers.

The Sin3-Rpd3 complex is a histone deacetylase enzyme with broad deacetylase activity on the histone tails of H3 and H4 [81]. The Rpd3 protein is responsible for the deacetylase activity of the complex and the artificial recruitment of Rpd3 to a promoter is sufficient for repression[52]. Furthermore, the deacetylase activity of the enzyme is essential for its activity as a single amino acid substitution in the core of the deacetylase motif eliminates its repressive function[52]. The deacetylase activity of Sin3-Rpd3 is highly localized to the

site of recruitment and the majority of deacetylation occurs within 1-2 nucleosomes[53], unlike long-range silencing mechanisms where deacetylation of histones can spread far from the site of nucleation [80].

The mechanism of repression by the Ssn6-Tup1 complex however is far from solved. The Tup1 protein is responsible for the majority of repression that is observed by the complex, as artificial recruitment of Tup1 to a promoter is able to mediate repression in the absence of Ssn6, whereas Ssn6 recruitment alone only represses transcription minimally[89]. The Tup1 domain has been proposed to repress transcription through a variety of mechanisms including: interactions with the mediator, recruiting histone deacetylases, stabilizing nucleosome positions, interfering with activators and more[94]. The most recent and compelling research to determine the mechanism of the Ssn6-Tup1 complex used the anchor-away technique, which can rapidly remove Tup1 from the nucleus. Using this technique, researchers conditionally removed Tup1 from the nucleus and by using ChIP, quantified components of the transcriptional machinery at Ssn6-Tup1 regulated promoters. Upon depletion of Tup1 from the nucleus, the researchers saw rapid assembly of the SWI/SNF, PIC, mediator and SAGA complexes at the promoters, as well as deactelyation of surrounding histones and increased transcriptional output[94]. Upon releasing Tup1 back into the nucleus, these same components dissociated from the promoter and transcription was repressed. The authors hypothesized that Tup1 likely masks activation domains and what previous researchers had identified as histone deacetylase activity, was actually a masking of activator mediated acetyltransferase activity[94].

Surprisingly, there have been few uses of natural repression domains in the field of synthetic biology. However, they have been used to perform a quantitative comparison of transcriptional repression and transcriptional silencing mechanisms [76]. Transcriptional silencing in yeast is a long range repressive structure that is prevalent at the telomeres and mating loci [80]. Silencing is mediated by the Sir2, Sir3 and Sir4 proteins of the SIR complex, which polymerizes along chromosomes. Researchers tagged TetR domains with

either the Sir3 protein, of the SIR silencing complex, or with the Ssn6 repression domain and recruited them to a promoter expressing a fluorescent reporter. By placing binding sites at various locations upstream of the promoter, they were able to measure how the repression strength changed as a function of distance. They observed that both silencers and repressors lost their repression ability in a graded manner as they were recruited to locations farther from the gene. However, the silencing protein retained its repression ability at greater distances [76]. However, they only recruited the transcriptional regulators to locations outside of the promoter and yeast repressors typically exert their influence within a few hundred base pairs [76, 95]. It still needs to be determined how repressors function based on their location within the promoter region.

Synthetic chromatin modifiers are becoming an emerging field in synthetic biology. Recent work has begun to uncover the transcriptional dynamics of different repressive chromatin marks [14] and how different chromatin modifying enzymes influence transcription when they are tethered near a promoter [55, 56]. A quantitative comparison between bacterial repression mechanisms and eukaryotic repression mechanisms will contribute to this emerging field.

1.1.4 Quantitative characterization of gene expression systems

The fundamental goal of synthetic biology is to develop a quantitative understanding of biological processes so that we can predictably build life from the bottom-up. The greatest success so far has been in the construction and characterization of synthetic transcriptional regulatory networks, which have begun to uncover the quantitative rules governing transcriptional regulation.

Gene transcription is largely controlled by interactions among promoter-bound transcription factors. By mapping promoter output as a function of its input, which is the concentrations of transcription factors bound to the promoter, a biological transfer func-

tion can be determined [69]. This is more commonly referred to as the gene regulatory function (GRF) [78]. The most common approach to measuring GRFs utilizes a promoter expressing a fluorescent reporter and transcription factors that are either inducible by small molecules [84, 68], or fluorescently tagged [78, 57]. In this manner, the fluorescent output from the promoter can be quantitatively measured using flow cytometry or fluorescent microscopy and the transcription factor concentration can be similarly quantified by its fluorescence output or by the concentration of its inducer.

This approach has been used to create thermodynamic models of transcriptional regulation, which can accurately predict the transcriptional output of a promoter from the concentration of one [57, 78, 70] or more transcription factors [84, 20, 76] that are bound to the promoter. What has become clear from these studies is that the steady state behaviour of a promoter can be accurately modelled by solely considering the DNA binding kinetics of the transcription factors and largely ignoring the complex mechanisms through which the transcription factors function [69, 35, 18].

The Hill function

Thermodynamic modelling applies the tools created by physical chemists for understanding enzyme kinetics, to transcriptional processes. The Hill function is a single parameter extension of Michaelis-Menten kinetics that enables steeper dose response curves and was first used to describe the sigmoidal response curve of oxygen binding to hemaeglobin [42]. It has been used extensively in thermodynamic models to describe the binding kinetics of transcription factors to DNA [3, 78, 57]. The basic Hill function for an inducible activator binding to a promoter is described in equation 1.1, where two parameters, Kd and n, are sufficient to define the sigmoidal input-output curve. As the concentration of inducer increases, the binding of the activator on the promoter also increases.

$$Promoter\ bound\ activator = \frac{[Inducer]^n}{[Inducer]^n + Kd^n} \tag{1.1}$$

By adding proportionality constants that define the basal transcription rate and maximal transcription rate (Equation 1.2), the Hill function can describe the transcriptional output of the promoter. A Hill function that has been solved for arbitrary values (black curve) is illustrated in every panel of Figure 1.2 b. Variations of the same Hill function with higher (red curve) or lower (blue curve) values for each parameter or constant are also plotted.

$$Transcriptional\ output = \ Basal + (Max - Basal)\frac{[Inducer]^n}{[Inducer]^n + Kd^n} \tag{1.2}$$

The parameter n describes the responsiveness or cooperativity of the dose response curve. Cooperativity is a phenomenon where the binding of one transcription factor on a promoter increases the likelihood that another transcription factor can bind a neighbouring site on the same promoter [3]. Cooperativity is dependent on TF-TF and TF-CRE interactions [18, 11] and increasing the number of DNA binding sites for an activator typically increases cooperativity. A higher value for n signifies positive cooperativity, which results in a steeper dose response curve, whereas a value of n lower than 1 signifies anti-cooperative interactions and a shallower curve. A value of n that is equal to 1 is equivalent to non-cooperative, Michaelis-Menten kinetics [3]. This is illustrated in Figure 1.2B where the Hill function becomes steeper as the value of n is increased and vice versa.

The maximal expression level represents the saturation of activation from the promoter. The maximal expression level is largely influenced by the strength of the core promoter [15] and is independent from the number of activator binding sites [11]. The maximal expression may also be affected by the type of activator that is recruited to the promoter [34].

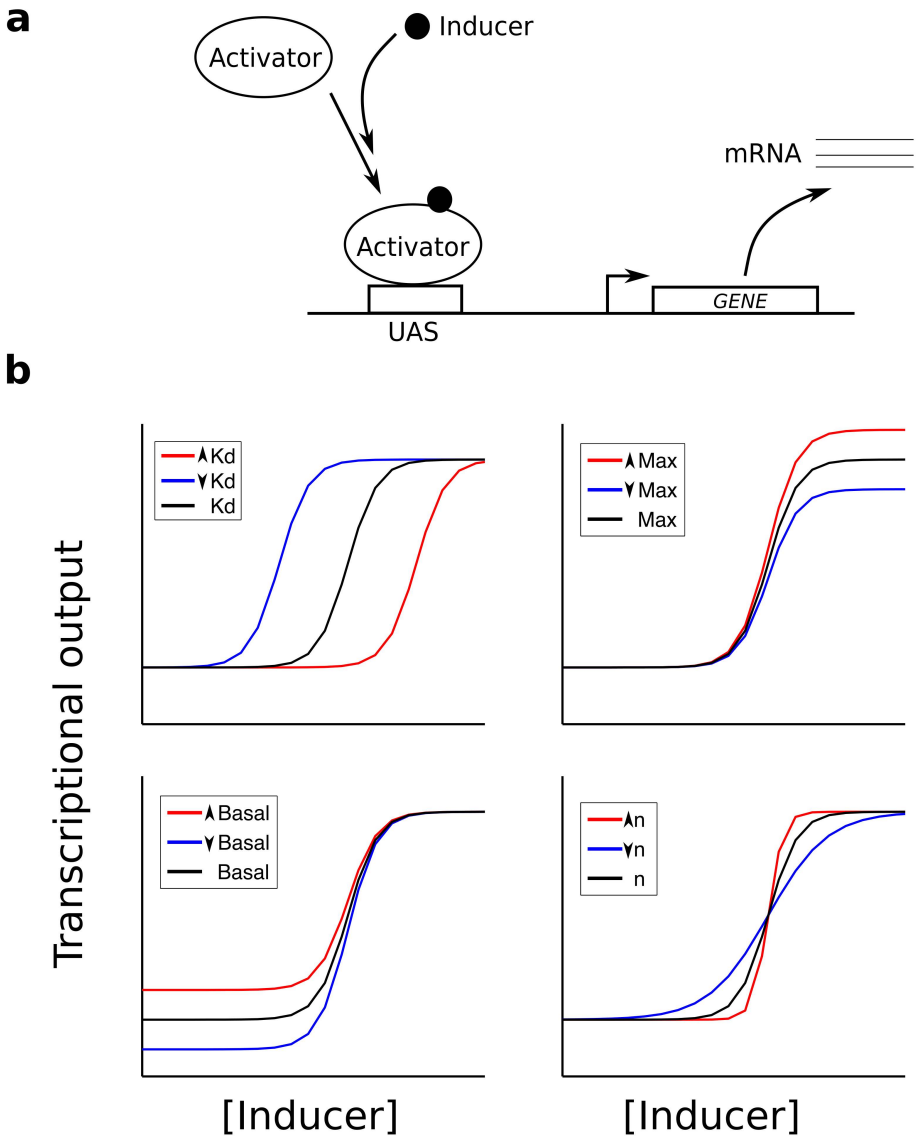

Figure 1.2: The four parameter Hill function can be used to fit gene regulatory functions. **a.** Schematic of an inducible transcriptional activator binding a UAS. **b.** Variations of the four parameter hill function. Each parameter value is either increased (red) or decreased (blue) in comparison to the standard model (black).

The basal expression level represents the amount of expression the promoter has without any activation. In an inducible system, an increase in basal activity could mean that the activator is able to bind the promoter in the absence of inducer, a common occurrence with tetracycline controlled systems [90]. Promoters can frequently initiate transcription

without any input from an activator, presumably because the TATA regions are accessible to the PIC, and this will also change the basal expression level.

The parameter K_d represents the dissociation constant of the inducer from the activator. This parameter is difficult to measure with *in vivo* assays and is typically replaced by the half maximal effective concentration (EC50) which is used to describe the inducer concentration required for half maximal activity [3]. The EC50 is a measure of the system's sensitivity, and a lower EC50 means the promoter is responsive to lower concentrations of inducer. This is illustrated in Figure 1.2 where the curve shifts left as the Kd value is decreased and vice versa.

EC50 is a good metric for the sensitivity of GRFs because even as other variables change, particularly the maximal and basal expression level, the EC50 remains the same. This is illustrated in Figure 1.2, where changing the parameter n or the constants Basal or Maximal, has no effect on the EC50. The EC50 captures the half maximal point of the sigmoidal curve, regardless of the values of n, max or basal and therefore provides a good indicator of the systems sensitivity to inducer.

1.2 TetR-based synthetic gene networks

1.2.1 The bacterial tet repressor (TetR)

The Tn10- tetracycline resistance operon is a transposable DNA element that endows bacterial species with resistance to the antibacterial chemical tetracycline [45]. Tetracycline is toxic to bacteria because it binds strongly to bacterial 70S ribosomes and prevents translational elongation [19].

The Tn10 operon has an interesting regulatory network, which is illustrated in Figure 1.3. It consists of a single promoter region that divergently expresses two proteins, the Tet repressor (TetR) and the TetA-tetracycline efflux pump. The promoter region has two

tet operators that serve as high affinity binding sites for the TetR regulator [44]. In the absence of tetracycline TetR binds as a dimer to its palindromic operator sites, which inhibits transcription of itself and the TetA efflux pump. In the presence of tetracycline, TetR undergoes a conformation change in its protein structure, which alleviates DNA binding and repression [46, 73]. The TetA efflux pump is then freely expressed, where it localizes to the membrane and exports tetracycline from the cell [44]. The key regulatory component of the system is the inducible TetR protein, which can selectively bind a unique 17 base pair operator sequence [45].

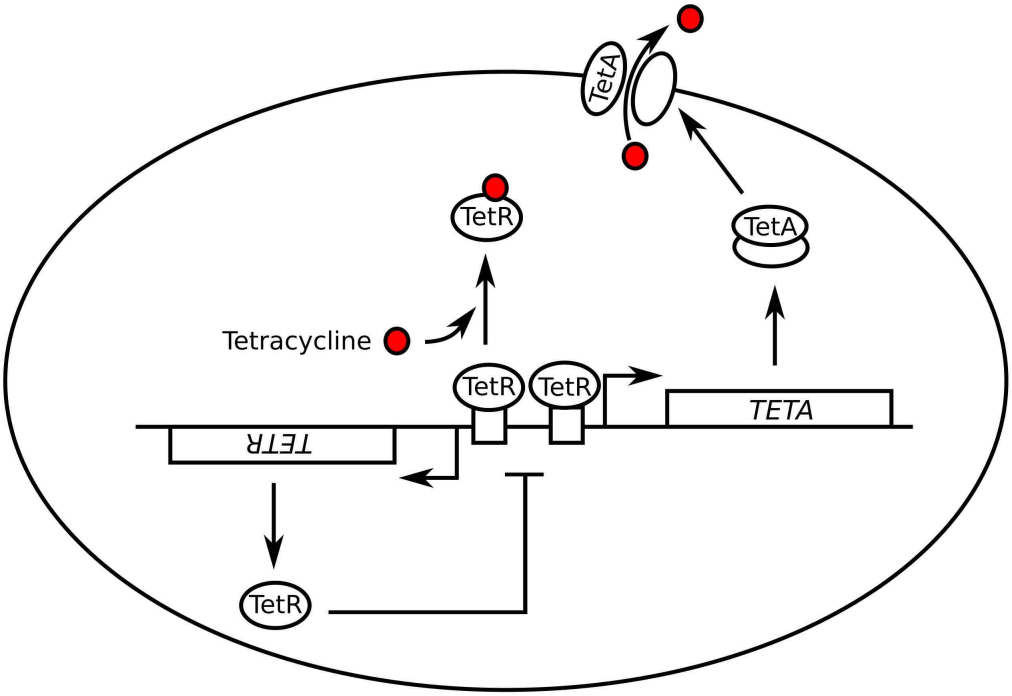

Figure 1.3: Schematic of the regulatory network of the bacterial tetracycline resistance transposon

The three dimensional structure of TetR has been solved in complex with DNA [73] and in complex with tetracycline [46]. This has elucidated the dynamic mechanism through which it conditionally binds DNA. The Tet repressor consists of ten α-helices and folds into two main domains, this is illustrated in Figure 1.4. The n-terminal α-helices (α-1 to α-3) form a helix-turn-helix (HTH) DNA binding domain which is connected to the core domain

of the protein (α-5 to α-10) by the linker helix α-4. In the absence of tetracycline, two TetR monomers can dimerize along their (α-7 to α-10) surfaces and bind the palindromic operator sequence. Essential to DNA binding is the separation of the monomers DNA binding domains, which are 36.6Å apart. The core domain of the protein contains a tetracycline-binding pocket. In the presence of tetracycline, hydrogen bonds are made with residues in α-5, α-7 and the C-terminus of α-4. Upon binding tetracycline, there is a confirmational change in the protein structure which results in α-4 swinging out like a hinge. This increases the separation of the DNA binding domains by 3Å to 39.6Å and TetR dimers can no longer bind DNA [73].

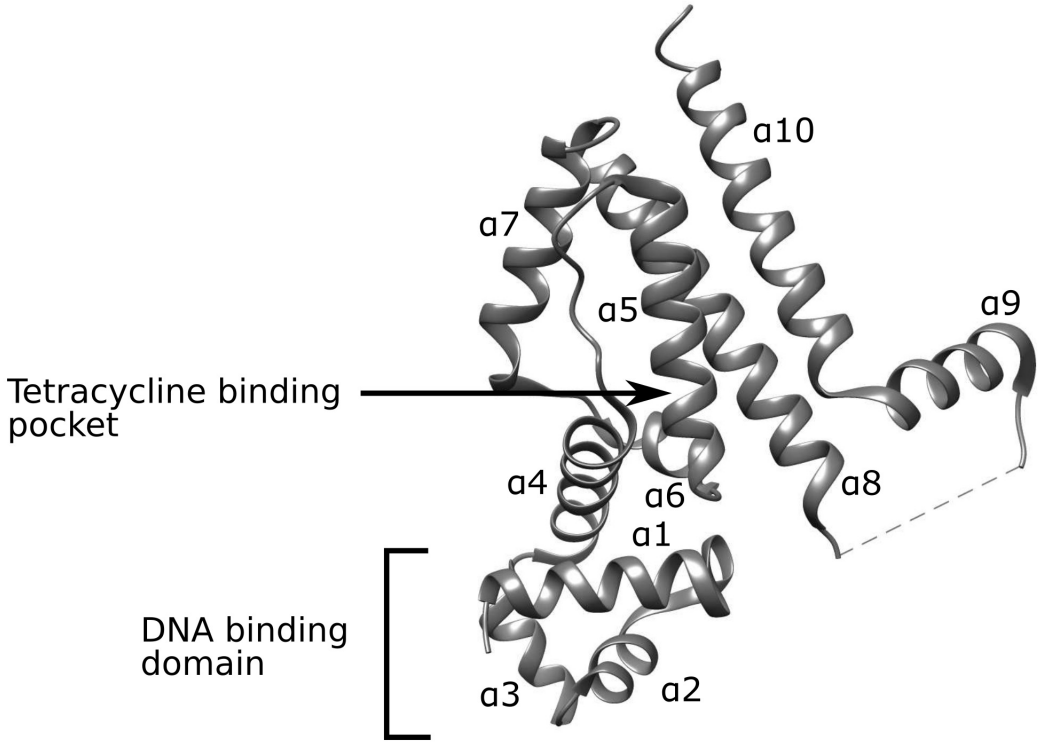

Figure 1.4: The crystal structure of the TetR protein. The α helices are labelled and the DNA binding domain and tetracycline binding pocket are identified

1.2.2 Development of tetracycline inducible transcriptional activators

The Tet repressor has been used extensively for conditional transcriptional repression in eukaryotes and its use has significantly accelerated the advancement of synthetic biology. As described in detail in section 1.1.3.

By adding a strong VP16 viral activation domain to TetR, researchers were able to convert the repressor into at tetracycline-controlled transcriptional activator (tTA). This activator was first tested in mammalian cells and could increase the expression of luciferase from a tet-responsive promoter, by up to five orders of magnitude. Importantly, the activation potential of the transactivator was lost by the addition of doxycycline (a tetracycline derivative) to the growth media, inducing dissociation of tTA from the DNA [39].

Conditional gene expression systems are frequently used to turn-on gene expression from a repressed state. If tTA were to be used for this purpose, it would require that cells be constantly cultured in doxycycline to maintain a repressed state. Although tetracycline is non-toxic at physiological concentrations, this would be undesirable and not practicable, particularly in the development of transgenic animals [40].

To address this, Gossen et al. created a reversible TetR (rTetR), which could bind DNA in the presence of doxycycline. To create rTetR they randomly mutated TetR by PCR. The mutants were then screened in a human cell line that contained a tet-repressible promoter expressing luciferase. By culturing the cells in doxycycline and measuring the luciferase activity with a high throughput plate based screen, they identified a TetR variant with a reverse DNA binding phenotype that repressed luciferase expression [40]. Sequencing analysis identified four amino acid substitutions in the TetR domain, E71K, D95N, L101S and G102D, though it is unclear what role these mutations have in reversing the DNA binding phenotype [73].

The VP16 activation domain was subsequently added to rTetR to create the reverse

tetracycline transactivator [40]. Although the original rtTA functioned as expected, it has very high basal activity, resulting in a low dynamic range, and it is not particularly sensitive to doxycycline [90].

The same research group repeated the assay, except the transactivators were screened in yeast cells [90]. This resulted in the rtTA-M2 variant which was also found to be functional in mammalian cell lines and has become the most popularly used rtTA variant [90]. Interestingly, the M2 variant shared none of the same mutations with the first generation rtTA. Sequencing identified five unique amino acid substitutions: S12G, E19G, A56P, D148E and H179R, and the mutations of E19G and A56P were found to be sufficient for the reverse phenotype [90]. The A56P mutation is particularly interesting because it is located in the hinge region of α-4. Unfortunately, the authors did not test a TetR mutant containing only the A56P mutation to see if that single mutation is sufficient for the reverse phenotype.

1.2.3 Optimization of rtTA

The rtTA system offers many advantages over other available conditional gene expression systems because it enables graded and reversible transcriptional control using a non-toxic inducer, the antibiotic doxycycline, that has few pleiotropic effects [93]. A disadvantage of the system is that the rtTA protein still retains some affinity for its DNA binding site in the absence of inducer resulting in leaky transcription of target genes [12, 48, 49, 1, 63, 90, 64].

Two properties of rtTA have been targets for extensive optimization: increasing the sensitivity to doxycycline and increasing its dynamic range by reducing its basal activity in the un-induced state.

Directed evolution has proven to be a powerful approach to improve the sensitivity of rtTA to doxycycline [26, 96]. This approach takes advantage of the high mutagenesis rate of HIV replication to rapidly mutate and select optimized rtTA variants. Specifically, researchers re-engineered an HIV virus so that its replication was dependent on rtTA

activity [26, 96]. By culturing the engineered virus in low levels of doxycycline, only viruses that introduced sensitivity enhancing mutations into rtTA would proliferate. This method produced two new rtTA variants, V16 and V10, which are more sensitive to doxycycline than the original M2 variant. The key TetR mutation that is found in these variants is F86Y. This residue makes direct contact with the doxycycline molecule in the TetR domain. The tyrosine mutation adds a hydroxyl group on the phenylalanine's side chain, which likely increases hydrogen bonding between the TetR binding pocket and doxycycline, thus increasing sensitivity [26].

While the viral optimization approach was successful in identifying variants with improved sensitivity, it has a few fundamental flaws. This assay does not impose a selective pressure on the inducible phenotype and could therefore select for variants that constitutively bind the operator site regardless of the presence of doxycycline. Furthermore, it would beneficial to the virus to select rtTA variants with increased leaky activity and therefore a decreased dynamic range. Testing of the V10 and V16 variants in mammalian cells lines did indeed show that there was significant leaky transcription in the absence of doxycycline [25]

Strategies that have been used to reduce the leaky transcription include combining rtTA with a tetracycline-responsive repressor [12, 79, 59], optimization of the rtTA target promoter [1, 63], and the introduction of positive feedback in the control of rtTA gene transcription [64]. However, none of these methods address the fundamental problem that the un-induced transactivator has affinity to its operator site.

The problem of high leaky transcription persists and continues to complicate the uses of the rtTA system. For example, when used in mammalian cells, it has been reported that multiple clones need to be examined to find one that has low target gene expression in the absence of inducer [63]. In a recent implementation of an rtTA system controlling CRISPR/Cas9, up to 33% of clones may have off-doxycycline mutagenesis caused by leaky Cas9 expression [27]. An optimized rtTA / rTetR would be an asset to synthetic and

molecular biology because it would allow for stringent, conditional DNA binding and reduce the amount of optimization needed to use these systems.

1.3 Research objectives

1.3.1 Objective I

While screening an rtTA system that had been constructed in yeast, I identified a single clone that had a greatly expanded dynamic range. The observed clone had no detectable rtTA activity in the absence of doxycycline but could still activate gene expression to the same maximal amount under conditions of full doxycycline induction. I hypothesized that the effect was due to one or more spontaneous mutations within the sequence of the rtTA gene, and tested this hypothesis by sequencing. The results suggested that the observed change in rtTA activity was due to a single amino acid substitution, namely G72V.

The first objective of this thesis is to investigate how this mutation impacts rtTA activity and use this knowledge to improve on the existing rtTA expression systems that are available.

1.3.2 Objective II

Using the improved rTetR DNA binding domains developed in Objective I, novel transcriptional repressors will be constructed by tagging various yeast repression domains to the rTetR domain. These synthetic repressors were recruited to various synthetic promoters to determine if the location of transcription factor binding affects their function.

The second objective of this thesis is to investigate the quantitative properties of bacterial and eukaryotic mechanisms of transcriptional repression using a synthetic promoter library.

Chapter 2

Materials and Methods

2.1 Strain construction

Overlap extension PCR and homologous recombination were used to assemble all synthetic DNA constructs. These techniques has been thoroughly described and their success rates have been quantified in comparison to other DNA assembly methods [4]. Briefly, these methods take multiple DNA parts that share sequence homology and put them together to build one DNA construct. Individual DNA parts are acquired by standard PCR protocols. The primers used for amplifying each part have overhangs which are homologous to the next DNA sequence that the part is going to be attached to. If the length of the homology between consecutive DNA pieces is greater than 40 base pairs the PCR products can be transformed directly (200ng of each DNA part, using a standard lithium acetate transformation [36]), where they will be assembled by natural yeast DNA repair mechanisms [85]. If the homology between DNA parts is less than 40 base pairs, PCR assembly can be done. To assemble DNA by PCR assembly, DNA parts with homologous regions to one another are added to the same PCR reaction ($\sim$20ng each) in the absence of primers. The first ten cycles of PCR are performed, where the parts can 'prime' each other and linearly expand. Primers are then added to the reaction and the remaining twenty cycles

of exponential amplification are performed. PCR was performed in a BioRad C1000 Touch or BioRad T1000 Thermal Cycler. Standard PCR protocol was: 30 reaction cycles with a denaturing temperature of 98 degrees celsius for 15 seconds, an annealing temperature of 55-60 degrees celsius for 20 seconds and an extension temperature of 72 degrees celsius for 30 seconds*(kilobasepairs of product). Reactions were set up as follows: 10uM of Primers (Invitrogen), 1 unit of Phusion High Fidelity Polymerase (M0530L, New England Biolabs), 20ng of template DNA, 200uM Deoxynucleotide mix (N0446S, New England Biolabs) and 1x High Fidelity Buffer (B0518S, New England Biolabs).

2.1.1 DNA construct design

The *GAL1* promoter with the *GAL4* UAS removed and *MIG1* sites scrambled served as the starting material for constructing the tet-responsive promoters. The P_{TET} promoters used in Chapter 3 were constructed by overlap extension PCR using primers that had tet operators on the overhangs. The P_{TET3} promoter was built by inserting tet operators at nucleotides -184, -267 and -351 from the *yeGFP* ATG. P_{TET4} had an additional site at nucleotide -417 from the *yeGFP* ATG. The synthetic dual input promoters that were used in Chapter 4 were ordered as synthetic dsDNA from Invitrogen GeneArt. The *GAL1* promoter with the *GAL4* UAS sequence removed and *MIG1* sites scrambled served as the base promoter. The three GEV sites were each separated by 2 base pairs and the three site cassette was placed at -300 from *yeGFP* ATG. The tet operators at position '1' were placed 2 base pairs away from the most proximal GEV binding site. The tet operators at position '2' were placed at nucleotide -100 from the *yeGFP* ATG. The tet operators at position '3' were placed 10 base pairs downstream of the TATA box.

2.1.2 Yeast cell transformation

DNA constructs containing the yeGFP, GEV and rTetR parts were sequentially integrated into the genome at the *ADE2*, *GAL4* and *ADE4* loci respectively, of *S. cerevisiae* strain BY4742 (MATα, his3Δ1, leu2Δ0, lysΔ0, ura3Δ0) (EUROSCARF). A standard lithium acetate transformation protocol[36] was used for integrating all constructs into the genome. Cells were selected after transformation on either YP media supplemented with 2% glucose, 2% adenine and appropriate antibiotic selection or on synthetic drop out media supplemented with 2% glucose and 2% adenine. Positive transformants were confirmed by PCR validation. PCR primers were designed on either side of the integration site in the genome and PCR was performed. The PCR reactions were then run on an agarose gel to visualize the size of the product and confirm the proper integration.

2.2 Cell culture and media

Single yeast colonies from antibiotic or auxotrophic selection plates were inoculated in synthetic media supplemented with 2% glucose and 2% adenine. Cells were maintained in logarithmic growth phase prior to and during induction with doxycycline and/or β-estradiol. Cells were grown under inducing conditions for 6 hours before measuring yeGFP fluorescence by flow cytometry or microscopy. All cultures were maintained at 30 degrees celsius and rotated at 200RPM.

2.3 Flow cytometry acquisition and analysis

Yeast cultures were diluted 1 in 10 in 50mM sodium citrate buffer prior to being sampled by the flow cytometer. An IntelliCyt HyperCyt autosampler attached to a Beckman Coulter CyAn ADP9 analyzer was used for the collection of all flow cytometry data. Yeast cells

were gated with a small forward and side scatter gate (∼40% of the population) to reduce variability from extrinsic sources. A 488nM laser and a 530/40 band-pass filter were used to excite and detect yeGFP fluorescence. FCS files were analyzed by custom scripts created in MATLAB R2013a (Mathworks Inc, Natick, Massachusetts). Mean fluorescent intensity values and standard error values were calculated from this data.

2.4 Fluorescent microscopy acquisition and analysis

Yeast cell cultures were concentrated by centrifugation at 4000RPM in an Eppendorf 5415D desktop centrifuge, re-suspended in sterile water, pipetted onto microscopy slides and immediately imaged. Fluorescent microscopy was performed with a Nikon Ti-E inverted fluorescent microscope with a Chroma HQ480/40x excitation and HQ535/50m emission filter cube. NIS Elements software was used to merge brightfield and fluorescent images.

2.5 Data fitting and simulations

Dose-response data was fit to standard hill type functions (as described in the introduction) in MATLAB R2013a (Mathworks Inc, Natick, Massachusetts) using the function 'nlinfit' from the statistics and machine learning toolbox. Simulations of competitive and non-competitive inhibition mechanisms were performed by evaluating the described equations using the parameters provided in table 4.1.

2.6 Western blotting

Western blotting was completed under the supervision of Dr. Rudner using his laboratory protocols that have previously been described[60]. The αTetR (Clontech), αCDK1 (Rabbit

Polyclonal) and αCLB2 (Covance) antibodies were used at dilutions of 1/1000, 1/1000 and 1/4000 respectively.

2.7 Protein structure modeling

The figure was generated using the structure of TETR(B) in complex with Minocycline and magnesium (4AC0.pdb)[92] and the PyMOL[83] software.

Chapter 3

Improvement of the reverse tetracycline transactivator by single amino acid substitutions that reduce leaky target gene expression to below detectable levels

3.1 Hypothesis

Hypothesis I

Introducing non-polar side chains by substituting G72 in the rtTA-M2 molecule with other amino acids reduces leaky target gene expression in a manner that depends on the size of the side chain.

Hypothesis II

Combining mutations at G72 with mutations that enhance sensitivity to doxycycline makes the rtTA-M2 system more robust with low leaky expression, high expression capacity and high doxycycline sensitivity across a broad range of rtTA expression levels.

3.2 Specific aims

Specific aim I - Characterize leakiness

A weakness of conventional rtTA systems is its ability to activate gene expression in the absence of tetracycline induction and this leaky expression has limited its utility. Leaky activity has been shown to be dependent on transactivator expression levels but this has not been investigated thoroughly. I aim to quantify how rtTA expression level impacts leaky target gene expression.

Specific aim II - Improve leakiness

Although the three-dimensional structure of rtTA has not been solved. The structure of TetR which shares 99% sequence identity has been. By investigating the location of the

mutation that we have identified, in the context of the three-dimensional protein structure, I aim to rationally introduce new mutations into the transactivator to further improve the dynamic range.

Specific aim III - Characterize sensitivity

We have identified that the single mutation decreases leaky target gene expression. It may have other influences on the activity of rtTA, such as decreasing its sensitivity to inducer. I aim to quantify the sensitivity of our novel rtTA variant to inducer by performing doxycycline dose responses and fitting the data to standard Hill-type functions.

Specific aim IV - Improve sensitivity

The initial rtTA variant that we began our experiment with was the most commonly used 'M2' variant, which has been available for years. Further research into rtTA has identified new mutations within the transactivator that can further improve its sensitivity to inducer. I aim to introduce sensitivity enhancing mutations into our novel rtTA variants to create an rtTA with low leaky activity and high sensitivity to inducer.

3.3 Results

Tetracycline inducible systems have been used extensively in yeast to study the control of gene transcription in single cells [9, 71, 88, 28, 16, 11, 97, 38]. To use rtTA in this context, we created two strong doxycycline-responsive promoters with either three (P_{TET3}) or four (P_{TET4}) rtTA binding sites, and used the optimized rtTA-M2 variant [90] to control the expression of yeast-enhanced green fluorescent protein (*yeGFP*) [23] from these promoters (Figure 3.1A). The rtTA / P_{TET}-*yeGFP* expression cassette was integrated in a single copy into the yeast genome. The M2 variant is identical to the variant found in ClonTech's Tet-ON Advanced expression systems.

3.3.1 Leaky rtTA activity is dependent on transactivator expression level

The problem of leaky target gene transcription is especially profound when rtTA is expressed at high levels. This is illustrated in Figure 3.1B which shows P_{TET3} doxycycline dose response curves when rtTA-M2 is expressed from the strong P_{TDH3} promoter. Fluorescence was very high when cells were exposed to saturating amounts of doxycycline. Nonetheless, because of leaky transcription from the P_{TET3} promoter, the dynamic range of the system was rather poor with the maximal expression ~17 times greater at full induction compared to the absence of doxycycline.

Consistent with a model where the transactivator has significant activation potential in its un-induced state, expression of rtTA-M2 from the weak P_{MYO2} promoter substantially reduced fluorescence in the absence of doxycycline. Although full saturation was not observed with this system, the reduction of leaky expression resulted in a drastic improvement in dynamic range and induction with doxycycline resulted in an ~200 fold increase in fluorescence. However, despite this improvement, both flow cytometry data (Figure 3.1C)

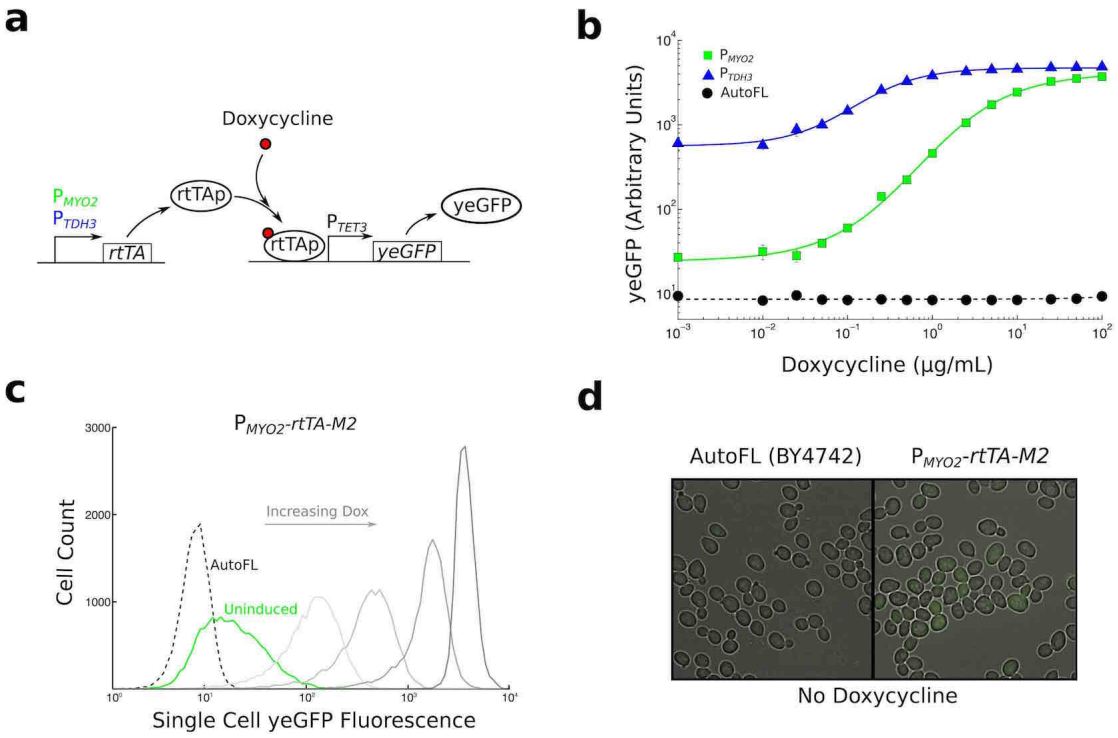

Figure 3.1: rtTA-M2 has significant activity in the absence of doxycycline induction. **a**, Schematic of the experimental setup where rtTA-M2 is expressed from the P_{MYO2} or P_{TDH3} constitutive promoters and *yeGFP* is expressed from a tet-responsive promoter. **b**, Doxycycline dose-dependent activation of rtTA-M2 expressed from either P_{MYO2} or P_{TDH3} (mean +/- s.e.m of three technical replicates). AutoFL is wildtype BY4742 yeast cells. **c**, Distributions of single-cell fluorescence obtained from cells carrying the P_{MYO2}-*rtTA* system at different doxycycline concentrations. Progressively darker shades of grey correspond to 0.25, 1, 5, and 100μg/mL of doxycycline respectively. AutoFL is wildtype BY4742 yeast cells **d**, Merged brightfield-fluorescence images of wildtype BY4742 yeast cells or uninduced cells carrying the P$_{MYO2}$-*rtTA* system.

and fluorescence microscopy data (Figure 3.1D) indicated that un-induced rtTA-M2 causes significant reporter gene expression even when the transactivator is expressed from a weak promoter.

3.3.2 A single amino substitution in rtTA diminishes leaky expression

During the initial testing of twelve clones carrying the *P_{TDH3}-rtTA-M2* system, we serendipidously discovered that one of them emitted unexpectedly low fluorescence in the absence of doxycycline but almost normal fluorescence at full induction (Figure 3.2A). Subsequent sequencing identified a single nucleotide mutation, guanine to thymine, that changes a glycine (GGG) to a valine (GTG) at residue 72 within the transactivator protein. Western blot analysis indicated that this substitution does not affect protein abundance (Figure 3.2A, Insert), and reconstructing the expression system confirmed that the single guanine to thymine mutation is responsible for the changes seen in the doxycycline dose response (data not shown).

The *P_{TDH3}-rtTA-M2(G72V)* system has a remarkable dynamic range and showed an ∼500 fold increase in fluorescence intensity when cells were induced with high doxycycline compared to no induction (Figure 3.2B). Moreover, the fluorescence emission detected by flow cytometry is indistinguishable from cellular auto-fluorescence in the absence of doxycycline, and the variant preserves the graded induction response of the original transactivator. Moreover, reporter expression from the un-induced rtTA-M2(G72V) strain was undetectable by fluorescence microscopy even under instrument settings where the un-induced rtTA-M2 strain gave a strong saturating signal (Figure 3.2C).

To gain insight into how the single mutation can have such a drastic impact on the dynamic range of rtTA-M2(G72V), we investigated where the residue was located in the context of the three dimensional structure of the protein. The structure of rtTA-M2 or

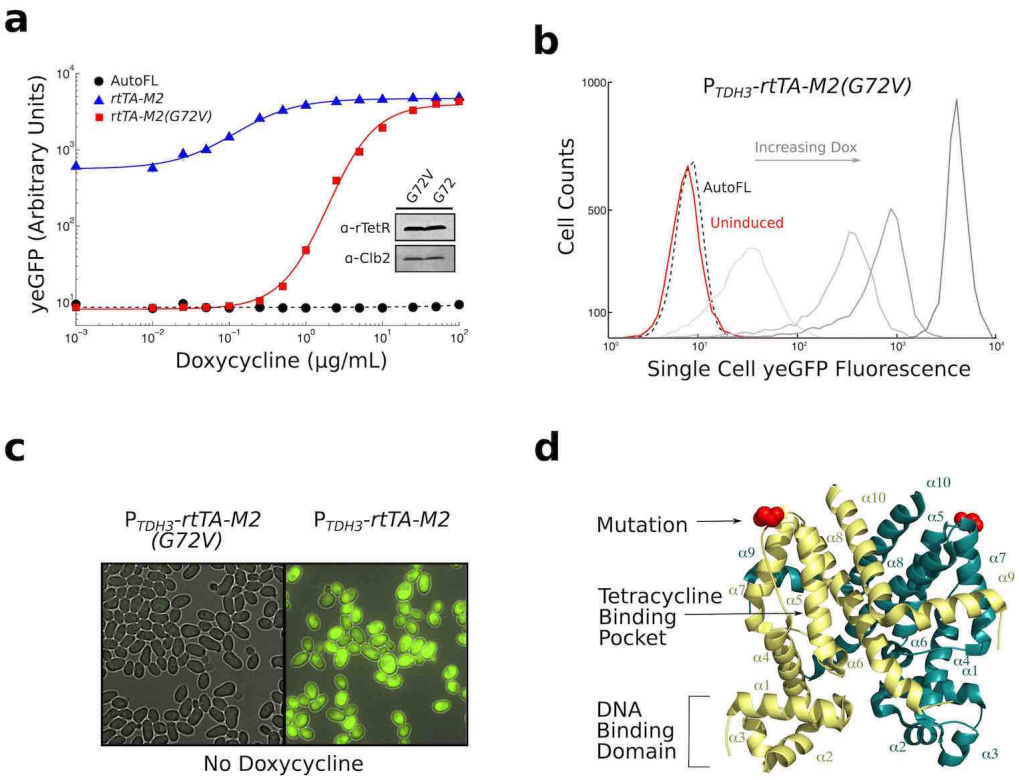

Figure 3.2: Mutation of glycine 72 in rtTA-M2 diminishes basal activity to undetectable levels. **a**, Doxycycline dose-dependent activation of rtTA-M2 variants expressed from P_{TDH3} (mean +/- s.e.m of three technical replicates). AutoFL is wildtype BY4742 yeast cells. In the inset, a western blot (cropped) comparing protein abundances of both rtTA-M2 and the G72V mutant. All samples were prepared and run under identical experimental conditions. **b**, Distributions of single-cell fluorescence obtained from cells carrying the *P_{TDH3}-rtTA(G72V)* system at different doxycycline concentrations. AutoFL is wildtype BY4742 yeast cells. Progressively darker shades of grey correspond to 0.1,1,2.5,5 and 100μg/mL of doxycycline respectively. **c**, Merged brightfield-fluorescence images of uninduced cells carrying the *P_{TDH3}-rtTA-M2* or *P_{TDH3}-rtTA(G72V)* systems. **d**. Cartoon representation of TetR dimer in which each protomer is colored different (yellow and turquoise). The secondary structure is labeled and G72 is shown as a sphere.

other rtTA variants has not been solved. However, with 203 out of 207 amino acids in the TetR domain of rtTA being preserved [90], the high resolution structure of TetR [92] gives insight into the structure of the rtTA DNA binding domain. In the TetR structure, the glycine residue 72 maps to a flexible loop located between α-helices $\alpha 4$ and $\alpha 5$, a region bridging the repressor DNA binding domain ($\alpha 1$-$\alpha 3$) and its tetracycline binding region ($\alpha 5$-$\alpha 9$) [92, 46](Figure 3.2D). This suggests that the substitution, which introduces a non-polar side-chain, triggers long range conformational changes that ultimately affect the activity of the transactivator, presumably by rigidifying its tertiary structure.

3.3.3 Leaky activity of rtTA is dependent on the size of the amino acid side chain at residue 72

To examine this hypothesis, we created two additional variants in which the glycine residue was mutated into alanine or proline. These mutations preserve the non-polar nature of the valine residue but introduce side chains of varying size. We anticipated that the single methyl side-chain of alanine would be less effective in preventing uninduced activity than the large cyclical side chain of proline. We also created a β-estradiol inducible expression system [31] to gain direct control of rtTA-M2 expression, and used the promoter with an extra TetR binding site, P_{TET4}, to enhance the ability of rtTA to bind DNA.

We first examined how the abundance of the un-induced rtTA variants impacted reporter gene expression by varying the concentration of β-estradiol in the absence of doxycycline (Figure 3.3D). In these experiments, the fluorescence of the four strains was indistinguishable from cellular auto-fluorescence in the absence of β-estradiol. A strain harbouring both the β-estradiol inducible transactivator and the tet-responsive promoter, but no rtTA, has reporter expression that is indistinguishable from a wildtype BY4742 yeast strain, demonstrating that there is no background reporter expression from P_{TET4} (Figure 3.4). At high β-estradiol induced rtTA expression, the strains with the G72A, G72V

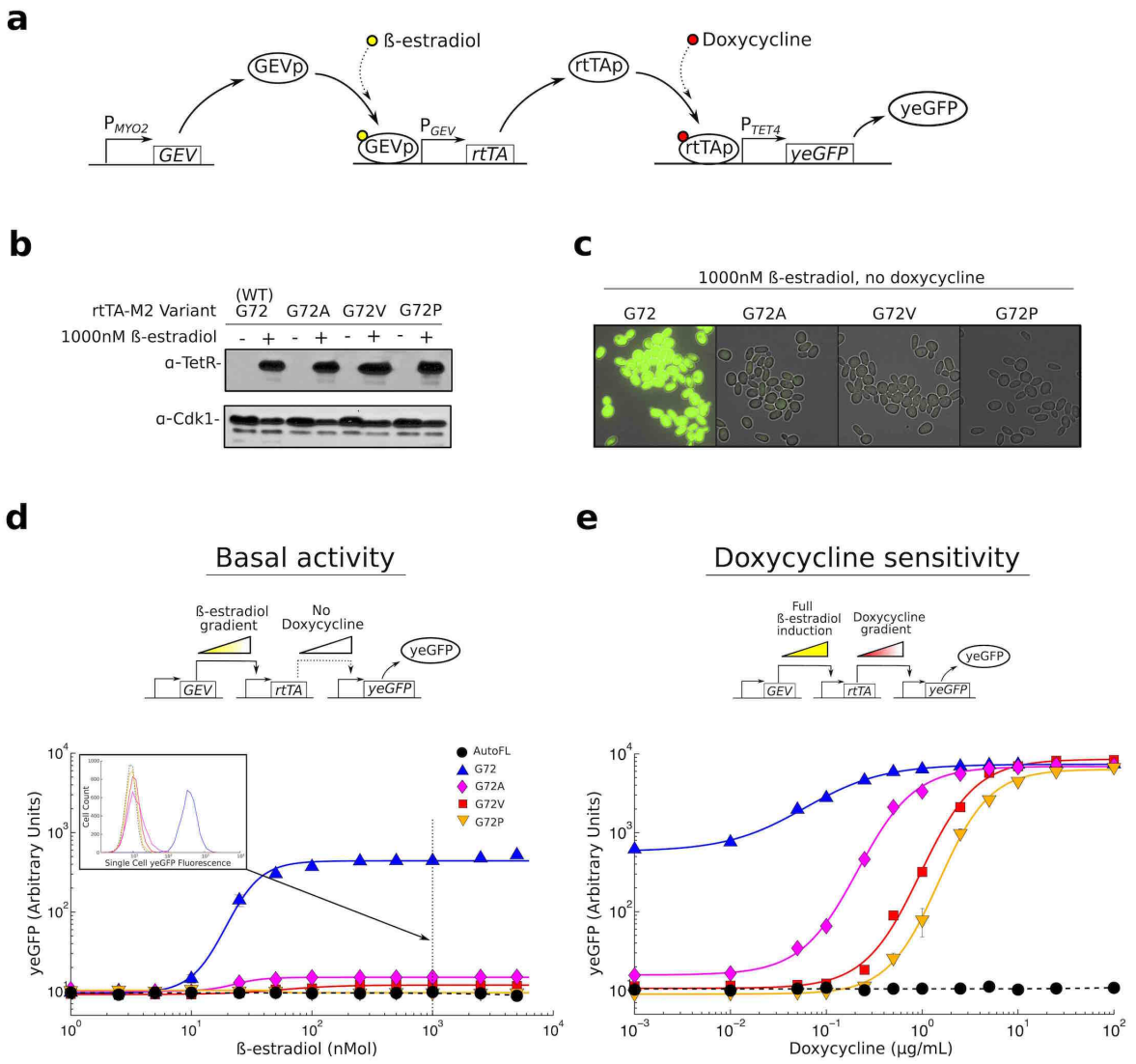

Figure 3.3: Non-polar side chains at residue 72 is a key determinant of basal activity. **a**, Schematic of the experimental setup where rtTA-M2 variants are expressed from a β-estradiol inducible promoter and yeGFP is expressed from a tet-responsive promoter. **b**, Western blot (cropped) comparing protein abundances of all four rtTA variants when induced by 1000nM of β-estradiol or no induction. All samples were prepared and run under identical experimental conditions. **c**, Merged brightfield-fluorescence images of all four rtTA variants induced by 1000nM β-estradiol with no doxycycline induction. **d**, The basal activity of rtTA variants as a function of their β-estradiol-dependent expression level (mean +/- s.e.m of three technical replicates). In the inset, histograms of reporter expression for the different rtTA variants. **e**, Doxycycline dose-dependent activation of rtTA-M2 variants expressed ubiquitously in 1000nM β-estradiol (mean +/- s.e.m of three technical replicates).

and G72P substitutions had significantly lower fluorescence than the original rtTA-M2 variant. We confirmed by western blot analysis that the four rtTA variants have similar abundances (Figure 3.3B).

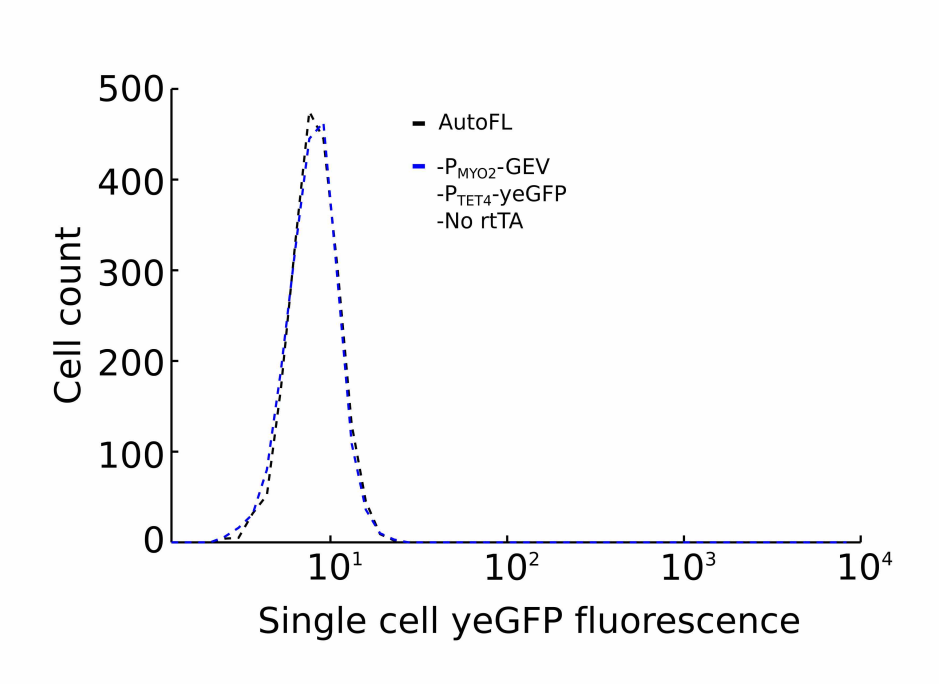

Figure 3.4: yeGFP expression from the P_{TET4} promoter and GEV cassette alone is indistinguishable from autofluorescence of wildtype BY4742 yeast cells when cells were cultured in 1000nM β-estradiol.

As expected, the alanine variant showed higher leaky expression than the valine variant, and the proline variant had fluorescence that was indistinguishable from cellular autofluorescence measured by flow cytometry (Figure 3.3D, Insert). This is also apparent from fluorescence microscopy images obtained with instrument settings where the strain harbouring the original M2 variant gives a strong fluorescence signal (Figure 3.3C).

Remarkably, the amino acid substitutions that significantly reduce basal reporter gene expression have minimal effect on rtTA transcriptional activation at full doxycycline induction. This is illustrated in Figure 3.3E, which displays the dose-response curves for the four G72-M2 variants measured when doxycycline is varied at full β-estradiol induction. Interestingly, the amino acid substitutions impact doxycycline sensitivity. In our

experiments (Figure 3.3E), the M2 variant had the highest sensitivity with a half maximal effective concentration (EC50) of $\sim$0.06μg/mL while the alanine variant (EC50 of $\sim$0.2μg/mL), the valine variant (EC50 of $\sim$1.0μg/mL) and the proline variant (EC50 of $\sim$1.5μg/mL) required progressively higher doxycycline concentrations to reach maximal expression capacity.

3.3.4 The doxycycline sensitivity of the novel rtTA variants can be improved by the addition of sensivity enhancing mutations

To counter this effect of the G72 mutation, we introduced additional mutations in the TetR domain that were recently shown to increase the sensitivity to doxycycline [26, 96, 25]. We specifically examined the effect of introducing the sensitivity enhancing (SE) mutations V9I, F67S, F86Y and R171K.

Figure 3.5A and 3.5B demonstrate that the SE mutations improve the doxycycline sensitivity of the SE-G72P and SE-G72A rtTA M2 variants. When the rtTA variants are expressed at high levels from the fully activated β-estradiol inducible promoter (Figure 3.3A), the introduction of the SE mutations reduces the doxycycline EC50 from $\sim$1.5μg/mL to $\sim$0.1μg/mL for the G72P variant (Figure 3.5A) and from $\sim$0.2μg/mL to $\sim$0.02μg/mL for the G72A variant (Figure 3.5B). This effect occurred without an appreciable change in reporter expression under full doxycycline induction, and did not compromise the dynamic range of the SE-G72P variant (Figure 3.6B). Interestingly, however, the SE mutations caused a significant loss of dynamic range for the SE-G72A variant by increasing reporter gene expression in the absence of doxycycline (Figure 3.6A).

To further explore the relationship between the level of rtTA expression, doxycycline EC50 and basal activity, we examined the effect on reporter gene expression of simultaneously varying β-estradiol and doxycycline induction. We investigated four different M2

variants; the original variant, the orginal variant with the SE mutations, the G72P variant and the SE-G72P variant.

The two-dimensional dose-response surface for the original M2 variant contains three distinct regions of reporter expression corresponding to low, intermediate and high reporter gene expression. These regions are labeled I, II and III in Figure 3.5C-3.5F. Region III is where reporter expression is maximal, which depends on both rtTA expression level and the level of doxycycline induction. Region II is where reporter expression is relatively high even when doxycycline induction is low. Region I is where reporter expression is low due to low β-estradiol or low doxycycline induction.

The SE mutations alone (Figure 3.5D) dramatically increase reporter expression in part of region I and the entire region II. This confirms that these mutations may enhance sensitivity in a narrow range of rtTA expression levels, but also cause a significant increase in the activity of rtTA in the un-induced state. Contrasting this, the G72P mutation alone (Figure 3.5E) dramatically reduces reporter expression in the entire region II and in part of region III. This confirms that the profound effect of this mutation on rtTA activity in the un-induced state is associated with a general loss of doxycycline sensitivity.

Figure 3.5F shows the effect of combining the SE and the G72P mutations. Compared to the SE variant (Figure 3.5D), adding the G72P mutation counters the increase in reporter expression caused by the SE mutations in region I and reduces reporter expression to undetectable levels in this region. Moreover, compared to the G72P variant (Figure 3.5E), adding the SE mutations almost completely restores the loss of reporter expression in region III. In other words, sensitivity is improved without introducing leaky target gene expression at any transactivator expression level.

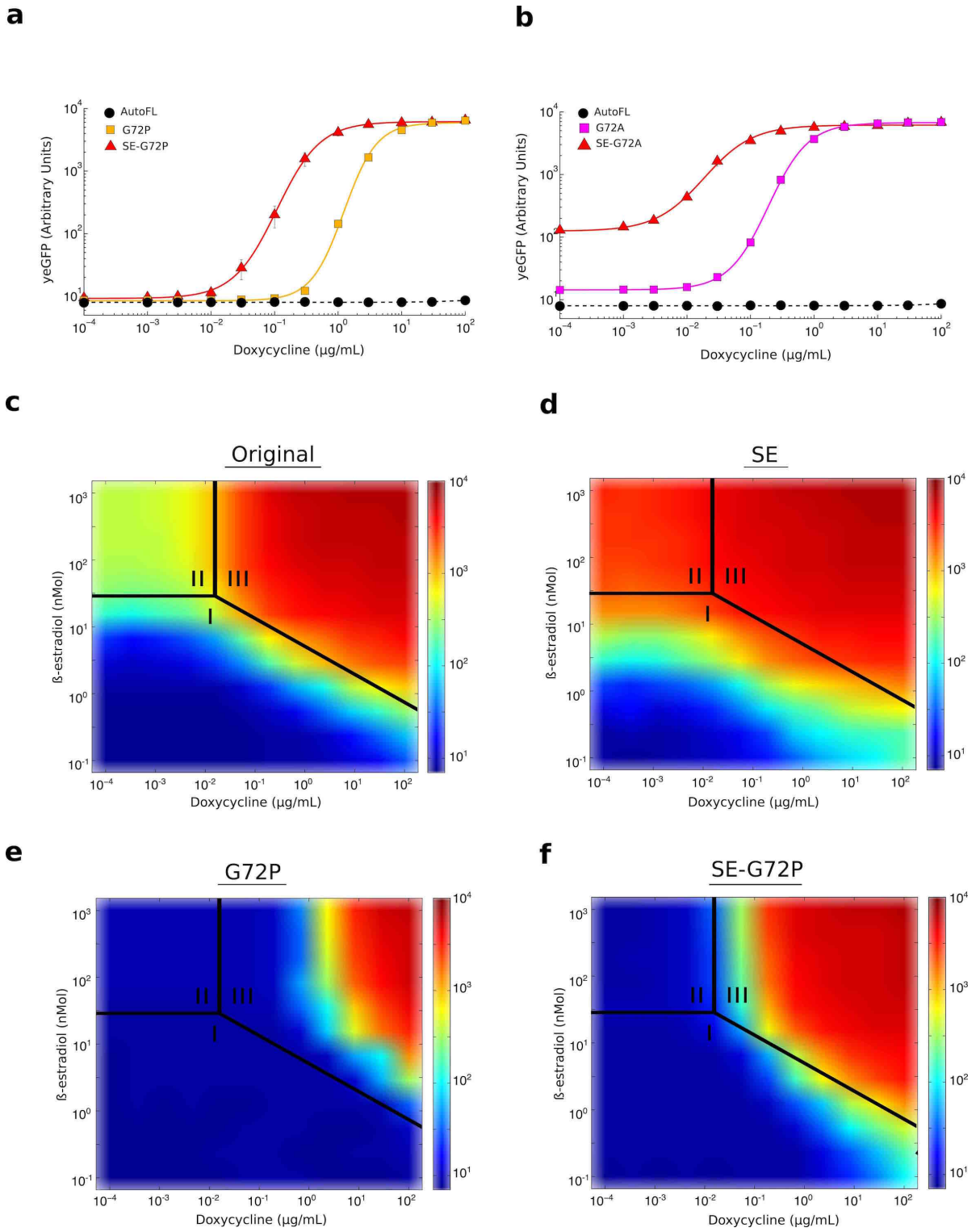

Figure 3.5: Doxycycline sensitivity of the novel rtTA variants can be improved by the addition of sensitivity enhancing mutations. **a,b**, Doxycycline dose-dependent activation of rtTA variants expressed ubiquitously in 1000nM β-estradiol (mean +/- s.e.m of three technical replicates). **c-f**, Heatmaps displaying reporter expression (Arbitrary units) as a function of β-estradiol-dependent transactivator expression and doxycycline induction.

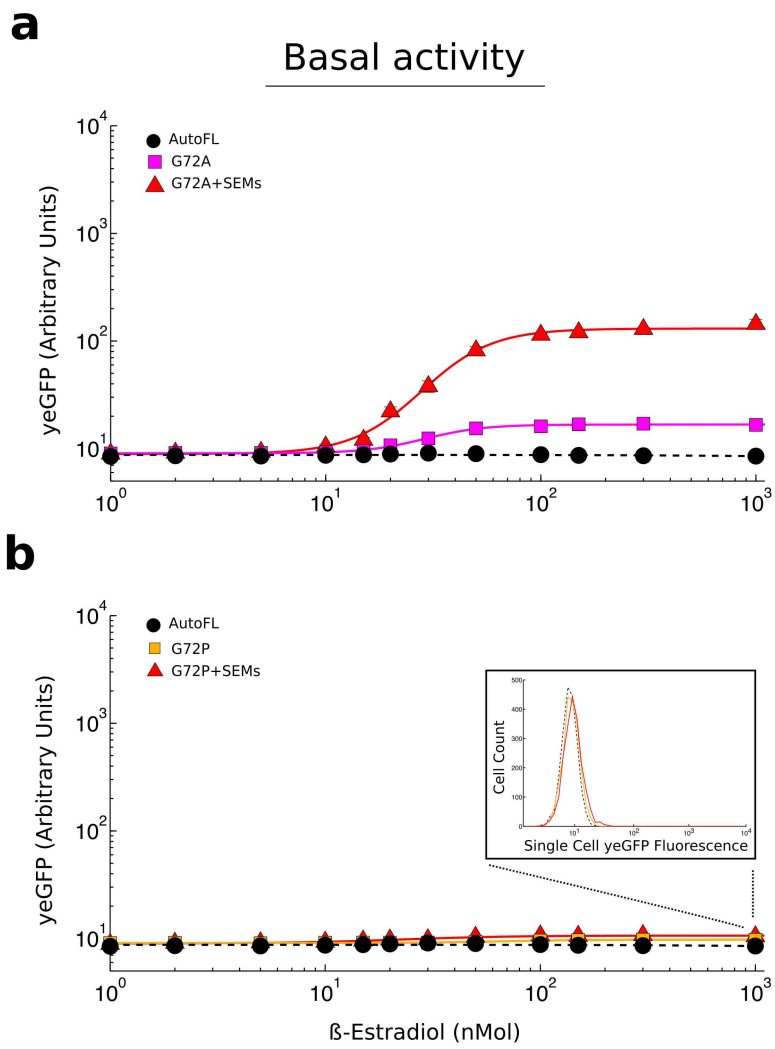

Figure 3.6: The basal activity of rtTA variants as a function of their β-estradiol-dependent expression level (mean +/- s.e.m of three technical replicates). In the inset of **b**, histograms of single cell fluorescence of the SE-G72P variant and a SE-G72P variant cultured in 1000nM β-estradiol.

Chapter 4

Comparison of eukaryotic and bacterial transcriptional repression mechanisms using a synthetic promoter library

4.1 Hypothesis

Hypothesis I

It has been shown that DNA binding proteins can repress transcription when they are recruited to the TATA region of eukaryotic promoters, presumably by inhibiting PIC assembly. I hypothesize that DNA binding domains targeted near activator binding sites can repress transcription in a similar manner, by preventing activator binding.

Hypothesis II

I hypothesize that natural transcriptional repressors that function by enzymatic activity do not need to be recruited near the TATA box or activator binding regions to repress transcription because their mechanisms of action affect local chromatin structure and are not dependent on DNA binding.

4.2 Specific aims

Specific aim I - Construct synthetic transcriptional repressors

Using an optimized rTetR domain from the previous chapter, I aim to construct inducible, synthetic transcriptional repressors by tagging the rTetR domain with an inert protein(BFP) or the enzymatic repression domains Sin3 and Ssn6.

Specific aim II - Construct a synthetic promoter library with binding sites for synthetic transcriptional activators and repressors

A synthetic promoter library will be designed and constructed based off the well studied P_{GAL1} promoter, using GEV activator binding sites and rTetR binding sites.

Specific aim III - Perform dose response experiments to quantify repression at different concentrations of activator and repressor

The genetic constructs created above will be integrated in single copy into yeast cells to generate strains containing each synthetic repressor with each of the promoters of the library. These yeast cells will be cultured in various amounts of the small molecule inducers for the GEV activator and rTetR repressors to alter the concentrations of the synthetic transcription factors on the promoter. High throughput flow cytometry will be used to quantify fluorescence expression from the synthetic promoters.

4.3 Results

4.3.1 Experimental overview

Bacterial transcriptional repression mechanisms have been studied extensively in yeast by recruiting TetR to the TATA region of promoters. Synthetic promoter libraries that vary the number and placement of TetR binding sites near the TATA box have shown how this type of repression can be predictably engineered [70, 28]. Eukaryotic repression domains, which repress transcription through fundamentally different mechanisms, have not been studied at varying locations in the promoter region, only at long distances [76].

In this context, we created a library of yeast strains that contains: a constitutively expressed β-Estradiol inducible activator (GEV), one of three constitutively expressed doxycycline inducible transcriptional repressors (rTetR-TF), and a yeGFP reporter expressed from synthetic promoters that respond to input from the activator and repressor (Figure 4.1A). Each component was integrated as a single copy into the yeast genome at the *GAL4*, *ADE4* and *ADE2* loci respectively.

The β-Estradiol inducible GEV system is identical to the one in Chapter 3. GEV

has been used extensively in molecular biology [68, 31] and activates transcription from promoters that contain *GAL4* binding sites distal to the core promoter. Upon β-Estradiol induction GEV localizes to the nucleus and activates transcription in a graded, reversible manner.

The yeast transcriptional repressors, Sin3 and Ssn6, and an enzymatically inert Blue fluorescent protein (BFP), were tagged to the C-terminus of the optimized rTetR-M2 (G72V) that was developed in Chapter 3 (Figure 4.1C).

A synthetic promoter library was constructed based on the core-*GAL1* promoter. Our base *GAL1* promoter has the native *MIG1* binding sites in the URS regions scrambled and the natural UAS region removed to prevent confounding transcription factor binding. A cassette consisting of three adjacent *GAL4* binding sites was placed at the native UAS region in all promoter variants. Two tet operators were inserted in the proximal, medial or distal regions of the promoter, as shown in Figure 4.1B. The proximal sites are located 10 base pairs downstream of the TATA box (3 base pairs from the TSS), the medial sites are located at the native URS region (100 base pairs from the TSS) and the distal sites are located adjacent to the activator binding sites (260 base pairs from the TSS). The promoters are named $P_{GT\#}$, indicating that they receive input from **G**EV and r**T**et, with the numbers indicating where the tet operators are located (1=distal, 2=medial and 3=proximal). Eight promoters with tet operators at all combinations of proximal, medial and distal locations were created. A control promoter with scrambled tet operators at all three locations served as a positive control for β-Estradiol inducible activation and a negative control for doxycycline inducible repression.

4.3.2 Strain construction and confirmation

The synthetic promoters were ordered as sequenced confirmed, double stranded blocks of DNA from Invitrogen. A Kanamycin-G418 resistance cassette was added 5‘ to the promoter

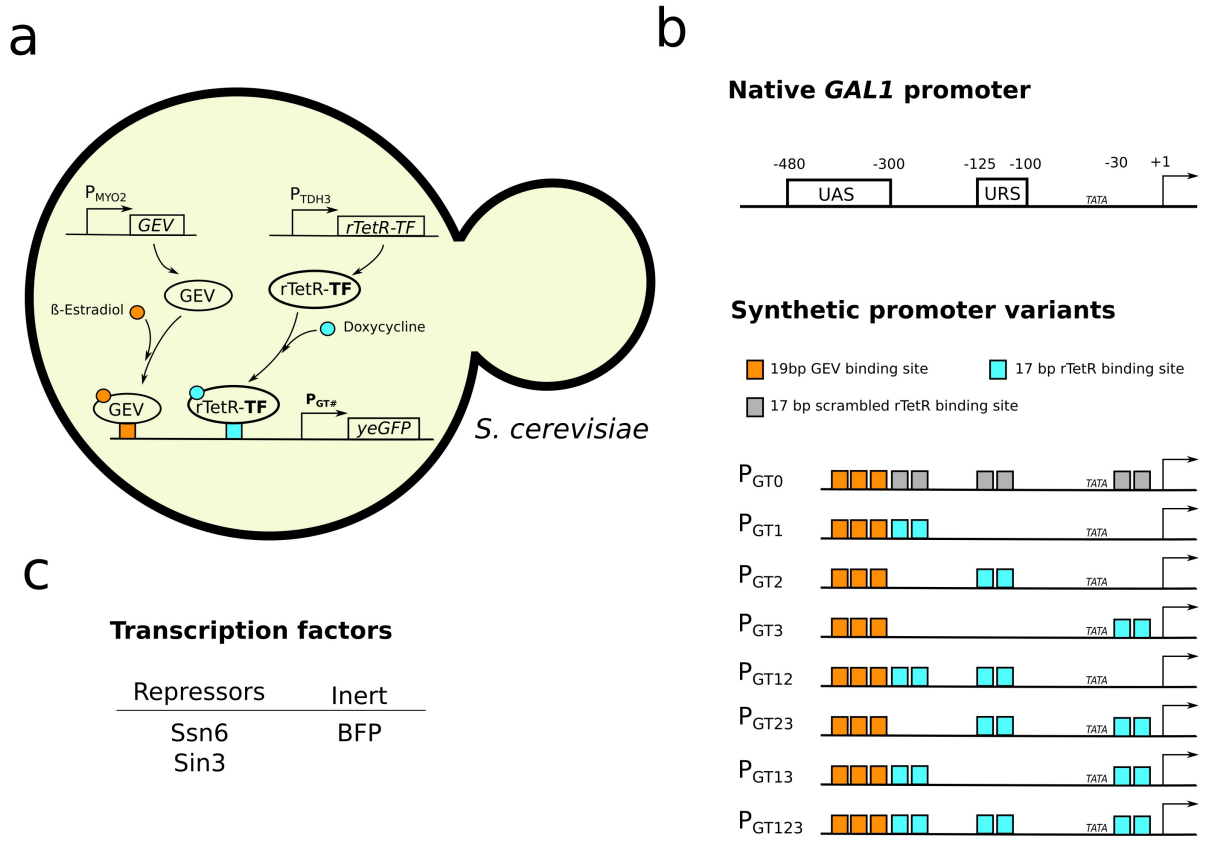

Figure 4.1: Schematic of the synthetic gene regulatory network and promoter library used in the study. **a.** Synthetic gene regulatory network that was constructed for testing the different repression mechanisms. **b.** The transcription factors that are tagged to the rTetR DNA binding domain. **c.** A detailed schematic of the native *GAL1* promoter and the synthetic promoter library. The location of the DNA binding sites for each synthetic transcription factor are shown.

and a yeGFP reporter was added 3‘ to the promoter using overlap extension PCR. The KanR-$P_{GT\#}$-yeGFP cassettes were integrated into the *ADE2* loci of yeast. Integration into the *ADE2* coding sequence causes a colour change in yeast, from white to red. The antibiotic selection confirms that the DNA was integrated, and the colour selection confirms that the DNA was integrated at the correct loci.

The GEV cassette from Chapter 3 was integrated into the *GAL4* loci, thus deleting the endogenous *GAL4* coding sequence, ensuring the activation of our promoters was dependent on the β-Estradiol inducible GEV transactivator.

We first confirmed that the GEV cassette and all eight promoters were functional. The eight strains containing the promoter variants and the GEV transactivator were cultured in saturating conditions of β-Estradiol (5000nM) and the yeGFP fluorescence was measured by flow cytometry (Figure 4.2A). All eight strains were responsive to β-Estradiol and had similar maximal reporter expression. Interestingly, integration of a tet or scrambled tet operator at the proximal location results in slightly lower maximal expression. Alteration of the core GAL1 promoter near the TATA box has been shown to decrease transcriptional output in other synthetic promoter libraries [70], likely by disrupting promoter proximal elements that contribute to PIC assembly.

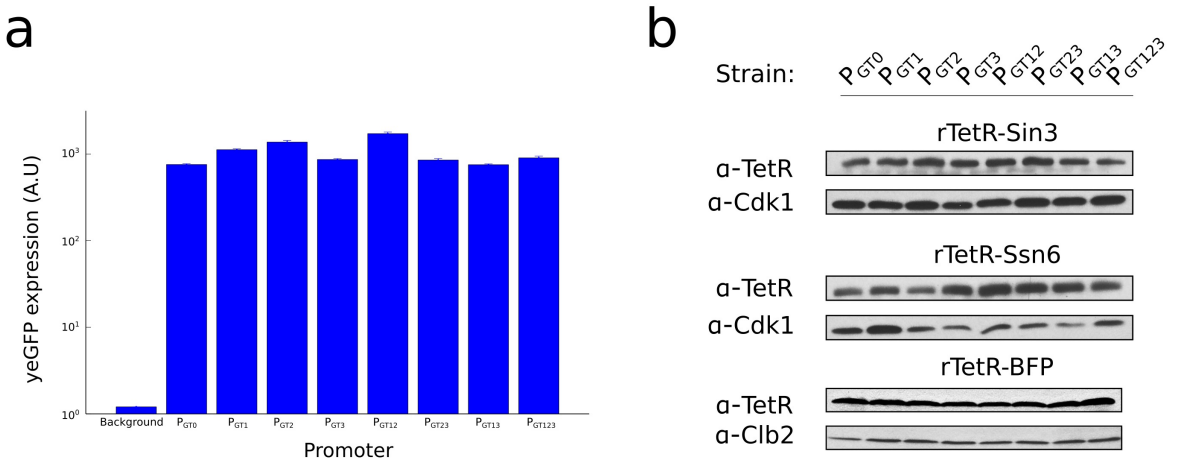

Figure 4.2: Validation of the β-Estradiol inducible promoters and synthetic transcription factors. **a.** The yeGFP expression (Arbitrary units) from each of the synthetic promoters under saturating conditions of β-Estradiol (5000nM) (mean +/- s.e.m of three technical replicates). **b.** Western blot analysis of the relative protein abundance of each of the synthetic transcription factors in all eight promoter backgrounds.

The synthetic transcriptional repressors were fused to the 3‘ end of the strong, constitutive *TDH3* promoter, ensuring the repressors would be expressed ubiquitously throughout the cell. A NatR resistance cassette was added for antibiotic selection to the drug Nourseothricin. The NatR-P$_{TDH3}$-rTetR-Repressor casettes were integrated into the *ADE4* loci of the yeast strains containing all eight promoter variants and a GEV transactivator.

Integration into the ADE4 coding sequence of a $\Delta ADE2$ strain causes a colour change in yeast, reverting the red cells to white. Once again, the antibiotic selection confirms that the DNA was integrated, and the colour change confirmed that it was integrated in the correct loci. Furthermore, we confirmed that the rTetR-Repressors were stably expressed in all 24 strains by performing western blot analysis with an α-TetR antibody (Figure 4.2B). The protein bands were found at the appropriate size for each of the three repressors and the relative band intensity was similar in all eight promoter strains that each repressor was transformed into (Figure 4.2B).

4.3.3 Repression of fully activated promoters

Single-input promoters

We first examined if the rTetR-Repressors could repress transcription from the fully activated promoters with distal (P_{GT1}), medial (P_{GT2}) and proximal (P_{GT3}) tet sites, while a promoter with no tet sites (P_{GT0}) served as a negative control. Dose-response curves were generated for all three rTetR-Repressors by varying the concentration of doxycycline in the presence of saturating β-Estradiol (5000nM) and measuring the reporter fluorescence by flow cytometry (Figure 4.3). The cells were cultured for six hours in the inducers to ensure steady state was reached. The yeGFP fluorescence values from the dose response experiments were normalized to each promoter‘s maximal expression value to correct for variations in promoter strength. The dose response data was fit to a simple Hill function to aid visualization.

The rTetR-BFP repressor should be enzymatically inert and repress transcription solely through its inducible DNA binding activity. We anticipated that it would repress transcription from the proximal location by interfering with the assembly of the PIC, and from the distal location by preventing activator binding.

Interestingly, rTetR-BFP was able to repress transcription of the P_{GT1}, P_{GT2} and P_{GT3}

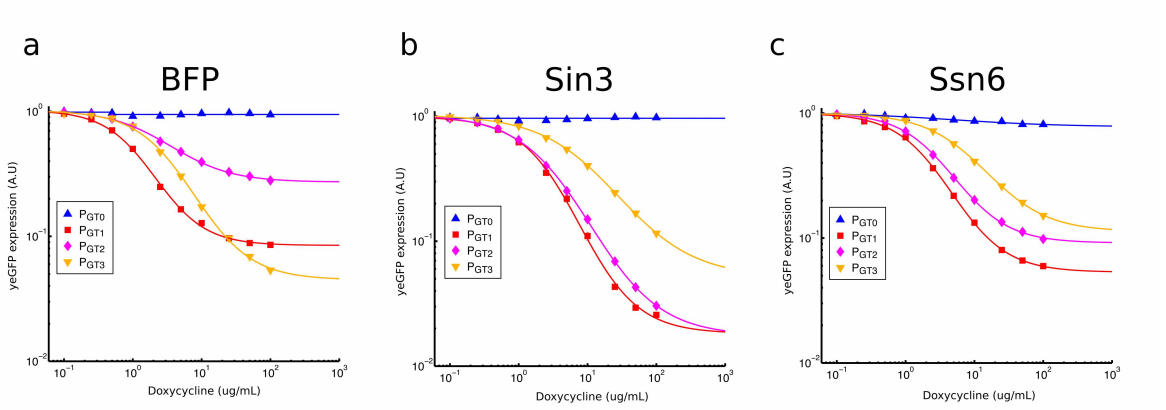

Figure 4.3: Doxycycline dose-response curves of the single-input promoters under saturating conditions of β-Estradiol (5000nM) (mean +/- s.e.m of three technical replicates), a. rTetR-BFP, **b.** rTetR-SIN3, **c.** rTetR-SSN6.

promoters (Figure 4.3A). Importantly, P_{GT0} had constant yeGFP expression at all doxycycline concentrations, confirming that rTetR-BFP only has repression capability when tet sites are in the promoter region and that doxycycline is not causing the observed decrease in fluorescence signal. The strength of repression at each promoter varied significantly. At maximal doxycycline concentrations, the P_{GT3} promoter enabled strong repression, the P_{GT2} promoter was minimally repressed and the P_{GT1} promoter was moderately repressed. However, the onset of repression from P_{GT1} occurred at lower doxycycline concentrations than the onset of repression from P_{GT2} and P_{GT3}, which occurred at similar levels of doxycycline. Notably, the repression from the P_{GT1} promoter was saturating at high doxycycline concentrations, whereas repression from the P_{GT3} promoter was not yet saturated.

We anticipated that the rTetR-Sin3 repressor would be stronger than rTetR-BFP at all three locations because it could repress transcription through DNA binding and through histone deacetylation. As expected, the rTetr-Sin3 repressor was able to repress transcription of the P_{GT1}, P_{GT2} and P_{GT3} promoters and there was no observable repression of the P_{GT0} promoter (Figure 4.3B). At full doxycycline concentration the repression of P_{GT1} and P_{GT2} was stronger than it was for rTetR-BFP, while the repression of P_{GT3} was slightly weaker. Overall the repression by rTetR-Sin3 was stronger at the distal and medial sites

than it was from the proximal sites. Notably, the onset of repression from P_{GT1} and P_{GT2} promoters were almost identical and occurred at lower dose of doxycycline than the onset of repression from the P_{GT3} promoter.

We expected that repression mediated by rTetR-Ssn6 would be similar to what was observed for rTetR-Sin3, with contributions from DNA binding and from its unknown enzymatic ability. As anticipated, the rTetR-Ssn6 repressor was able to repress transcription of the P_{GT1}, P_{GT2} and P_{GT3} promoters and there was no observable repression of the P_{GT0} promoter (Figure 4.3C). The repression curves looked similar to what was observed for rTetR-Sin3, with stronger repression of the P_{GT1} and P_{GT2} promoters than the P_{GT3} promoter. Once again, the onset of repression from the P_{GT3} promoter occurred at higher doxycycline concentrations than it did for the P_{GT1} and P_{GT2} promoters.

Multi-input promoters

I next examined how the rTetR-Repressors repressed transcription when they were recruited to multiple locations within the same promoter region. The multi-input promoters have tet sites at the: distal and medial promoter regions (P_{GT12}), medial and proximal promoter regions (P_{GT23}), distal and proximal regions (P_{GT13}) and the distal, medial and proximal regions (P_{GT123}). Dose response curves were once again generated for all three rTetR variants by varying the concentration of doxycycline in the presence of saturating β-Estradiol (5000nM) and measuring the reporter fluorescence by flow cytometry (Figure 4.4).

Repression of the multi-input promoters by rTetR-BFP shared many characteristics of the repression that was observed at the single-input promoters (Figure 4.4A). All promoters that contained a distal '1' site (P_{GT12}, P_{GT13} and P_{GT123}) had an earlier onset of repression than the P_{GT23} promoter. At full doxycycline concentration the repression from the multi-input promoters was generally stronger than it was for the single-input promoters, except

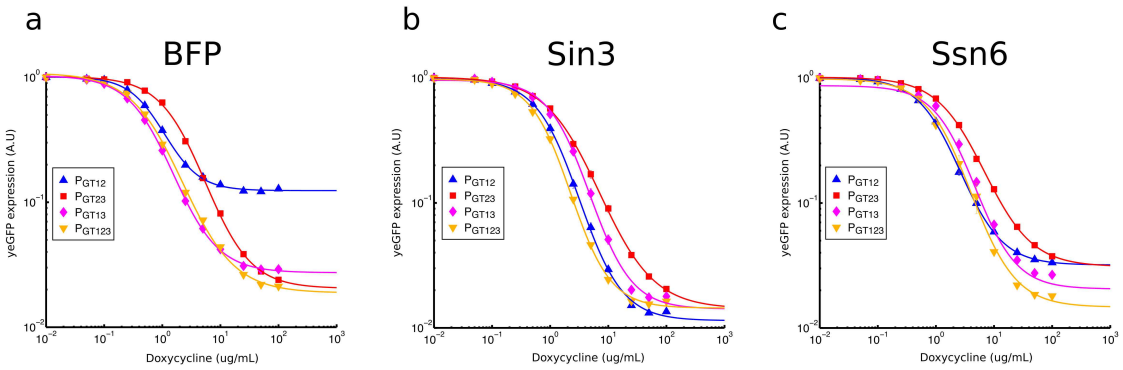

Figure 4.4: Doxycyline dose-response curves of the multi-input promoters under saturating conditions of β-Estradiol (5000nM) (mean +/- s.e.m of three technical replicates), a. rTetR-BFP, **b.** rTetR-Sin3, **c.** rTetR-Ssn6.

for P_{GT12}, which had reporter expression between that of P_{GT1} and P_{GT2}.

Repression of the multi-input promoters by the enzymatic repressors rTetR-Sin3 and rTetR-Ssn6 were very similar (Figure 4.4B,C). Strong repression was observed at all four promoter variants for both repressors. There were minimal differences in the concentration of doxycycline required for the onset of repression, although P_{GT23} required slightly higher doxycycline, particularly for rTetR-Ssn6.

Despite the three different repressors having different mechanisms of action, similar trends were observed in the repression curves at each promoter. We hypothesized that the steady state repression observed by these repressors, at the specific promoter regions, was largely dependent on DNA binding kinetics.

4.3.4 Competitive and non-competitive inhibition models

It is well established that dose response curves of transcriptional repressors can be accurately fit using approaches that were originally developed for understanding enzyme kinetics [70]. Competitive and non-competitive inhibition models of enzyme activity are classic biochemistry textbook examples for introducing mathematical modelling [91]. We

wondered if the DNA binding kinetics of transcriptional repression could be explained by similar mathematical models. We hypothesize that the repression at the distal site is largely mediated by competitive DNA binding interactions between the GEV activator and the rTetR DNA binding domain, as illustrated in Figure 4.5A, whereas repression from the proximal and medial promoter region is non-competitive (Figure 4.5B).

Standard competitive and non-competitive inhibition models are presented in equations 4.1 and 4.2, and equation 4.3, respectively. The competitive inhibition model resembles the Hill function where K_m is a product of the activator's dissociation constant (K_d) and doxycycline-dependent repression. The K_i parameter is the dissociation constant for the repressor on DNA. The non-competitive inhibition model is simply the product of a Hill function for activator binding and the inverse of a Hill function for repressor binding. Modelling the activity of these repressors will allow us to differentiate between fundamentally distinct biological mechanisms of repression.

These models were evaluated with biologically realistic parameters (Table 4.1) and plotted in Figure 4.5A,B. The canonical approach for comparing competitive and non-competitive inhibition, visualizes the enzyme activity as a function of substrate, in the presence of various amounts of inhibitor. Similarly, we can plot the promoter activity (yeGFP expression) as a function of β-Estradiol concentration, at various doxycycline concentrations.

Competitive inhibition model

$$Transcription = \ Basal + (Max - Basal)(\frac{[Estradiol]^n}{[Estradiol]^n + {K_m}^n}) \tag{4.1}$$

$$K_m = K_d(1 + \frac{[Doxycyline]^{n_2}}{[K_i]^{n_2}}) \tag{4.2}$$

Under a competitive inhibition model, a repressor competes with an activator for the

same DNA region, thereby directly affecting the dissociation constant of the activator. As the concentration of repressor is increased, it would increase the concentration of activator needed to activate transcription. On a plot this would appear as a "flattening" out of the dose response as the repressor concentration is increased, as shown in Figure 4.5A. However, the maximum transcription rate would not be affected, and the extrapolation of all curves should still approach the same maximal output (though practically this extrapolation would require concentrations that are not biologically possible for some of the curves).

Non-competitive inhibition model

$$Transcription = \ Basal + (Max - Basal)(\frac{[Estradiol]^n}{[Estradiol]^n + {K_\mathrm{d}}^n})(\frac{[K_\mathrm{i}]^{n2}}{[Doxycycline]^{n2} + {K_\mathrm{i}}^{n2}}) \tag{4.3}$$

Under a non-competitive inhibition model, the repressor and activator do not compete for the same DNA region and each can be modelled as an independent DNA binding event. As the concentration of repressor is increased, it would decrease the maximal transcription rate of the promoter. On a plot this would be evident by a downward shift of the maximal expression "plateau", as shown in Figure 4.5B. However, the dissociation constant would not be affected, because the two regulator binding events are independent, and therefore the half-maximal point of all dose-responses would remain constant.

Parameter values

Two dimensional dose responses of single-input promoters

Quantitative transcriptional repression studies typically study the effect of changing repressor concentration at a fully active promoter and fitting the one-dimensional data, similar to what was done in subsection 4.3.3. However, one-dimensional data is not sufficient to determine if repression is competitive or non-competitive. We performed two-dimensional

Table 4.1: Parameters and constants used for evaluating the models of inhibition

Parameter/Constant Name	Value
Basal	10 Arbitrary units
Max	630 Arbitrary units
K_d	30nMol [68]
n	4 [3]
K_i	1000ng/mL [26]
n_2	4 [3]

dose responses of β-Estradiol and doxycycline to control the amount of activator and repressor that were bound at the promoter region. The cells were cultured for six hours in the inducers to ensure steady state was reached. The unrepressed promoter's activity (red curves) were compared against the promoter's activity at increasing amounts of repression (increasing shades of grey), by plotting all the dose response curves on the same axis.

I first performed the two-dimensional dose response curves for rTetR-BFP when recruited to P_{GT0}, P_{GT1}, P_{GT2} and P_{GT3}. Importantly, there was no observable repression when the repressor was recruited to P_{GT0}, as evidenced by the dose response curves overlapping at all doxycycline concentrations (Figure 4.6A). Interestingly, at the P_{GT1} promoter, the dose response curves become more linear at higher doxycycline concentrations and the steepness of the dose response curve is lost, similar to a competitive inhibition model (Figure 4.5A). There also appears to be a shift in the half maximal concentration as doxycycline is increased (Figure 4.6B). This is not observed when the repressor is recruited to the P_{GT2} and P_{GT3} promoters. At the P_{GT2} promoter, there is little repression even at high doxycycline concentrations (Figure 4.6C), similar to what was observed in Figure 4.3A. Interestingly, the dose-response curves for the P_{GT2} and P_{GT3} promoters are qualitatively very similar, with the maximal yeGFP expression of the promoters decreasing as doxycycline is increased. Notably, there is no observable change in the half maximal concentrations as repression is increased (Figure 4.6C,D). Repression from the P_{GT2} and P_{GT3} promoters is similar to a non-competitive inhibition model (Figure 4.5).

I next performed the two-dimensional dose response curves for rTetR-Sin3 when recruited to P_{GT0}, P_{GT1}, P_{GT2} and P_{GT3}. Qualitatively, the dose response curves looked very similar to those of rTetR-BFP in Figure 4.6. There was no observable repression when the repressor was recruited to P_{GT0} (Figure 4.7A). Similar to rTetR-BFP, repression of P_{GT1} by rTetR-Sin3 causes the dose response curve to become less steep as the doxycycline concentration increases, resembling competitive inhibition. In contrast, repression of the P_{GT2} and P_{GT3} promoters resembles non-competitive inhibition, with the half maximal concentration staying the same and the maximal expression level decreasing as repression increases. Moreover, repression from the P_{GT2} promoter is much stronger than it was for rTetR-BFP, similar to what was observed in Figure 4.3B.

Finally, I performed the two-dimensional dose responses for rTetR-Ssn6 when recruited to P_{GT0}, P_{GT1}, P_{GT2} and P_{GT3}. Repression by rTetR-Ssn6 was qualitatively very similar to rTetR-Sin3, as shown in Figure 4.8. Interestingly, the maximum expression of the promoters, even in the absence of doxycycline, is strikingly lower than it is in the rTetR-BFP and rTetR-Sin3 strains and the onset of activation occurs at higher β-Estradiol concentrations.

Combined competitive and non-competitive inhibition

The single tet site promoters displayed characteristics that are similar to competitive and non-competitive models of enzyme inhibition. We wondered if this could be taken a step further for the multi tet site promoters that repress transcription presumably by both mechanisms, as shown in Figure 4.9. The mathematical model presented in equations 4.4 and 4.5 is a combination of the two inhibition mechanisms where the addition of doxycycline can inhibit activator binding in a competitive manner, and PIC formation in a non-competitive manner. The combined model was evaluated with the same parameters as before (Table 4.1) and plotted in Figure 4.9.

Under a combined competitive and non-competitive model, the repressor would com-

pete with the activator for the same DNA region and would also bind non-competitively at another location. As the concentration of repressor is increased, it would increase the concentration of activator needed to activate transcription and it would decrease the maximal transcription rate. Visually, the dose response curves would become less steep and the maximal expression plateau would decrease, as shown in Figure 4.9.

$$Transcription = \ Basal + (Max - Basal)(\frac{[Estradiol]^n}{[Estradiol]^n + {K_\mathrm{m}}^n})(\frac{[K_\mathrm{i}]^{n_2}}{[Doxycycline]^{n2} + {K_\mathrm{i}}^{n_2}}) \tag{4.4}$$

$$K_\mathrm{m} = K_\mathrm{d}(1 + \frac{[Doxycyline]^{n_2}}{[K_\mathrm{i}]^{n_2}}) \tag{4.5}$$

Two dimensional dose responses of multi-input promoters

I first performed the two-dimensional dose response curves for rTetR-BFP when recruited to P_{GT12}, P_{GT23}, P_{GT13} and P_{GT123}. The dose responses for all promoters with a distal '1' site exhibit an increase in half maximal concentration as the repressor concentration is increased (Figure 4.10A,C,D), suggesting there is some competitive inhibition occurring. Notably, this shift is absent when P_{GT23} is repressed (Figure 4.10B), with all curves having a half maximal concentration of ∼100nMol, similar to what was observed for the P_{GT2} and P_{GT3} single input promoters (Figure 4.6C,D), indicating that the inhibition is non-competitive. The dose response curves for the P_{GT12} promoter are harder to interpret because of the noisy reporter expression at low β-Estradiol concentrations. However, the dose response appears to become shallower at higher repressor concentrations which is indicative of competitive inhibition (Figure 4.10A). The repression of P_{GT13} and P_{GT123} causes a noticeable increase in the half maximal concentration and a significant downward shift in maximal reporter expression (Figure 4.10C,D), suggesting that there are combined competitive and non-competitive interactions occurring.

I next performed the two-dimensional dose response curves for rTetR-Ssin3 and rTetR-Ssn6 when recruited to P_{GT12}, P_{GT23}, P_{GT13} and P_{GT123} (Figure 4.11, 4.12). Qualitatively, the dose response curves for both repressors looked very similar to those of rTetR-BFP in Figure 4.10. All promoters that contained a distal '1' tet site had higher half maximal concentrations as repression was increased. The promoters that did not contain a distal tet site (P_{GT23}), had no shift in K_d and displayed more non-competitive behaviour. The activation of all promoters that contained an rTetR-Ssn6 repressor once again occurred at higher β-Estradiol concentrations and the maximal promoter output was lower than strains with the rTetR-Sin3 and rTetR-BFP repressors.

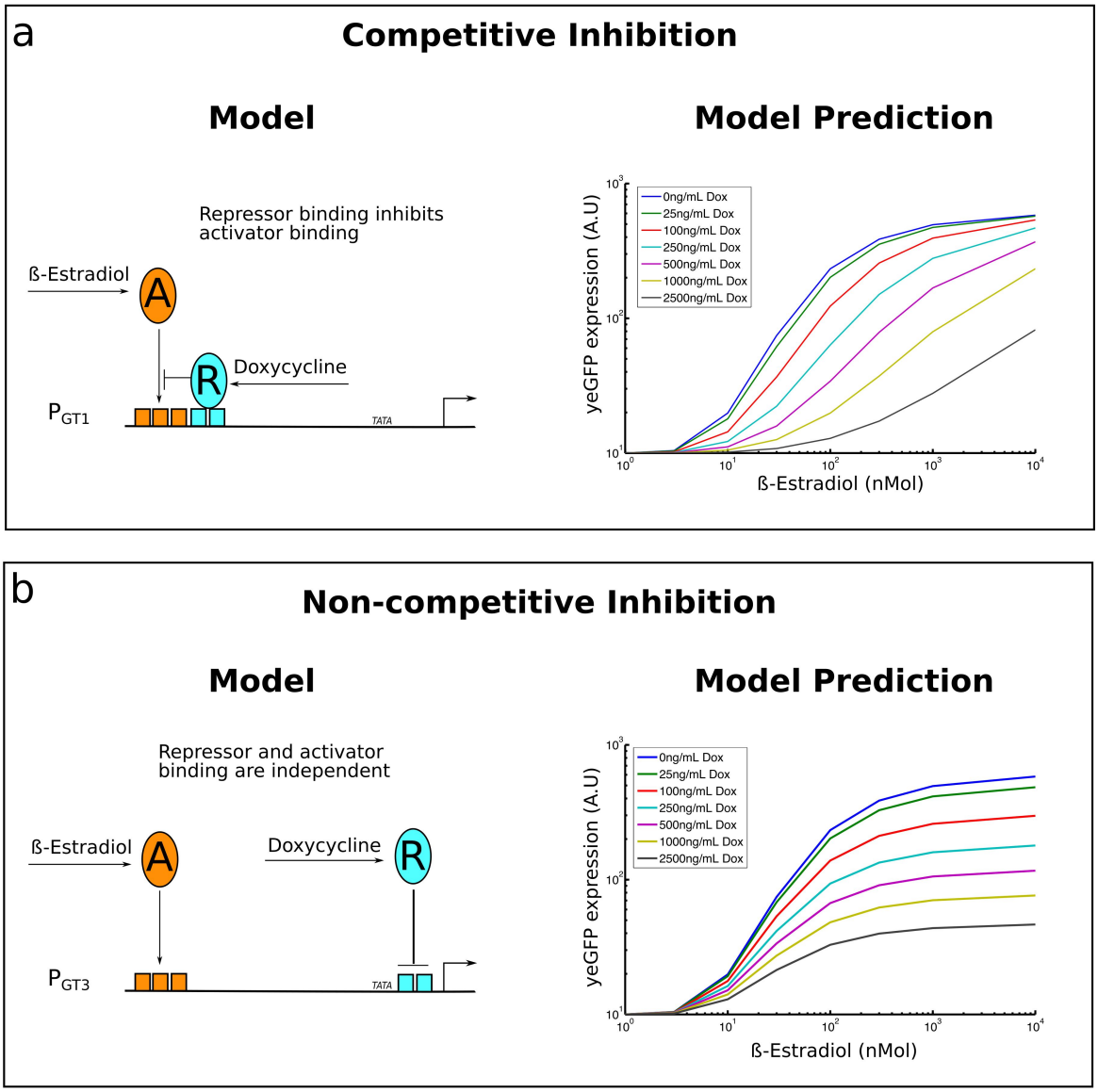

Figure 4.5: Models of sterical hindrance based repression at the activator binding region or TATA box. **a.** A model of competitive inhibition evaluated using equations 4.1, 4.2 and the parameters in table 4.1. **b.** A model of non-competitive inhibition evaluated using equation 4.3 and the parameters in table 4.1.

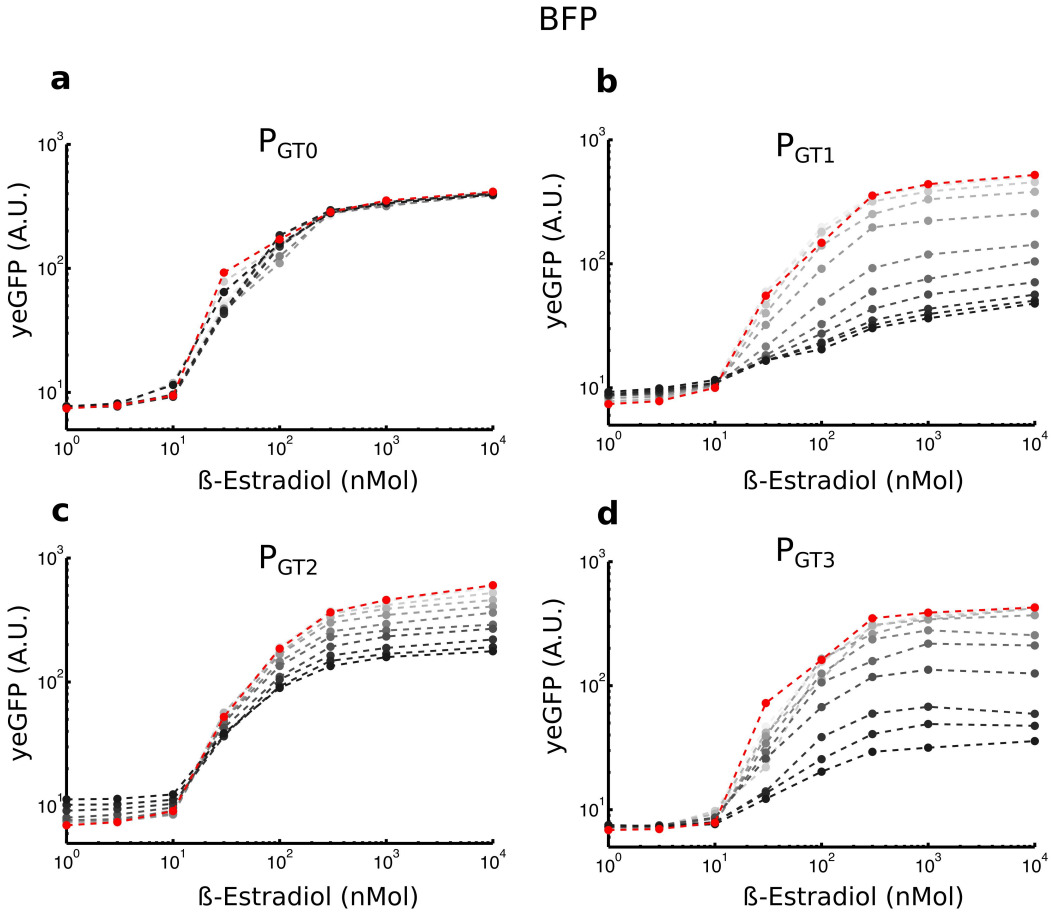

Figure 4.6: Transcriptional activation of single-input promoters while repressed by various amounts of an inert (BFP) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT0}, **b.** P_{GT1} , **c.** P_{GT2}, **d.** P_{GT3}

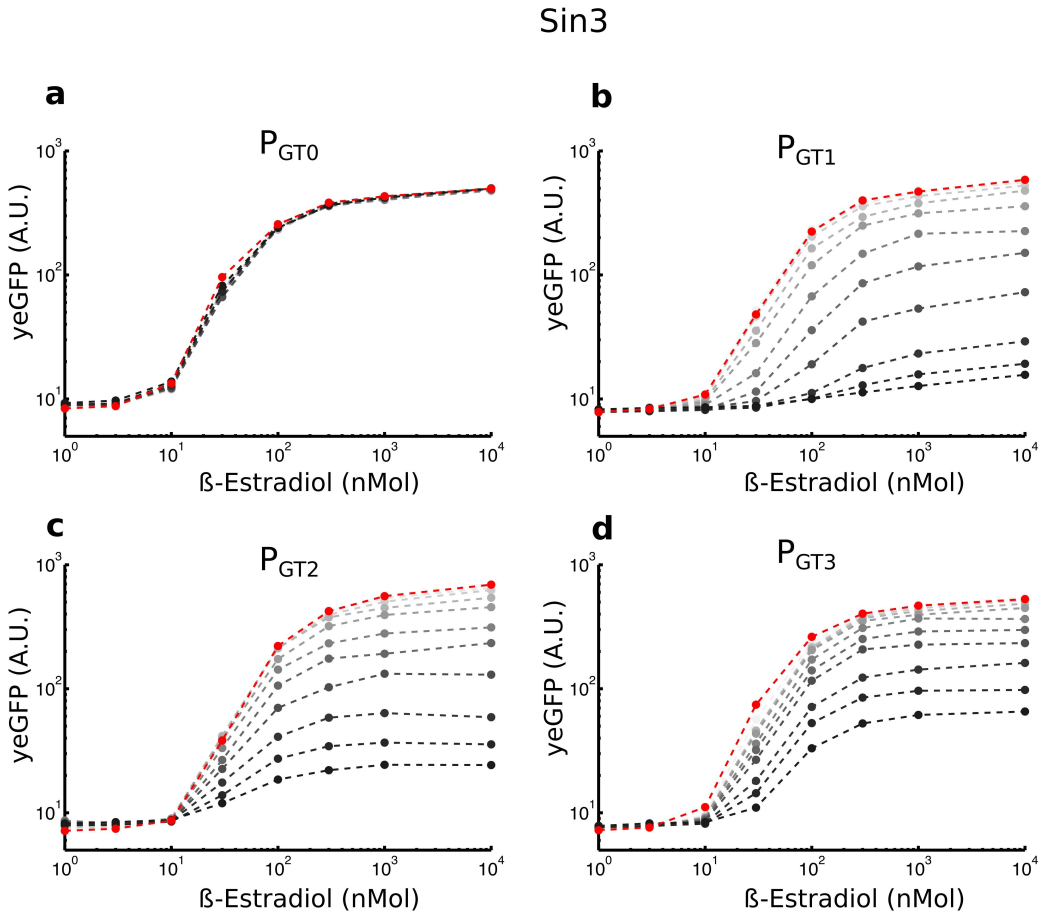

Figure 4.7: Transcriptional activation of single-input promoters while repressed by various amounts of an enzymatic (Sin3) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT0}, **b.** P_{GT1} , **c.** P_{GT2}, **d.** P_{GT3}

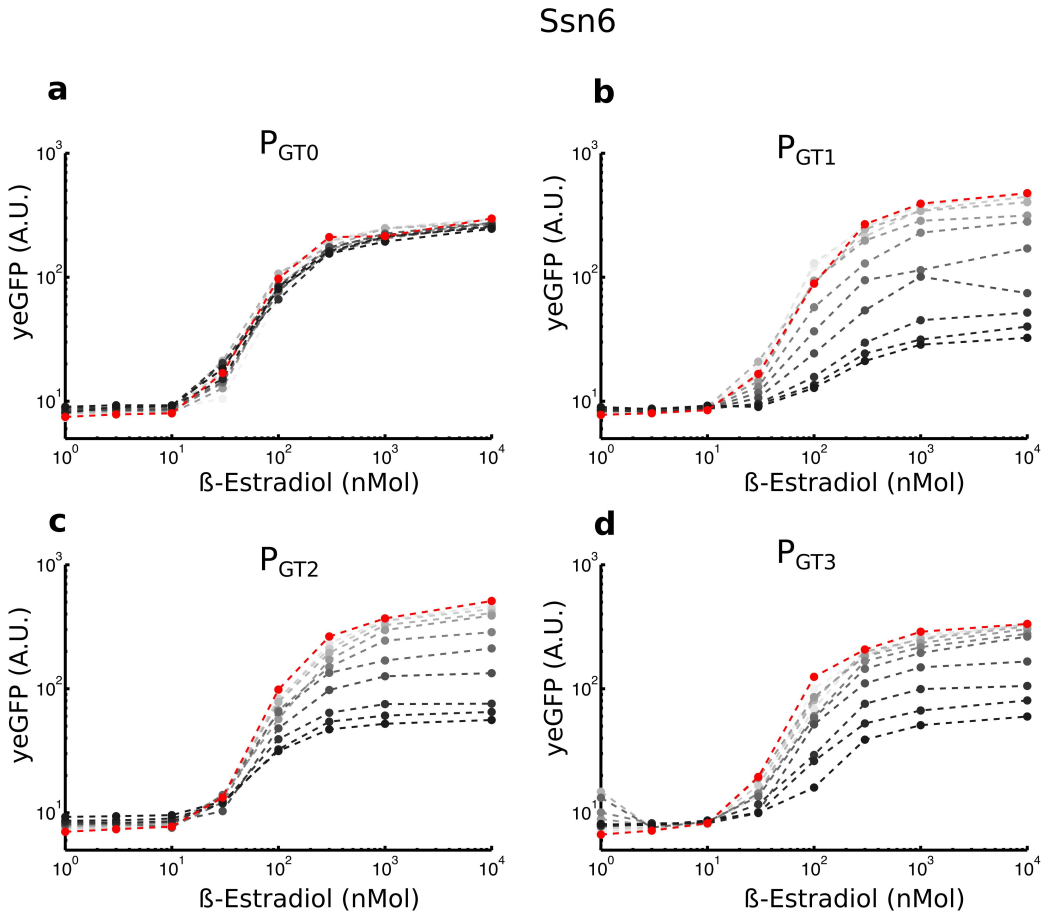

Figure 4.8: Transcriptional activation of single-input promoters while repressed by various amounts of an enzymatic (Ssn6) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT0}, **b.** P_{GT1} , **c.** P_{GT2}, **d.** P_{GT3}

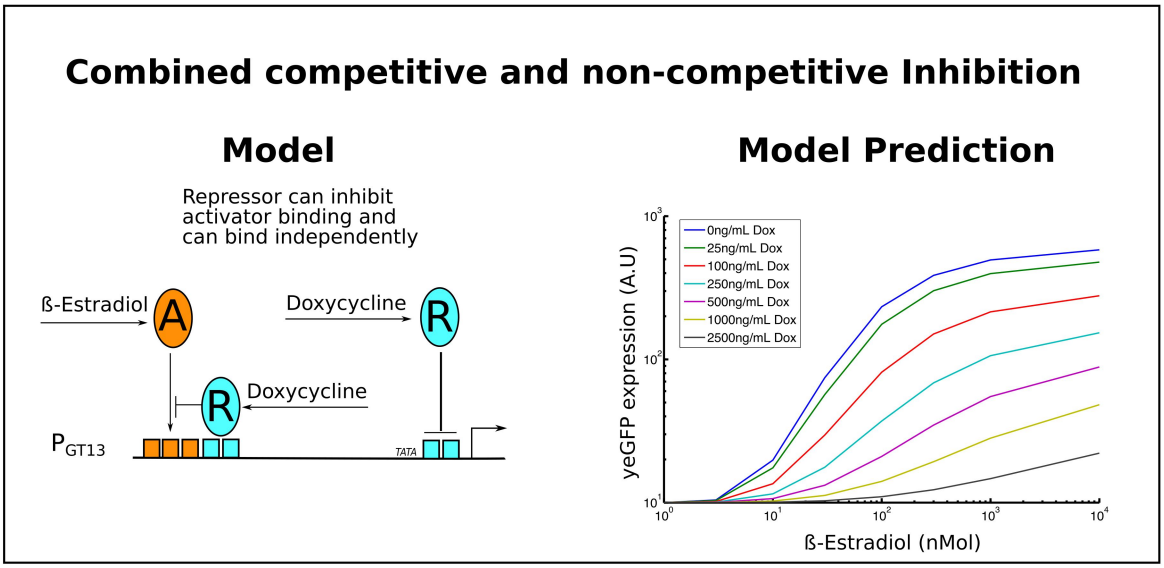

Figure 4.9: A combined model of sterical hindrance based repression at the activator binding region and the TATA box. **a.** The model was evaluated using equations 4.4, 4.5 and the parameters in table 4.1

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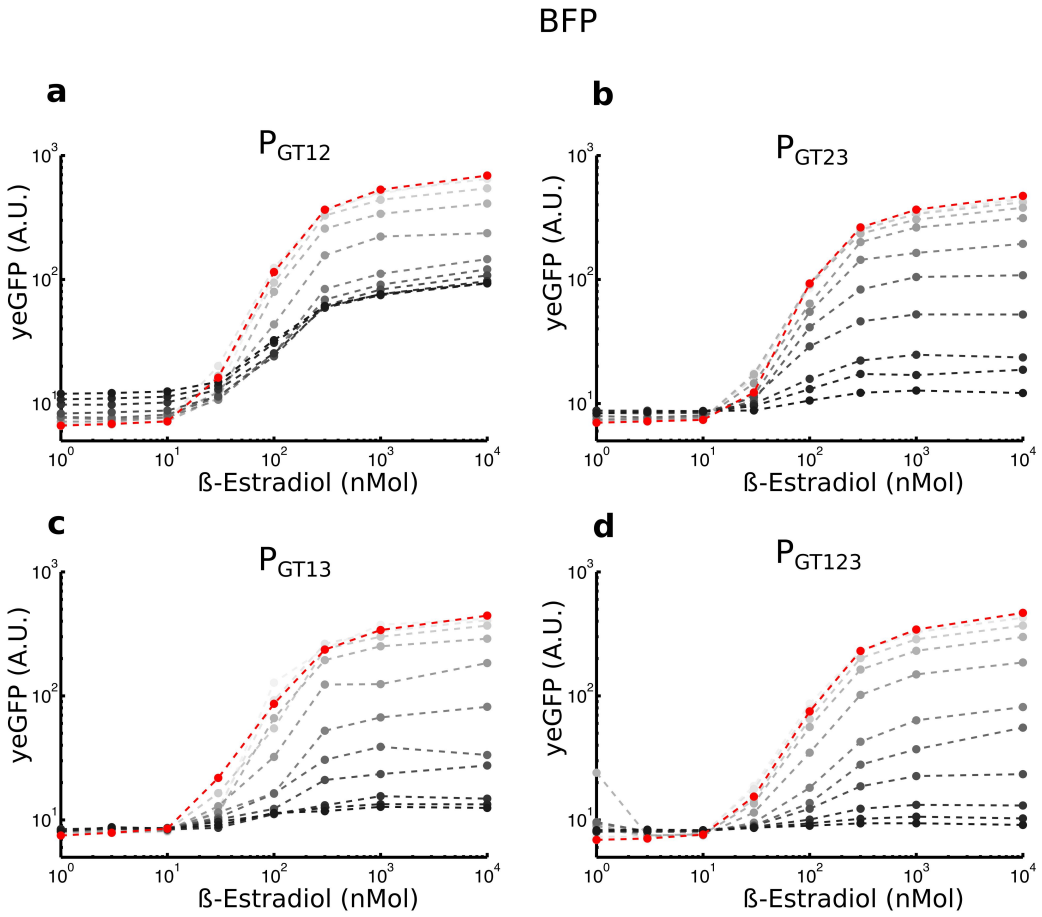

Figure 4.10: Transcriptional activation of multi-input promoters while repressed by various amounts of an inert (BFP) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT12}, **b.** P_{GT23} , **c.** P_{GT13}, **d.** P_{GT123}

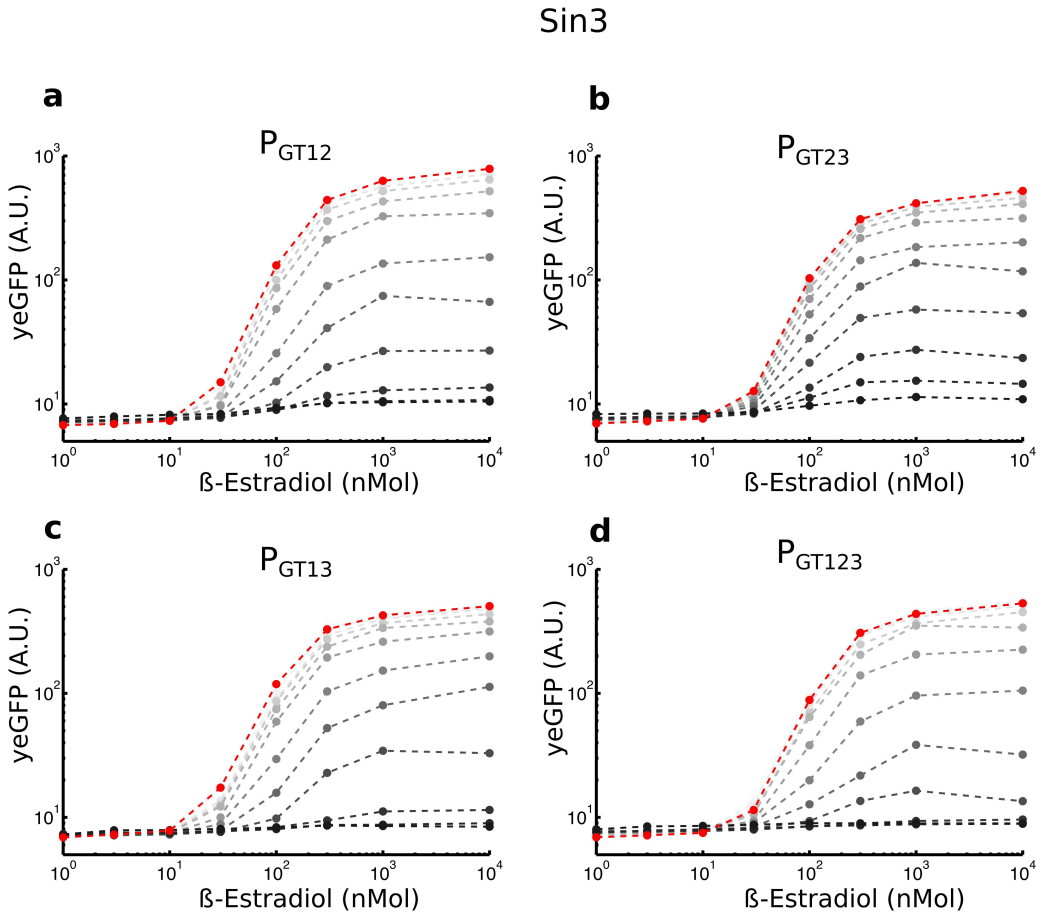

Figure 4.11: Transcriptional activation of multi-input promoters while repressed by various amounts of an enzymatic (Sin3) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT12}, **b.** P_{GT23} , **c.** P_{GT13}, **d.** P_{GT123}

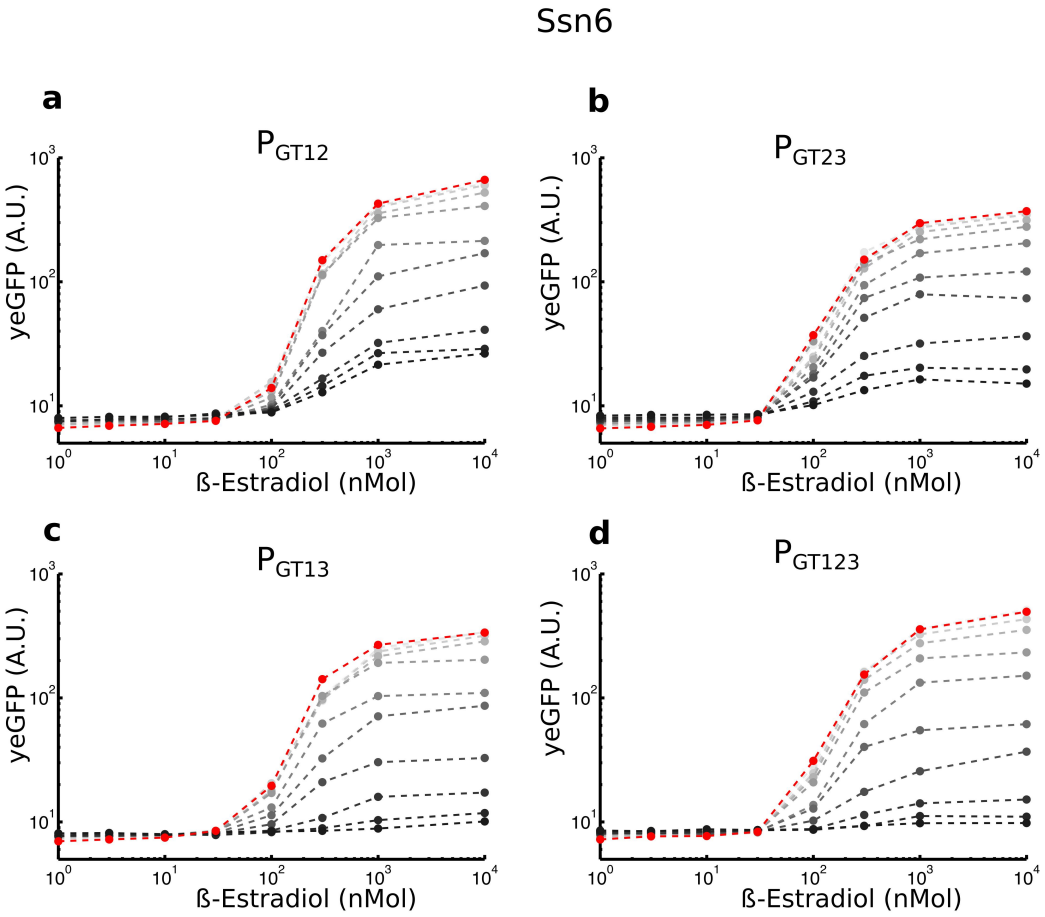

Figure 4.12: Transcriptional activation of multi-input promoters while repressed by various amounts of an enzymatic (Ssn6) repressor. A two dimensional dose response of β-Estradiol and doxycycline was performed. Progressively darker shades of grey correspond to 25,100, 250, 500,1000, 2500, 5000, 10000, 25000, 50000 and 100000 ng/mL of doxycyline. The red curve is 0ng/mL and displays transcriptional activation in the absence of repression. The promoters used are: **a.** P_{GT12}, **b.** P_{GT23} , **c.** P_{GT13}, **d.** P_{GT123}

Chapter 5

Discussion

5.1 Optimization of an rtTA variant with undetectable leaky activity

5.1.1 Summary of findings

We have identified single amino acid substitutions in the widely used doxycycline-inducible transactivator (rtTA) that significantly improves dynamic range without compromising maximal expression capacity. Replacing a single glycine (G72) in the rtTA-M2 variant with residues that introduce non-polar side chains reduces its ability to activate transcription in the absence of doxycycline in a manner that depends on the size of the side chain. This suggests that the reduction in leaky target gene expression may be due to an increased rigidity of the tertiary structure of rtTA that reduces its DNA binding affinity in the absence of induction.

The mutations that reduce leaky target gene expression also reduce the sensitivity to doxycycline. This loss of sensitivity depends on the size of the side chain introduced at G72, suggesting a complex interplay between the rtTA DNA binding domain and its doxycycline

binding pocket.

The loss of sensitivity could be a problem in applications where it is not possible, or not practicable, to use high doxycycline concentrations. We have demonstrated, however, that introducing additional mutations into the G72P M2 variant can improve the sensitivity to doxycycline without introducing leaky target gene expression. The variant with high sensitivity and undetectable leaky target gene expression carries five mutations (V9I, F67S, G72P, F86Y, R171K) in the TetR domain of rtTA-M2 and the G72P mutation appears to be critical. A G72A variant has higher doxycycline sensitivity (lower EC50) than the G72P variant, but also substantially higher activity in the absence of doxycycline. These observations suggest that the combined effect of mutations that impact rtTA DNA binding and doxycycline induction are highly nonlinear.

5.1.2 Implications and future work

Applications for reduced leaky activity

Improved rtTA systems with low leaky activity will enable studying biological processes that are particularly sensitive to low levels of gene expression. Two lab groups at the Ottawa Institute of Systems Biology have already used our new variant successfully where the conventional rtTA system or other conditional gene expression systems have failed due to leaky expression.

In our study we created our own tet-responsive promoters by simply placing tet sites in regions of the *GAL1* promoter that were known to bind transcription factors. There was no further optimization of these promoters. Most tet-responsive promoters that are commonly used with rtTA have been optimized extensively to reduce leaky expression while still allowing high maximal expression. Often, these approaches mutate a core promoter randomly and insert tet operators upstream [63]. By testing the promoters in high throughput they can identify promoters with low leaky activity that still retain high max-

imal expression. However, these approaches do not address the fact that the rtTA is still weakly binding its operator. Our variants with decreased leaky expression, presumably by weaker uninduced DNA binding, will allow for more flexibility in creating tet-responsive promoters. One can imagine that tet operators could simply be inserted upstream of any promoter region, to upregulate that gene's expression, in a doxycycline dependent manner. This would allow for true upregulation of gene expression from its natural basal expression level because the insertion of tet operators could be done with out interrupting the core promoter. Furthermore, multiple endogenous promoters could be upregulated simultaneously, by simply have tet operators inserted upstream. Stringent conditional rtTA variants will ease synthetic promoter construction and optimization.

The rtTA system is used extensively in mammalian cell lines and whole animal models. Although rtTA systems that were developed in yeast have been successfully deployed in mammalian cell lines [90], it is essential to test our new variants in various mammalian cell lines. Improved rtTA systems with low leaky expression would be very useful for mammalian molecular biology. Transient transfections are often used to introduce gene constructs into cell lines, however, there is little control of the amount of DNA that is being transfected into each cell across the population. This could cause variability in rtTA activity across the population. We've showed that leaky rtTA activity is highly dependent on the transactivator's expression level, which could vary significantly in transiently transfected cells. Leaky rtTA activity has been shown to be dependent on the amount of transiently transfected DNA [25] and our new variants with decreased leaky activity could be useful for these applications to ensure populations of cells have a homogenous response to doxycycline. Furthermore, rtTA is used for conditional gene expression in animal models including: mice, plants and drosophila [7]. In multicellular systems, promoter strength can vary from tissue to tissue. New rtTA variants that are robust to variable expression levels would simplify the creation of whole animal models because the transactivator could be expressed at various levels in different tissues without any leaky activity.

Quantifying leaky activity

Leaky rtTA activity has been observed extensively since its inception [12, 48, 49, 1, 63, 90, 64]. It has been repeatedly hypothesized and it is generally accepted that leaky activity is dependent on rtTA expression level, though it has never been experimentally shown. Our study is the first to quantify how rtTA transactivator levels influence leaky activity. Similar approaches could be made when creating or using rtTA systems in mammalian cells to quantify the leaky activity of the transactivator. Although the β-Estradiol inducible GEV system cannot be used in mammalian cell lines, the transactivator's expression level could be experimentally varied with other inducible systems. Moreover, various concentrations of rtTA DNA can be transfected and the leaky expression measured. In this manner, the leaky activity could be plotted as a function of transfected DNA. We hope that our work will influence other researchers to study rtTA in a similar quantitative manner.

Conditional DNA binding and synthetic chromatin modifiers

The fundamental mechanism of the rtTA system is conditional DNA binding of the rTetR domain to the tet operator in the presence of doxycycline. The rTetR domain does not necessarily need to be fused with activation domains and can be fused to other proteins, as we did in Chapter 4.3. The conditional targeting of enzymes to specific regions of DNA is an emerging field in synthetic biology [55, 56, 14] and stringent, inducible, DNA binding is essential. Inducible, targeted chromatin modifiers would allow for studying chromatin modifications in a concentration and temporally regulated manner [14]. For example, an rTetR domain tagged with a DNA methyltransferase would allow for conditional and permanent silencing of transcription near a tet operator. Any leaky rTetR binding would be undesirable as it could cause uninduced silencing. Improved rTetR domains with reduced basal binding would be essential for mammalian genome engineering applications such as this.

5.2 Comparison of eukaryotic and bacterial transcriptional repression mechanisms

5.2.1 Summary of findings

Bacteria and eukaryotes repress transcription in fundamentally different ways [87]. Bacteria repress transcription with DNA binding proteins that interfere with the assembly of the transcriptional machinery, while eukaryotes repress transcription through a variety of mechanisms including masking activation domains and changing local chromatin structure. It is well established that bacterial mechanisms of repression are functional in eukaryotes, however, a side-by-side comparison of bacterial and eukaryotic mechanisms of repression has never been done.

We have created novel synthetic transcriptional repressors that can inhibit gene expression in a doxycycline dependent manner. These repressors were built using an optimized rTetR that was developed in Chapter 4.3 and contain either the natural yeast repression domains, Sin3 or Ssn6, or an inert blue fluorescent protein (BFP). Furthermore, we have also created a synthetic promoter library that can receive input from a synthetic transcriptional activator and the novel doxycycline responsive repressors. We characterized these new components in a comprehensive manner by measuring the expression output from the synthetic promoters at various concentrations of the activator and repressor.

We have shown that DNA binding proteins that bind near upstream activating sequences (UAS) or the TATA box can repress transcription without additional enzymatic activity. However, these repressors are only able to mildly repress transcription from a region medial to both the UAS and TATA box. On the other hand, repressors with natural yeast repression domains are able to strongly repress transcription wherever they are recruited in the promoter region.

Furthermore, we showed that repressors that bind in the distal promoter region, near

the UAS, exhibit behaviour that is consistent with a competitive inhibition model. In contrast, repressors that bind the medial or proximal regions exhibit behaviour that is consistent with a non-competitive inhibition model.

5.2.2 Steady state analysis of bacterial and eukaryotic repression mechanisms

Western blotting confirmed that each of the repressors were expressed similarly amongst the eight different promoter strains into which they were integrated (Figure 4.2). This allows us to directly compare the doxycycline dependent activity of each repressor at all promoter variants without being concerned that the repressor is differentially expressed. However, we believe the repressors are differentially expressed in relation to each other and we did not make any direct comparisons between the doxycycline concentrations required for repression from the different repressors.

To generate steady state dose responses of the repressors turning off gene expression, we varied the concentration of doxycycline when the promoters were fully activated with saturating concentrations of β-Estradiol (5000nM). From this data we can determine the strength of repression at each region of the promoter and the concentration of doxycycline needed for the onset of repression.

Bacterial mechanisms of repression

We anticipated that the inert (BFP) repressor would be able to repress transcription at the distal and proximal regions by interfering with activator binding or pre-initiation complex (PIC) assembly, but not at the medial site which is located away from both the activator binding sites and the TATA box. Interestingly, the inert repressor was able to repress transcription at all three promoter locations (Figure 4.3A).

Repression through the inhibition of activator binding has never been explored in synthetic biology, though it appears to repress transcription quite effectively. We observed significant promoter repression by targeting repressors to proximal sites within the promoter region. However, the promoter was not fully repressed even under conditions of maximal repressor induction. We believe this is likely due to the specific promoter architecture used in this study. The tet sites in the P_{GT1} promoter are located 2 base pairs away from the proximal activator (GEV) binding site, as shown in Figure 4.1. We hypothesize that the binding of the repressor to these sites is likely preventing activator binding at the most proximal GEV sites but not at the distal site which is 44 base pairs away. Stronger repression could likely be achieved by placing the tet sites between or around the GEV sites, which would increase the proximity of the repressor to all activator sites. Interestingly, repression at the P_{GT1} promoter occurs at lower doxycycline concentrations than it does at the P_{GT3} promoter. This could indicate that preventing a relatively small transcriptional activator from binding requires lower repressor concentrations than preventing a large multi subunit complex such as the PIC.

We did not anticipate that rTetR-BFP would be able to repress transcription from the medial tet sites, which are 70 base pairs upstream of the TATA box and 160 base pairs downstream of the activator binding sites. We predict that the repression that we observed indicates that the repressor is still able to interfere with the assembly of the general transcriptional machinery. Although the TATA box is the scaffold for assembling the PIC at the promoter, the PIC is quite large and it is possible that DNA binding 70 base pairs upstream of the TATA may still interfere with its assembly.

Our data supports the hypothesis that rTetR-BFP is able to disrupt PIC assembly from at least 70 base pairs away, as the mechanisms of repression at P_{GT2} and P_{GT3} appear similar. The onset of repression occurs at the same doxycycline concentration at both P_{GT2} and P_{GT3}, and the initial slope of repression is very similar (Figure 4.3). This suggests that similar repressor concentrations result in inhibition of PIC assembly, at both medial

and proximal promoter regions. We therefore suspect that repression of P_{GT2} and P_{GT3} by rTetR-BFP occurs through a similar mechanism, while this repression is weaker than when binding sites are located 10 base pairs away from the TATA box.

It would be interesting to build promoters with tet sites located between the medial and proximal regions to quantify how the repression strength is lost as the repressor site is moved farther away from the TATA box. Similar approaches have been done with tet sites at various locations between the TATA box and transcriptional start sites (TSS) [70], but never upstream of the TATA box.

Eukaryotic mechanisms of repression

We anticipated that the enzymatic yeast repressors, Sin3 and Ssn6, would be able to repress transcription from any location in the promoter region because their mechanisms of action have local effects on the promoter region and are not solely dependent on DNA binding. The Sin3-Rpd3 complex deacetylates histones and alters local chromatin structure [52] whereas the Ssn6-Tup1 complex is currently believed to mask activation domains [94].

As anticipated, both repressors were able to repress transcription at all three promoter locations (Figure 4.3B,C). Our steady state approach to studying the various mechanisms of repression did not elucidate much information about the fundamental mechanisms of either Sin3 or Ssn6. However, a consistent aberration in our data was the low reporter expression from any strain that contained the rTetR-Ssn6 repressor. We believe this is consistent with the latest model of Ssn6 function that suggests it masks activation domains [94]. The VP16 activation domain on GEV is an acidic activation domain, much like natural yeast activation domains. We hypothesize that the overexpressed rTetR-Ssn6 is able to interact and quench the free floating GEV molecules, independent of co-localization to the same promoter. This would explain why higher β-Estradiol concentrations are required to turn on the promoters in the strains containing the rTetR-Ssn6 repressor and also why the

promoters in these strains don't reach the same maximal expression level.

The dose response curves for the rTetR-Sin3 and rTetR-Ssn6 repressors were very similar and can be analyzed simultaneously (Figure 4.3B,C). Enzymatic repression was strongest at the distal and medial promoter regions and the weakest at the proximal promoter region. The onset of repression at the proximal promoter occurred at notably higher doxycycline concentrations than at the distal and medial sites. This is unlike what was observed for rTetR-BFP where only distal repression occurred at low concentrations.

We hypothesize that at the proximal promoter the repressors are competing with the PIC for access to the promoter, whereas the medial and distal sites are comparatively easier for the repressor to access. This would explain why repression at the distal and medial regions is strong and comparable whereas high concentrations of repressor are needed for the onset of repression at the proximal region.

Contributions of DNA binding and repression mechanisms to the strength of steady state repression

By analyzing the dose response curves we've shown that the onset of repression varies significantly based on the mechanism of the repressor and the location of its binding. Bacterial repression is mediated solely by DNA binding whereas enzymatic repression is mediated by both DNA binding and higher order interactions [87].

We think there are two primary considerations that explain the observed differences in the onsets of repression.

1. The ability of the repressor to access/bind its operator site on the DNA.
2. The concentration of repressor required for repression.

With these considerations we can explain the experimental observations as follows (Summarized in Table 5.1):

At the distal and medial regions of the promoter, the repressor has easy access to its respective binding site. This allows the repressor to bind the DNA at low concentrations of doxycycline. At the proximal promoter region, the repressor has to compete with the PIC for access to its repressor site which is between the TATA box and TSS. This requires more doxycycline for the repressor to bind the DNA.

Repression by DNA binding at the distal region can occur at low repressor concentrations because the activator protein is relatively small and easy to outcompete. This means less doxycycline would be required for repression. At the medial and proximal promoter regions, the DNA binding protein has to exclude a large multi component complex from the promoter region: this would require more repressor and therefore higher concentrations of doxycycline.

Repression by enzymatic activity can occur at low concentrations of repressor because its repressive effect is not dependent on the strength of DNA binding and outcompeting other complexes recruited to the promoter. At the distal and medial promoter regions, this would require low concentrations of doxycycline. At the proximal promoter region, high concentrations of the enzymatic repressor is required for it to outcompete the PIC and bind its cognate site. Therefore, proximal promoter repression would require higher doxycycline concentrations.

Table 5.1: Concentration of doxyxycline required for repressor function at the three promoter regions

	Concentration of repressor required for:			
Tet site location	Binding DNA	Steric hindrance	Enzymatic activity	**Repression**
Distal	Low	Low	Low	**Always low**
Medial	Low	High	Low	**Low for enzymatic, high for bacterial**
Proximal	High	High	Low	**Always high**

These considerations hold true for the multi tet site promoters as well (Figure 4.4). For the inert rTetR-BFP, the onset of repression occurs at lower doxycycline concentrations for promoters that contain a distal ('1') tet site. Repression from the P_{GT23} promoter requires the most doxycycline because it depends on outcompeting PIC assembly. For the rTetR-Sin3 and rTetR-Ssn6 enzymatic repressors, the onset of repression is quite similar for all multi input promoters but it is slightly higher for the promoters that contain a proximal ('3') tet site, which we hypothesize that the repressor has a harder time binding.

Competitive and non-competitive models of transcriptional repression

We hypothesized that the repression at the distal promoter region is due to a direct competitive interaction between the binding of the transcriptional activator and the binding of the transcriptional repressor. Furthermore, we hypothesized that the repression from the medial and proximal promoter regions would be non-competitive as the repressor binding sites are far from the activator binding sites and should be independent.

The hallmark of competitive enzyme inhibition is an increase in the concentration of substrate required to reach half maximal activity in the presence of inhibitor [91]. In the context of our assay this would be an increase in the concentration of β-Estradiol required to elicit half maximal gene expression in the presence of repressor (doxycycline). This is indeed what we observe. As the concentration of doxycycline is increased, the amount of β-Estradiol needed to reach half maximal expression is increased. This trend is observed from any repressor at all promoters that contain the distal tet sites. However, our results do not qualitatively appear to fit this model exactly. This is likely due to the incomplete repression of the distal activator sites as describe above in subsection 5.2.2. To combat this we are creating new promoters with distal tet sites that are located between the GEV binding sites. This should increase the competitive repression because the repressor will bind closer to all the activator binding sites.

The hallmark of non-competitive inhibition is a decrease in the maximal enzyme activity in the presence of inhibitor [91]. In the context of our assay this would be a decrease in the maximal promoter output in the presence of repressor. This model qualitatively appears to fit the repression observed from any repressor at all the promoters that contain tet sites at the medial and/or proximal region.

5.2.3 Implications and future work

This study contributes to the field of synthetic biology by creating new genetic parts and characterizing promoter-targeted transcriptional repression mechanisms. A major goal of synthetic biology is to decrease the amount of time spent in the Design-Build-Test cycle to allow for easier construction of novel, useful organisms. One of the most laborious tasks in synthetic biology is building synthetic gene regulatory components such as transcription factors and promoters that can be used to predictably build transcriptional controllers. Here we have designed, built and tested new synthetic transcriptional regulators using an rTetR domain with very low uninduced binding, and a library of synthetic dual input promoters. These components can be rearranged and combined to build larger gene regulatory networks such as cascades and feed forward loops. Multiple projects using these components are currently underway in the lab.

One of the more interesting findings of our study was that transcriptional repressors have different onsets of repression depending on their mechanism of action and their location of recruitment. Notably, all repression in the proximal promoter region required high concentrations of repressor binding. Currently, most synthetic gene regulatory networks built in eukaryotic synthetic biology projects use proximal repression. It is therefore intriguing to observe that distal repression, through inhibiting activator binding or enzymatic activity, can be achieved at much lower repressor concentrations. Synthetic biology could adapt this mechanism to be able to engineer transcriptional regulation with lower

expressed transcription factors. The limitations of sequence engineering within the proximity of the TATA box should therefore be kept in mind when designing novel repression or activation systems.

It has been previously reported that mutations near the TATA box can effect variability in gene expression [15]. Furthermore, we observed that inserting operators near the TATA box had an effect on the maximal transcriptional output of the promoter (Figure 4.2). Core promoter regions are likely sensitive to sequence changes that may alter strand separation, bending of the DNA or the assembly of the general transcriptional machinery. Distal promoter engineering that occurs much farther from the essential, proximal promoter elements may therefore be desirable, as it would be less likely to cripple promoter strength and alter noise properties. Eukaryotic transcriptional regulators are typically recruited upstream of the core promoter. It is compelling to think that eukaryotes adapted these mechanisms over simpler DNA binding based bacterial mechanisms because it allows for more experimentation with its transcriptional regulators. By swapping or mutating cis regulatory elements at the distal promoter region, the promoter is available for experimental sampling by novel DNA-binding regulators with minimal effect on core promoter properties such as promoter strength and the variability in expression.

Our work suggests that repression by DNA binding can function through competitive or non-competitive inhibition models. By placing repressor binding sites near the activator binding sites, competitive inhibition can be engineered. As the repressor sites are moved away from the activator sites, this competitive behaviour is lost and repression resembles non-competitive inhibition. An interesting effect of competitive inhibition is the "flattening" out of the dose response when repressor is present. This makes the promoter respond to activator in a linear manner, and reduces the steep dose response curve. Linearity in input-output relationships is associated with lower variability in the output response [71]. Increasing linearity in dose response curves has been accomplished in synthetic biology by the addition of negative feedback loops [71]. Its exciting that it can now be engineered

through competitive activator-repressor interactions. The finding that repression outside of the distal region is mediated through non-competitive interactions was also promising. This means that the transcriptional output is a function of two independent DNA binding events. If two dose responses are performed, one for the activator and one for the repressor, the transcriptional output at every combination of activator and repressor concentration can be calculated and predicted. This predictability is highly valued when constructing synthetic gene regulatory networks.

Future work

To improve on the competitive inhibition data, we will build a new synthetic promoter with distal tet sites located closer to all the activator binding sites. We hypothesize that the repression of this new promoter will fit to a competitive inhibition model better than the repression from the current P_{GT1} promoter. Moreover, we will need to mathematically fit the two-dimensional dose response curves to the equations describing competitive and non-competitive inhibition in Chapter 4.3.

The approach we took in this study relies on steady state data. Although steady state data can be used to predict dynamical behaviour, current work in our lab is investigating these repression mechanisms dynamically using microfluidics-enabled timelapse microscopy. This approach will elucidate more insightful information on the mechanism of action of the enzymatic repressors and whether different repression domains have unique timescales of action.

Chapter 6

Conclusion

The rtTA expression system serves a critical role in biological and biomedical research as a means to precisely control target gene expression in a graded and gratuitous manner. It also has many potential applications in gene therapy. In most applications, an optimal inducible gene expression system has undetectable target gene expression in the absence of inducer and sufficiently high target gene expression at non-toxic and achievable inducer concentrations. Usually, there is a trade-off between low leakiness and high expression capacity [25]. Our sensitivity-enhanced G72P M2 variant retains low leakiness and high expression capacity over a broad range of expression levels in yeast. Such robustness of function may be particularly advantageous in experiments where transactivator expression levels may vary, such as transient transfection or random integration of an expression cassette. Although past studies have shown that other rtTA variants first characterized in yeast preserve their function when expressed in mammalian cell lines and transgenic animals, it will be important to further investigate if the improved function of the sensitivity-enhanced G72P M2 variant extends from yeast to higher organisms. We have further shown that these enhanced conditional DNA binding domains can be fused with repression domains to create inducible transcriptional repressors. The function of these transcriptional repressors is dependent on the location of their recruitment and their

mechanisms of action. Enzymatic repression domains, such as SIN3 and SSN6 are able to strongly repress transcription from any region of the promoter whereas an inert DNA binding protein can only repress transcription from sites adjacent to activator binding sites or the TATA box. Repression of activator binding qualitatively agrees with a competitive inhibition model, whereas repression at any other promoter region agrees well with a non-competitive inhibition model.

Chapter 7

Other contributions

I have also made numerous contributions to science in addition to the work presented in this thesis.

Publications in print

- Azizi, A., Lam, W., Phenix, H., Tepliakova, L., **Roney, I.J.**, Jedrysiak, D., Power, A., Gupta, V., Elnour, N., Hanzel, M., Tzahristos, A.C., Sarwar, S., Kaern, M. (2015) No training required: experimental tests support homology-based DNA assembly as a best practice in synthetic biology. Journal of Biological Engineering. 9:8.
  *** I performed the flow cytometry which was used to quantify the cloning success rate. I contributed to data analysis and writing of the paper.**

Publications that are under review or submitted

- **Roney, I.J**., Rudner., A., Couture J.F., Kaern, M. (2016) Improvement of the reverse tetracycline transactivator by single amino acid substitutions that reduce leaky target gene expression to below detectable levels. Under review at Scientific Reports
  *** I performed all experiments and analysis, and wrote the paper along with my supervisor Dr. Mads Kærn. The paper is currently under review for publication.**

- Jing, W., Camellato, B., Azizi, A., **Roney, I.J**., Kaern, M., Godin, M. (2016) Microfluidic Measurements of Single-Cell Fitness Show Bet-Hedging in Eukaryotic Multidrug Resistance. Submitted to Biophysical Journal.

  *** I contributed to the experimental design and performed preliminary experiments on the strains that were used for the study. I optimized the experimental conditions that were used to collect the flow cytometry data.**

Patents

- **Roney, I.J**., Kaern, M. (2015) Reverse tetracycline transactivator mutants. US Provisional Patent Filing.

Oral and poster presentations

- **Roney, I.J**., Phenix, H., Kaern, M. (2015) Predictable outputs: A comprehensive study of combinatorial TF recruitment using a synthetic promoter library (Oral). qBio Winter Meeting (Kaanapali Beach, USA).

- **Roney, I.J**., Kaern, M. (2015) Construction and characterization of synthetic gene regulatory networks (Oral). Ottawa Institute of Systems Biology Retreat (Mont Tremblant, CAN).

- **Roney, I.J**., Kaern, M. (2014) Construction and characterization of synthetic dual-input promoters (Poster). Synthetic Biology: Engineering, Evolution Design(SEED) (Manhattan Beach, USA).

- **Roney, I.J**., Charlebois, D., Kaern, M. (2013) Implications of gene network architecture on variation in gene expression (Poster). 3rd Annual Computational Biology and Bioinformatics Poster Day (Ottawa, CAN).

Appendix

By plotting the data from Figure 3.5c-f as a surface plot instead of as a heatmap, the non-linearity is more apparent.

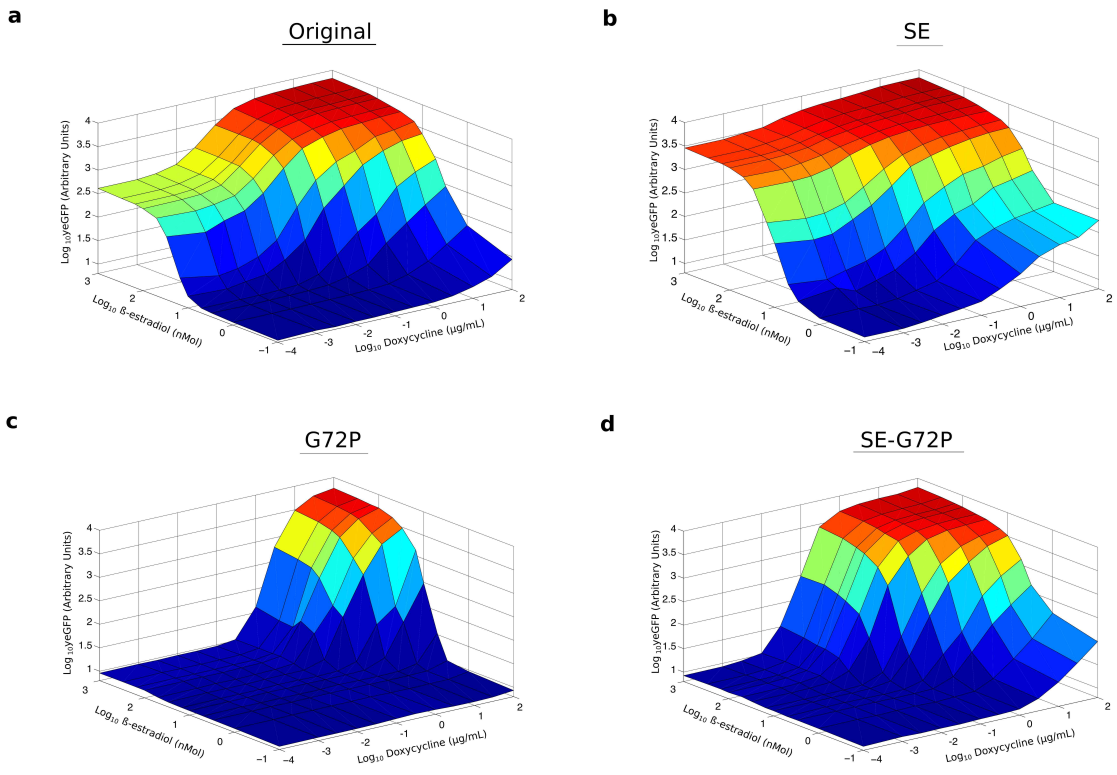

Figure 1: Surface plots of the data presented in Figure 3.5c-f. **a-d**, Surface plots displaying reporter expression (Arbitrary units) as a function of β-estradiol-dependent transactivator expression and doxycycline induction.

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