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Optimization of the Tet-On system in *Saccharomyces cerevisiae*

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by

Quoc B. Tran

Committee in charge:

Professor Jeff Hasty, Chair
Professor Nan Hao, Co-Chair
Professor Lorraine Pillus

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The Thesis of Quoc B. Tran is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABBREVIATIONS

dox	doxycycline
MoClo	Modular Cloning System
MoClo YTK	Modular Cloning System Yeast Toolkit
rtTA	reverse tetracycline-controlled transactivator
Tc	tetracycline
TRE	Tet Response Element
tetO	tetracycline operator
tTA	tetracycline-controlled transactivator

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ABSTRACT OF THE THESIS

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Synthetic biology in *Saccharomyces cerevisiae* or budding yeast has proved indispensable in metabolic engineering, gene circuit engineering, and molecular and cell biology. These applications require robust “plug-and-play” inducible promoter systems for tight and tunable control of gene expression, but current inducible systems have limitations. We have incorporated the parts of the Tet-On system into the MoClo YTK system. With these parts we have built and optimized the Tet-On system in *S. cerevisiae* through multiple design iterations. Through different combinations of the rtTA transactivators and constitutive promoters, we have created strains with varying

responses to doxycycline induction that will be useful for different applications. These systems boast extremely tight control over basal activity indistinguishable from the background strain, nanomolar sensitivities to doxycycline, and maximum output surpassing the strong pTDH3 promoter. Not only are these systems well-characterized and ready-to-use but the incorporation of the parts into the MoClo YTK allows them to be easily customized for specific applications.

INTRODUCTION

Yeast Synthetic Biology

In the past decade, the field of synthetic biology has moved from predominantly using bacterial cells toward eukaryotic organisms with yeast and mammalian cell cultures. *Saccharomyces cerevisiae* or budding yeast, especially, offers a wide variety of applications for synthetic biology, ranging from biosynthesis of metabolites to multiplexed control of genes in molecular biology experiments (Rahmat and Kang 2020).

Complex and layered synthetic circuits have been incorporated in *S. cerevisiae* for the metabolic engineering of diverse products previously only available from direct chemical synthesis or natural products such as the chemotherapy medication paclitaxel (Benedikt et al. 2008), the antimalarial drug artemisinin (Paddon et al. 2013), and the antioxidant resveratrol (Li et al. 2015).

Beyond metabolic engineering, budding yeast serves as a model eukaryotic organism and has been used to explore important principles in cell and molecular biology. Synthetic biology has played a major role in molecular biology tool development, such as the creation of yeast two-hybrid screening assays (Brückner et al. 2009), to probing of core principles of eukaryotic cell biology, like the understanding aging as a cell fate process (Li et al. 2020).

All of these applications require tunable control of gene expression, which is often achieved using inducible promoter systems (Da Silva and Srikrishnan 2012). A few exist for use in *S. cerevisiae* each with their own advantages and disadvantages.

Two of the most popular inducible promoter systems in budding yeast are the progesterone and β-estradiol-inducible promoters. The progesterone-inducible system requires expression of a progesterone receptor which, in the presence of progesterone, binds to a response element upstream cloned upstream of the gene of interest (Poletti et al. 1992). The β-estradiol system works similarly with a Gal4dbd.ER.VP16 activator and the Gal4p DNA sequence upstream of a promoter (McIsaac et al. 2011). These systems are generally chosen for their tight regulation of gene expression and fast response to induction. However, for use in microfluidic experiments with polydimethylsiloxane of PDMS, the material tends to absorb small hydrophobic molecules such as progesterone and β-estradiol, which makes these systems imprecise and ineffective when studying small colonies or single cells in microfluidics (Regehr et al. 2009, Wang et al. 2012).

Other commonly used tunable promoters include the galactose-inducible system and a copper-inducible system. Both systems make use of transcriptional activators endogenous to *S. cerevisiae* so only induction of gene only requires a gene circuit consisting of a synthetic promoter driving the gene itself (Bram et al. 1986, Etcheverry 1990, Griggs and Johnston 1991). The galactose-inducible system co-opts the cell's response to presence of galactose in its environment with promoters that mimic those that drive galactose metabolism genes. The copper-inducible system works similarly with the cell's machinery for the detoxification of free copper ions (Butt et al. 1984). This mechanism of action is a double-edged sword. While it allows for the easier cloning of only one expression cassette, its reliance on endogenous systems in yeast can lead to crosstalk with other pathways (Mortimer and Hawthorne 1966). In addition, the change of a carbon source or addition of copper in the media can result in

altered growth rate (Rutherford and Bird 2004). There is a need for a wider range of inducible promoter systems in yeast as tools to understand cell and molecular biology in a variety of contexts and conditions.

With the advent of larger and more complex engineered genetic circuits, there has also been a push for the standardization of biological parts. Well-characterized and interchangeable parts allow for rapid iteration of genetic circuits, accelerating the build-design-test-learn cycle of synthetic biology (Canton et al. 2008).

The basis of any gene circuit is an expression cassette that regulates a gene. In *S. cerevisiae*, this consists of a promoter sequence, the gene itself, and a transcription termination sequence. The promoter's main role is to recruit transcription factors and RNA polymerase, either constitutively or when induced by a transactivator, to transcribe the gene. In more complex gene circuits, multiple expression cassettes can be combined and then transformed into yeast either integrated into the genome or maintained as an extrachromosomal plasmid.

Cloning methods such as Gibson Assembly or restriction-ligation cloning prove to be inefficient at building these constructs. Restriction-ligation cloning involves the amplification of parts to add restriction enzyme sites at the ends, a restriction digest, followed by ligation of the parts to one another. Gibson Assembly has become a widespread method of cloning through isothermal recombination of linear DNA fragment overhangs. In the usual Gibson Assembly workflow, the constitutive parts of a final construct are first amplified in a PCR to add matching overhangs with their adjacent parts and combined into a single circular piece with a Gibson Assembly mix of exonucleases, polymerases, and ligases (Gibson et al. 2009).

In building even the simplest of gene circuits consisting of one expression vector, each of the necessary parts — the promoter, coding sequence, terminator, and integration and selection vector — will require two PCR primers to add either the Gibson overlap or restriction enzyme sites each, totaling eight primers. Even more inconvenient is the fact that changing out a single part — expressing the same gene under a different promoter, for example — requires not only new primers for that part, but the adjacent parts as well, in a Gibson Assembly. These methods do not scale well as gene circuits get larger and the need for specific single-use PCR primers slows down the iterative design cycle of gene circuit engineering.

The Modular Cloning System (MoClo) offers standard parts and standard Golden Gate assembly overhangs allowing reuse of standard biological parts to build an expression cassettes and plasmids consisting of multiple expression cassettes (Weber et al. 2011).The Golden Gate assembly method makes use of Type IIS restriction enzymes which cut DNA adjacent to the recognition site, allowing assembly of a plasmid through repeat cycles of digestion and ligation of constitutive part plasmids (Engler et. al 2008).

This allows the parts of the MoClo system to be rapidly reused, in contrast to other cloning methods. Because the same restriction digest can produce different overhangs depending on the sequence of the part and because the overhangs are standardized by part type — promoters, coding sequences, terminators, the various parts needed for assembly of multiple gene cassettes, and integration vector parts —, one part can be substituted for another of the same type without any other

modifications to the reaction. Once a part is incorporated into the toolkit, it can be easily used in any gene circuit.

The MoClo system has been expanded and there exist several toolkits making use of the system in various organisms. In *S. cerevisiae*, the yeast toolkit, MoClo YTK, prescribes standards for part overhangs that allow gene circuits to be cloned in bacteria and transformed into yeast in various ways as well as the characterization of many biological parts, including a wide range of constitutive promoters with varying strengths (Lee et al. 2015).

In yeast synthetic biology, the MoClo YTK system is widely used, and many other toolkits make use of the same system. There have been parts made for the system for applications as diverse as optogenetic control of genes (An-Adirrekkun et al. 2020) to reconstruction of the yeast α-factor signaling pathway (Shaw et al. 2019). However, current applications have mainly focused on utilizing existing parts from the yeast MoClo kit. Thus, there exists a need to interface the standardized framework of the MoClo YTK system with an expanded parts list of both new and existing genetic components. Toward this end, we set out to build, optimize, and characterize inducible promoter systems within the MoClo YTK system, allowing for ease of use in many different applications.

Tet-On System

The Tet-On system is a widely used inducible promoter system in many eukaryotic systems, built from *E. coli* regulatory elements. The first inducible Tet systems were built from the tetracycline (Tc) resistance gene network of *E. coli* where tetracycline resistance genes of the *Tn10* operon are transcriptionally blocked by the

tetracycline repressor (tetR) bound to tetracycline operator (tetO) DNA sequences. However, when tetracycline binds to TetR, the complex does not bind as efficiently to tetO, allowing the expression of the *Tn10* operon. This system was first isolated to build the Tet-Off system in mammalian cells where the removal of Tc or its analog doxycycline (dox) can induce gene expression through tetR fused with a virion protein 16 (VP16) a eukaryotic activation domain to create the tetracycline-controlled transactivator (tTA) (Gossen and Bujard 1992).

Four amino acids in the tetR domain of the tTA transactivator were mutated to create a reverse tetracycline-controlled transactivator (rtTA) which can only bind to tetO sequences in the presence of Tc or dox (Gossen et al. 1995). In the Tet-On system, rtTA in the presence of tetracycline binds to the Tet Response Element (TRE), which consists of seven tetO sequences upstream of a minimal promoter and drives a gene of interest.

Initial efforts to optimize the rtTA transactivator through both random mutagenesis and viral evolution by two different groups were able to identify three amino acid substitutions. The efforts to optimize the rtTA transactivator were able to identify a single amino acid mutation to create rtTA2^{S}-M2 (rtTA$_{S12G}$), which had higher transcriptional activity and sensitivity than the wild-type rtTA (Urlinger et al. 2000). Building from rtTA2^{S}-M2, viral evolution was able to discover two more amino acid mutations to create an rtTA that was even more transcriptionally active and sensitive to lower dox concentrations. The resulting mutant, since termed rtTA3 (rtTA$_{S12G\ F86Y\ A209T}$), is one of the most widely used rtTA transactivators in Tet-On systems (Das et al. 2004).

Viral evolution has been used to identify additional amino acid mutations that could improve the activity and sensitivity of the rtTA transactivator. Further work with viral evolution iterating off rtTA2^{S}-M2, was able to identify nine naturally evolved variants, termed rtTAv2 through rtTAv10, with mutations at five distinct amino acid residues (Zhou et al. 2006). The best of these systems, rtTAv10 (rtTA$_{S12G\ F67S\ R171K}$), boasts around a 7-fold increase in transcriptional activity and a 23-fold increase in dox sensitivity. rtTAv10 is commonly sold as part of the Tet-On 3G system by Clontech, which includes rtTAv10 renamed as the Tet-On 3G transactivator (Tet3G) and a P_{TRE3G} promoter with a modified minimal CMV promoter in ready-to-use plasmids with multiple cloning sites to express a gene of interest in mammalian cells.

Combinations of those same mutations found through viral evolution created even more variants of the transactivator that maintained high transcriptional activity and led to increases in dox sensitivity. Among these was rtTAv16 (rtTA$_{V9I\ S12G\ F67S\ R171K}$) with a 7-fold increase in transcriptional activity and a 111-fold increase in sensitivity (Zhou et al. 2006).

Optimization of the Tet-On system has mainly been focused on creating better versions of rtTA. Because of the system's applicability in all eukaryotes, only the rtTA transactivator protein and tetO sequences will remain consistent. While there are commercial standards in mammalian cell lines, like Clontech's Tet-On 3G system, standardized Tet-On systems for use in *S. cerevisiae* are still lacking and scientists that use the Tet-On system for gene expression to study molecular and cell biology do not have readily available and well-characterized kits for making use of the variousTet-On promoter systems. While doxycycline-inducible systems have been extremely popular as tools in molecular biology research, the wide variety of combinations

available for different transactivors, constitutive promoters driving those transactivators, and the Tet-On promoters themselves has led varied adoption of different Tet-On systems among different experiments (Janevska et al. 2017, Prasai et al. 2017).

RESULTS

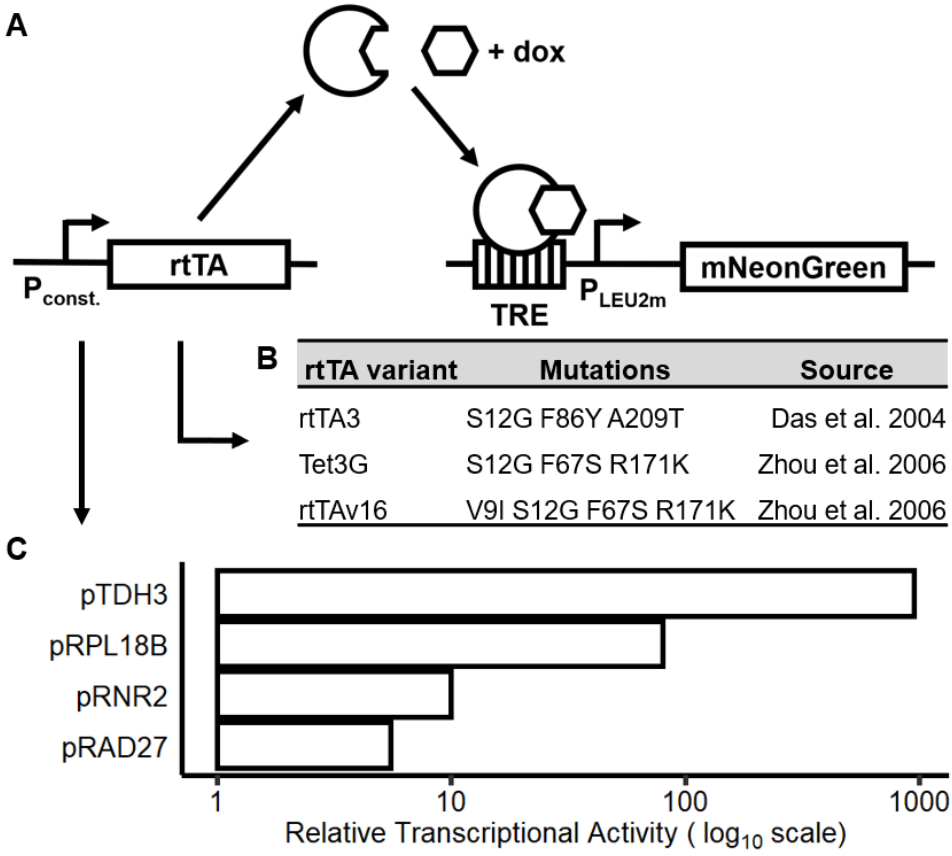
Building the initial constructs

We first set out to incorporate the necessary parts for a Tet-On system into the MoClo YTK system. Any Tet-On system consists of the same basic components: a constitutive promoter driving rtTA expression and a promoter with a TRE driving a gene of interest (figure 1A). Of the popular rtTA variants, we decided to focus on the widely used rtTA3 and Tet3G transactivators, as well as the rtTAv16 transactivator which promised to have very high transcriptional activity and sensitivity (figure 1B). These transactivators were all incorporated into the MoClo YTK system as type 3 parts.

In addition to the rtTA transactivator, we also needed to choose which yeast promoter to use to drive its expression. The MoClo yeast toolkit offers a variety of well-characterized constitutive promoters taken directly from the yeast genome already integrated into the toolkit as parts. The pRPL18B promoter offers a medium level of expression at around a tenth the expression of the pTDH3 promoter, one of the strongest available native yeast promoters, as well as very low noise in gene expression, making the pRPL18B promoter a good choice for the first iteration of our inducible Tet-On systems (Lee et al. 2015), allowing us to raise or lower the expression levels of the rtTA gene as needed in future iterations (figure 1C).

The three rtTA variants that we chose (Tet3G, rtTA3, and rtTAv16) were put under the pRPL18B promoter from the MoClo yeast toolkit, creating the pRPL18B-Tet3G, pRPL18B-rtTA3, and pRPL18B-rtTAv16 systems. Alongside a constitutively expressed rtTA gene in these systems, each construct also consisted of a promoter built from a TRE with seven tetO repeats downstream of a minimal promoter, pLEU2m, driving an mNeonGreen fluorescent reporter, each incorporated into the toolkit as type

2a, 2b, and 3 parts, respectively (Lee et al. 2015). Each system was cloned in *E. coli* and transformed into *S. cerevisiae* BY4741 (Brachmann et al. 1998).



rtTA variant	Mutations	Source
rtTA3	S12G F86Y A209T	Das et al. 2004
Tet3G	S12G F67S R171K	Zhou et al. 2006
rtTAv16	V9I S12G F67S R171K	Zhou et al. 2006

Figure 1. Construction of the Tet-On inducible system
(A) The rtTA transactivator is expressed by a constitutive promoter. When the transactivator is complexed with doxycycline (or another tetracycline analog) the complex can bind to the Tet Response Element (TRE) and promote the expression of mNeonGreen through the minimal pLEU2m promoter. (B) The rtTA variants that are used in this work, alongside their mutations from the first version of rtTA (Gossen et al. 1995) and their source, are shown in a table. (C) The constitutive promoters from the MoClo Yeast Toolkit used in this work are shown in a bar plot of their relative transcriptional activity as fold over background fluorescence from experiments done in Lee et al. 2015.

Characterizing the constructs

These three versions of the Tet-On system in *S. cerevisiae* were tested for bulk fluorescence in a microplate reader. Addition of dox led to dose-dependent expression of our mNeonGreen fluorescent reporter as expected. However, all three systems showed markedly distinct responses to the addition of the inducer (figure 2A).

These differences in inducer responses can be quantified as tightness, maximum output, dynamic range, sensitivity, and operational range. Different applications of an inducible promoter system like our Tet-On systems can inform the decision on which of these properties to prioritize.

Looking at the output of the system, tightness or basal activity is the measurement of gene expression in the absence of inducer, while maximum output is the highest possible level of gene expression, and dynamic range is the fold change between these two states. Tight control of gene expression is necessary to ensure transcription only occurs when induced and a high maximum output is required to reach levels of gene expression that match or exceed those of native promoters (Razo-Mejia et al. 2018, Shaw et al. 2019).

Sensitivity and operational range both quantify the amount of inducer needed in a system. Sensitivity is the measurement of how low of a concentration is needed to induce gene expression and operational range is the span of concentrations of inducer between minimum and maximum expression. Though doxycycline appears to not affect the growth of *S. cerevisiae*, a higher sensitivity allows for the use of less inducer to change gene expression without affecting the cell state in other ways. Operational range is based on the Hill slope and a smaller operational range results in a steeper

more digital induction while the opposite can lead to more analog and finer control of gene expression (Razo-Mejia et al. 2018, Shaw et al. 2019).

To determine each of these system properties the induction data was fitted to a Hill model and the fitted parameters were used to find all five of the properties for each of our systems.

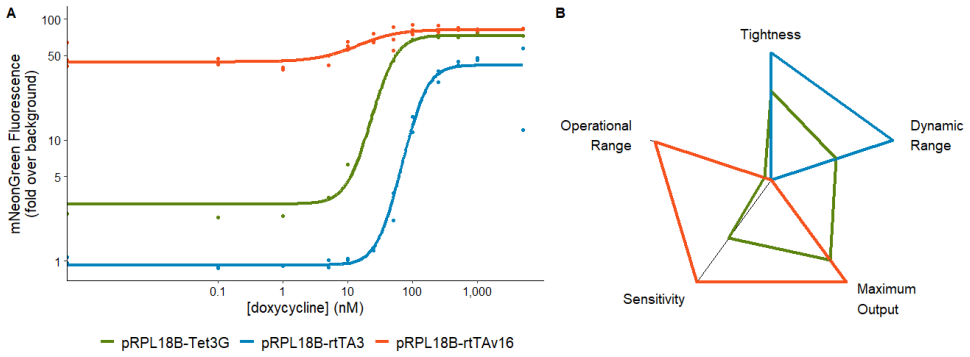


Figure 2. Characterization of the initial Tet-On constructs
(A) Fluorescence, normalized as fold over background, is plotted against doxycycline concentrations in the 96-well plate characterization experiments. Both axes are in $\log_{10}$ scale. Lines corresponding to the fitted hill curve are plotted alongside each data point and the hill curve was used to determine the system characteristics in the radar chart in (B). The exact values of each characteristic are listed in Table 1. Some of the values were rescaled as described in the methods section and all were scaled between the lowest and highest values of all the systems tested in this work then plotted on the radar chart. The colors used for each system are consistent throughout all figures and panels.

The system with the highest maximum output was pRPL18B-rtTAv16, inducing to 82-fold fluorescence over background compared to the 55-fold reached by mNeonGreen driven directly by the strong pTDH3 promoter, but with the tradeoff of very high basal activity at 44-fold over background and low dynamic range. The system induces to less than twice its basal expression already at 44-fold over background,

nearly indistinguishable from constant high expression by a strong native yeast promoter.

On the other hand, pRPL18B-rtTA3 had extremely tight control over expression. In the absence of dox, the system expressed mNeonGreen at 0.92-fold over background with the compromise of a relatively low maximum output at 42-fold and reduced sensitivity and operational range. The basal expression at background suggests that no gene expression occurs without induction.

In between the two, the pRPL18B-Tet3G system had slightly higher basal activity than pRPL18B-rtTA3 at 3.0-fold over background but with higher sensitivity and the ability to reach near the maximum output of the pRPL18B-rtTAv16 system at 73-fold (figure 2B).

Reducing basal activity of pRPL18B-rtTAv16

Our initial results showed that, while all three of these systems worked as expected in yeast, there was room for improvement. The initial priority was to decrease the basal activity of the pRPL18B-rtTAv16 system. We hypothesized that this could be most easily accomplished through reducing rtTAv16 protein copy number in the cell. With less of the transactivator in the cell, we reasoned that there would be less stochastic binding of the protein to the tetO sequences in the absence of the dox inducer, reducing the basal activity while hopefully maintaining the high maximum output of the system. This was done through driving rtTAv16 with the pRNR2 and pRAD27 promoters which both have around ten-fold reduction in transcriptional activity compared to pRPL18B, creating the pRNR2-rtTAv16 and pRAD27-rtTAv16 Tet-On systems.

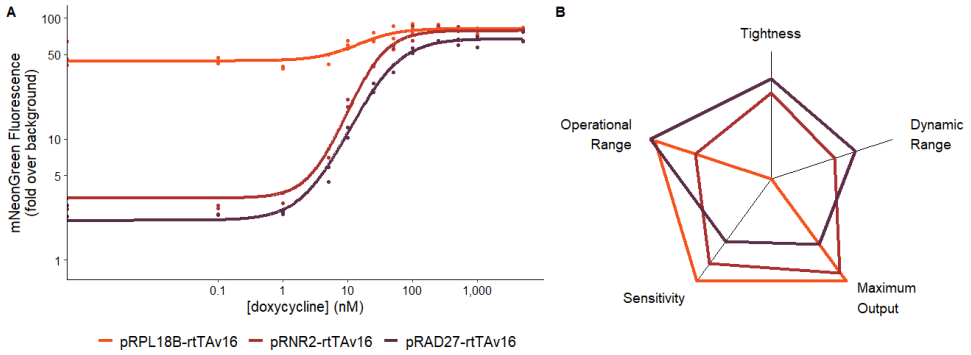


Figure 3. Characterization of the optimized rtTAv16 constructs
The normalized fluorescence responses from the optimized rtTAv16 systems are plotted against the concentration of doxycycline in (A) and characteristics were determined from the fitted hill curve and plotted in (B). The exact values are listed in Table 1. Some of the values were rescaled as described in the methods section and all were scaled between the lowest and highest values of all the systems tested in this work. The colors used for each system are consistent throughout all figures and panels.

Reducing the level of rtTAv16 transactivator expression led to a significant decrease in the basal activity compared to the pRPL18B-rtTAv16 system in both the pRNR2-rtTAv16 and pRAD27-rtTAv16 systems without a proportional decrease in the maximum output (figure 3A). pRNR2-rtTAv16 had a basal expression of 3.2-fold over background while still reaching a maximum of 79-fold over background, nearly matching the very high maximum output of pRPL18B-rtTAv16. It also maintains a relatively high sensitivity and operational range. These improvements make pRNR2-rtTAv16 a better option over pPRL18B-rtTAv16, bringing down basal expression without many sacrifices.

pRAD27 is a slightly weaker promoter than pRNR2 and pRAD27-rtTAv16 showed both lower basal activity at and maximum output than pRNR2-rtTAv16, as expected, at 2.1-fold over background and 67-fold over background, respectively. Therefore, modulating the expression of the rtTA transactivator is an effective way to

lower the overall expression of an inducible system and, in the case of pRPL18B-rtTAv16, to significantly increase tightness without sacrificing a high maximum output (figure 3B).

Increasing maximum output of pRPL18B-Tet3G and pRPL18B-rtTA3

We next sought to increase the maximum output of the pRPL18B-Tet3G and pRPL18B-rtTA3 systems using the same methods. Each of the two transactivators were driven with pTDH3, the strongest promoter available in the MoClo yeast toolkit, creating the pTDH3-Tet3G and pTDH3-rtTA3 systems. In line with our previous results, the increased presence of the transactivators led to both higher basal activity and higher maximum output with a disproportionate fold change affecting the basal activity of the system (figure 4A).

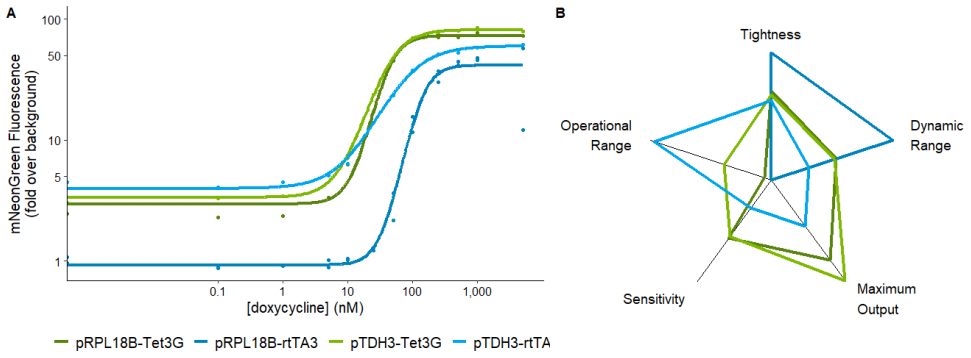


Figure 4. Characterization of the optimized Tet3G and rtTA3 constructs
The normalized fluorescence response from the optimized rtTA3 and Tet3G systems are plotted against the concentration of doxycycline in (A), and characteristics were determined from the fitted hill curve and plotted in (B). The exact values are listed in Table 1. Some of the values were rescaled as described in the methods section and all were scaled between the lowest and highest values of all the systems tested in this work. The colors used for each system are consistent throughout all figures and panels.

The pTDH3 promoter has around a tenfold increase in transcriptional activity compared to pRPL18B, but compared to pRPL18B-Tet3G, the pTDH3-Tet3G system only showed slight changes in basal activity from 3.0 to 3.4-fold over background and maximum output from 73 to 81-fold over background without any meaningful changes in sensitivity or operational range.

On the other hand, pTDH3-rtTA3 showed a very distinct response to dox compared to the pRPL18B-rtTA3 system. Basal expression increased from 0.92 to 4.0-fold over background while maximum output only increased from 42 to 60-fold, resulting in a marked decrease in dynamic range from 45 times over basal, the best of all the systems that we built, to only 15 times over basal, among the lowest. The system also showed a large increase in operational range (figure 4B).

Table 1. Inducible system characteristics
Inducible promoter system characteristics from four parameter Hill curves fitted to the normalized fold-over-background fluorescence induction data. Basal activity and maximum output are the lower and upper asymptotes, respectively, and dynamic range is the fold change between them. Sensitivity is the half maximal induction dox concentration and operational range is the Hill Slope.

Strain	Basal Activity	Max. Output	Dynamic Range	Sensitivity (nM)	Op. Range
pRPL18B-rtTA3	0.92	41.53	44.92	135.72	0.36
pRPL18B-Tet3G	2.96	73.26	24.78	42.25	0.38
pRPL18B-rtTAv16	44.25	82.03	1.85	17.39	0.73
pRNR2-rtTAv16	3.24	78.7	24.27	24.84	0.60
pRAD27-rtTAv16	2.12	67.31	31.70	38.68	0.75
pTDH3-Tet3G	3.36	81.47	24.28	43.26	0.51
pTDH3-rtTA3	3.97	59.91	15.09	77.38	0.73

DISCUSSION

We have built and extensively characterized seven distinct variants of the Tet-On system in budding yeast using the MoClo system. Each of these inducible systems have distinct responses to doxycycline induction and can be used in different applications.

The extremely low basal activity and low operational range of pRPL18B-rtTA3 makes the system ideal for applications when very tight control of gene expression is needed. With basal activity very close to background fluorescence at low dox concentrations, the system can be used to prevent genes from being expressed until they are needed. In combination with tight control, the low operational range, and more switch-like nature of dox induction in pRPL18B-rtTA3 can lead to faster induction.

In situations where cell state and stress are concerns, the pRAD27-rtTAv16 system is a good choice. The constitutive promoter pRAD27 in the MoClo yeast toolkit has been shown to have around half the activity of pRNR2, a tenth of pRPL18B, and one two-hundredth of pTDH3 (Lee et al. 2015), leading to a relatively low level of the rtTAv16 expression and concentration of the transactivator in the cell, reducing the transcriptional and translational burden on the cell. In addition, the system is also very sensitive and can reach its maximum output at lower concentrations of dox in the same period as most of the other systems that we built. Though doxycycline does not generally affect global gene expression in *S. cerevisiae* (Wishart et al. 2005), it has been found to enrich expression of specific genes involved in DNA replication and repair (Sanchez et al. 2020) as well as inhibition of mitochondrial protein translation in

high concentrations (Moullan et al. 2015). The highly sensitive rtTAv16 systems can avoid or minimize these effects.

Not only can these systems be used as is but with all the necessary parts already incorporated into the MoClo system as parts, they can be easily recombined to build Tet-On systems for specific use cases. While we only explored three different rtTA transactivators under a few different constitutive promoters. The system can be easily rebuilt with any constitutive promoter or transactivator incorporated into the toolkit.

While increasing the amount of rtTA generally increased both the basal activity and maximum output, it did not do so in a completely predictable way. Changing the pRPL18B-rtTA3 and pRPL18B-Tet3G systems to both use the pTDH3 promoter did not have consistent effects across both systems. pTDH3-rtTA3 had a drastic increase compared to pRPL18B-rtTA3 probably because pRPL18B-rtTA3 had a very low activity to begin with.

The convergence of both systems to the same levels of activity in both low and high dox conditions suggests either a saturation of the binding of the transactivator to the TRE or of the transcriptional machinery to express the mNeonGreen gene, which could be determined by increasing the copy number of the mNeonGreen gene under the tetO7pLEU2m promoter.

While a single timepoint induction was sufficient to determine the tightness, dynamic range, maximum output, sensitivity, and operational range, experiments can be performed to understand more properties of each inducible system. Doxycycline

induction through the Tet-On system has also been shown to be reversible and time-course experiments to monitor fluorescence following addition and removal of dox will reveal the kinetics of each system and how they vary based the type of rtTA transactivator and its copy number in the cell.

While we have not developed a complete understanding of how expression of the rtTA transactivator affects the Tet-On system's response to induction, this method has created inducible systems that work very well in *S. cerevisiae*. We have also incorporated and characterized the basic parts for these inducible promoter systems into the MoClo YTK system, providing a robust and tunable toolkit for inducible expression with doxycycline in budding yeast.

MATERIALS AND METHODS

Cloning of Parts

Sequences were ordered as gBlocks from Integrated DNA Technologies (IDT) with MoClo compatible overhangs or amplified using PCR adding the overhangs. The parts were incorporated into the pYTK001 entry vector with a BsmBI golden gate, transformed into DH5α competent cells, plated onto LB plates with chloramphenicol. Colonies were picked and verified by sequencing (Eton Bioscience). Parts were incorporated into gene cassettes or multigenes with the MoClo YTK system in a BsaI or BsmBI golden gate, respectively (Lee et al. 2015). All constructs were miniprepped (Qiagen) and verified with colony PCR and restriction digest.

Golden Gate Assembly Protocol

An equimolar solution of 20 fmol of each insert part was combined with 10 fmol of the vector plasmid in a PCR tube alongside 0.5 µL T7 DNA Ligase (NEB), 0.5 µL BsmBI-v2 or BsaI (NEB), and 1 µL T4 DNA Ligase (NEB) to a total volume of 10 µL. Reactions were run in a thermocycler for 25 cycles of digestion (42°C for 2 minutes) and ligation (16°C for 5 minutes) then a final digestion step (60°C for 10 minutes) and heat inactivation step (80°C for 10 minutes) (Lee et al. 2015).

Yeast Transformations

Yeast colonies were grown overnight in YPD (Thermo Fisher) then diluted 1:00 in 50 mL YPD and grown for 5-6 hours. Cells were spun down at 2,000 RPM for 10 minutes then resuspended in 25 mL of 0.1 M lithium acetate (Sigma-Aldrich) then pelleted again and resuspended in 1 mL of 0.1 M lithium acetate. 100 µL of each cell resuspension was used per transformation. To each transformation, 10 µL of freshly boiled and cooled salmon sperm DNA was added and mixed followed by up to 100 µL

of transformation DNA (500 ng to 2,000 ng total). The transformation mixture was incubated at room temperature for 30 minutes and 900 µL of a 30% PEG-3350 (Sigma-Aldrich), 0.1 M lithium acetate, 10% DMSO (Thermo Fisher) mixture was added. The mixture was incubated for 30 minutes, then heat shocked at 42 degrees Celsius for 14 minutes and spun down for 2 minutes at 8,000 RPM. The supernatant was removed, and the pellet resuspended in 250 µL 5 mM calcium chloride. The entire mixture was plated on the correct dropout plates.

Characterization of Inducible Promoters

Deep round-bottom 96-well plates were inoculated with single colonies of each strain from a YPD plate in 500 µL of SC media (US Biological). The colonies were grown overnight at 750 RPM and 30°C for 18 hours and then diluted 1:100 in fresh media and induced with dox to a final volume of 500 µL (Lee et al. 2015). After 6 hours at 750 RPM and 30°C, 200 µL from each well was transferred to a black-walled, clear-bottom 96-well plate (Tecan) and was read using a Tecan Infinite 200 microplate reader. mNeonGreen fluorescence was measured with 500 nm excitation and 535 nm emission and normalized by OD600. Fluorescence and OD600 values for blank media induced with dox were subtracted from their respective measurements and the resulting fluorescence was divided by fluorescence of the background BY4741 strain. To normalize data between separate runs, a strain of BY4741 transformed with mNeonGreen driven constitutively by pTDH3 (pTDH3-mNeonGreen) was run with each plate and all fluorescence values by dox concentration were scaled to the average pTDH3-mNeonGreen value for that concentration.

Calculation of System Characteristics

Normalized inducible promoter fluorescence data was fitted using R and the "drc" package (Ritz et al. 2015). The data was fitted to a 4-parameter log-logistic model, also known as a Hill curve (Equation 1), where x is the dox concentration, b is the Hill slope, d is the upper asymptote, a is the lower asymptote, and c is the inflection point. These values were then used to calculate the system characteristics.

Tightness was calculated as the negative $\log_{10}$ of the lower asymptote (d) and maximum output was the upper asymptote (a). Dynamic range was the maximum asymptote divided by the minimum asymptote. Sensitivity was the negative $\log_{10}$ of the inflection point or half maximal induction dox concentration (c) and operational range was the negative reciprocal of the Hill slope (b). The data was rescaled between the minimum and maximum of each characteristic for all systems tested before being plotted on a radar chart (Razo-Mejia et al. 2018, Shaw et al. 2019).

Equation 1. $$f(x) = \frac{a-d}{1+(\frac{x}{c})^b} + d$$

The material used in this thesis is currently being prepared for submission for publication of this material alongside Richard O'Laughlin and Andrew Lezia. The thesis author was the primary investigator and author of this material.

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