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Drug-Responsive Regulation and Comparison of Engineered Transcription Factors Containing Different Activation Domains

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

The pursuit of effective tools for probing biological systems has led to a growing interest in approaches that offer both rapid, reversible, and accurate methods. Specifically, the ability to perturb the function of specific proteins with precision is highly sought after, as it contributes significantly to our capacity to unravel the intricacies of complex biological processes. Effective methods to regulate gene expression or protein function within mammalian hosts are essential for understanding basic biological mechanisms (Banaszynski 2008). Examples of these methods include generating animal knockouts, siRNA, the Cre-loxP system, and small molecule activators or inhibitors. However, these methods are limited because they may not be reversible, are non-specific, or—in the case of animal knockouts—the gene being knockout out is essential for development thus confounding the study of the protein's function. Another method involves synthetic transcription factors paired with destabilizing-domain fusion proteins that target a gene of interest. The destabilizing-domain are stabilized in the presence of a ligand, thus providing the ability to control and regulate gene expression. This technique can provide the regulatability, specificity, and ease of use in understanding complex biological mechanisms. One component of synthetic transcription factors is the design of activation domains utilized. Activation domains promote recruitment of chromatin modifiers, which cause chromatin decondensation and accumulation of histone marks, thus promoting transcription (Martella 2021). The variability of transcription efficacy is based on the properties of different activation domains. In this study, three different

domains are investigated: VPR, VP64, and p65, with the goal of determining which one provides the highest dynamic range in a lentiviral-transduced mammalian cell system using a synthetic transcription factor fused to a drug-dependent destabilizing protein domain.

Dedication

I would like to dedicate this work to my husband, Lee Nave Jr. for encouraging and motivating me to complete my degree. I would never have been able to do this without his support. I also would like to dedicate this to my cat, Oreo Smoothie Pie, for spending long nights on my lap supervising my studies as well as Zayne Nave, my son, for bringing me joy through his smiles.

Acknowledgments

I would like to acknowledge Obsidian Therapeutics for giving me the time and opportunity to do my research in the lab. I would also like to acknowledge Mara Inniss, my supervisor and one of my thesis directors, for her support and guidance. Also, I would like to acknowledge Professor Amy Tsurumi, my second thesis director, for being so helpful and encouraging.

I would also like to acknowledge the following co-workers at Obsidian Therapeutics for their assistance. Firstly, Grace Olinger for teaching me so many protocols from how to run FACS, transduce lentivirus, and so many other lab techniques. Secondly, Andrew McNeal for helping me find the right antibody to use in my Western Blot.

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Chapter I.

Introduction

The synthetic transcription factor is not a new concept; it was mentioned in 1995 by Pomerantz, et al. in his paper "Structure-Based Design of Transcription Factors". In the paper, the most basic structure of a synthetic transcription factor was outlined. A fusion protein made up of zinc fingers (ZFHD1), VP16 activation domain, a short polypeptide linker, and a homeodomain from Oct-1 was designed. Zinc fingers were used because they are small and the most prevalent DNA-binding domain in human transcription factors. They are also easily reprogrammable to recognize new motifs and can be concatenated to bind to longer DNA sequences (Israni 2021). The constructs used in this study also utilizes zinc fingers as binding sites to a gene encoding green fluorescent protein (GFP). In the most basic concept, the transcription factor construct is introduced into a mammalian cell line in vitro, where the cell line contains a different reporter construct that has a DNA-binding sites for a given sequence of zinc fingers, a promoter domain, and the gene of interest. When both constructs are present within the cell, the transcription factor is transcribed and binds to the reporter construct, thus initiating transcription of the gene of interest. In Burrill (2012), there was an attempt to design this system using a single plasmid where both the transcription factor and reporter constructs were combined. However, this system demonstrated constitutive reporter gene of interest expression in the absence of the activating ligand, likely due to cis-activation caused by enhancer elements in the promoter. For similar reasons, in this study the constructs were separated. The separation of plasmids also allowed for a more modular

system where different transcription factors can be tested in a single cell line. The method in which to introduce these non-native constructs into a mammalian cell line like HEK293 plays an important role in seeing any changes in expression and regulation. In this study, a transient transfection of the two plasmids were first done as a pilot experiment to check the proper function of the plasmids. However, transient transfection has a broader range of expression versus viral transduction and slight increases in residual fluorescence can be seen in the absence of drug (Banaszynski 2006). We can see this in the results of this study, where the basal levels of expression are much higher in transient transfection, thus elevating the maximum expression level to a degree where the GFP fluorescence exceeded the detection limit of the flow cytometer. Viral transduction, such as the use of lentivirus, allows for a more stringent test in gene expression because of the lower protein basal expression. The use of stable cell lines expressing the construct of interest are commonly used in applications involving gene expression and, in this study, this was achieved by transduction of lentivirus to provide optimal conditions for observing the broadest range of gene expression.

Comparison to Cas9 and RNAi

Effective methods to regulate gene expression or protein function within mammalian hosts are essential for understanding basic biological mechanisms, as well as for directing gene-based therapies (Banaszynski 2008). To date, there are a variety of different ways to achieve this purpose using technologies such as RNAi and CRISPR. However, both methods have their own drawbacks. In both, significant off-target activity is a problem. There are a variety of different ways to mitigate this. In siRNA, the careful

design of sequences can help address both the off-target activity and increase the success rate of silencing. In CRISPR, modified Cas9 proteins with low off-target activity can be used, as well as modifying the gRNAs used to guide the endonucleases to increase specificity. Synthetic regulatory programs can be used to support these technologies by providing the capability to accurately regulate and control gene expression. The four basic principles for clinically-driven design of synthetic regulatory programs would be (1) human based: prioritizing human-derived proteins, when possible, to minimize immunogenic potential, (2) orthogonal: components with programmable, unique specificities that minimize cross-talk with native regulation, (3) regulatable: can be controlled with safe, clinically suitable molecules, (4) compact: minimized genetic footprints for efficient delivery (Israni 2021). The optimization of drug-responsive synthetic transcription factor systems will be invaluable in providing a programmable tool to modulate gene transcription that can be used to support other technologies already available.

Drug-Responsive Destabilizing Domains

The power of a synthetic transcription factor system that can be tightly controlled lies in the concept of the destabilizing domain (DD). These dimerizers can provide a means of triggering specific signal transduction, protein recruitment, or gene transcription events independently of other cellular activities (Amara 1997). The concept of drug responsive domains (DRD) is based on the knowledge that a protein's stability is dependent on its N-terminal residue. Thus, small peptide sequences can be fused to the N-terminus of the protein of interest, thus conditionally targeting this fusion protein for

degradation through induced localization to either an E3 ligase complex or to the proteasome itself (Banaszynski 2006). The introduction of a drug or ligand stabilizes the fusion protein, thus protecting it from degradation and allowing it to perform its function. Another way of designing DRDs is by perturbing the subcellular localization of the protein where without the presence of the drug, the fusion protein cannot enter the nucleus of the cell and initiate transcription. Such examples of DRDs include ER50 (estrogen receptor) and DHFR (dihydrofolate reductase). The drugs 4-hydroxytamoxifen and trimethoprim (for ER50 and DHFR, respectively) are both clinically proven small molecule drugs that are used to activate the DRDs, stabilizing the domains and allowing normal function of the fusion protein (Maji 2016).

Promoters

While not the focus of this study, it is still important to explain the significance of promoters in gene expression because in this study, two different types are used to find the optimal gene expression range. The promoters used are minCMV and YB_TATA. Promoters are the genomic sequences where the transcriptional machinery assembles, and core promoter activity is conferred by the presence of short sequence motifs at specific positions relative to the transcription start site (TSS) (Yella 2016). The TATA-box sequence is the most well-studied core promoter element and its sequence "TATAAAA" is variable in natural promoters (Savinkova 2013). YB_TATA is a synthetic minimal promoter that is a 25-base pair sequence containing a TATA box with flanking spacers. It was shown to combine low basal expression with high transcription rate in the induced state to achieve significantly higher fold-induction ratios (Ede 2017). MinCMV promoter

is derived from cytomegalovirus (CMV), and it is one of the ‘leakiest’ promoters. It is used to drive low-to-moderate levels of gene expression, thus the high basal expression from the promoter can serve as a reliable means for tuning constitutive gene expression. It has the largest increase in absolute gene expression when induced, but its fold-change was relatively small due to its high basal expression level and is limited in applications that require a tightly “off” state in regulated gene expression (Ede 2017). Both minCMV and YB_TATA were used in the reporter constructs in this study to compare between the two promoters. While theoretically, YB_TATA seems to be the optimal choice for observing low basal expression and high dynamic range, the addition of minCMV as comparison served as a control to confirm its function.

Activation Domains

Activation domains play a pivotal role in gene expression. Transcriptional initiation occurs through the coordinated recruitment of necessary machinery by several locally concentrated transcription factor activation domains (ADs) (Chavez 2015). They promote recruitment of chromatin modifiers, which cause chromatin decondensation and accumulation of histone marks promoting transcription (Martella 2021). A common activation domain used in artificial systems is VP16, derived from the herpes simplex virus protein. Tandem fusion of multiple ADs such as VP16 would increase transcriptional activation by mimicking the neutral cooperative recruitment process (Chavez 2015). Such combinations include VP64, which contains four copies of VP16. A human-derived activation domain, p65, is derived from NF-kB. However, it has been observed that p65 were not stronger activators than VP64 (Chavez 2015). Another

activation domain is a combination of three different activation domains, known as VPR. It is a tripartite activator made up of VP64-p65-Rta. This hybrid was shown to have 22-to-320-fold improved activation of endogenous targets and allowed for robust multi-locus activation, exhibiting several-fold higher expression levels than VP64 across a panel of genes (Chavez 2015). Out of the three activation domains tested and compared in this study, the order of strength from highest to lowest appears to be VPR, VP64, and p65. However, higher activation strength is not necessarily the characteristic that allows it to be the most optimal in a regulated transcription factor system. There needs to be a balance between basal expression and highest expression to have the optimal and highest range. Thus, in this study, all three activation domains are tested and compared in a synthetic transcription factor system.

Chapter II.

Materials and Methods

The following section outlines the materials and protocols used for the study. In summary, tissue culture techniques were used to create and maintain HEK293 cell lines and both plasmid transfection and lentivirus transduction were used to introduce constructs into the cells for expression. The final readout consisted of both flow cytometry to measure fluorescence of GFP and other markers, and Western Blot to detect protein expression. All experiments were carried out in a BSL-2 level lab.

Plasmid Design and Production

DNA sequences for the ybTATA and minCMV reporters as well as the VPR, VP64, and P65 GFP-targeting transcription factors were designed on Benchling. Sequences were given to Quintara Biosciences (Cambridge, MA) and the plasmids were manufactured for the experiments.

Tissue Culture (HEK293 Cells)

HEK293 cells were maintained in culture with DMEM + GlutaMax (Gibco 10569-010) media with 10% Heat Inactivated FBS (Gibco A38400-01) added, and then filtered through a 0.22 um Millipore Express Filter (Millipore S2GPU05RE). When removing cells for passaging or flow cytometry, media was removed from the adherent cells, washed with PBS (ThermoFisher 10010049), and Trypsin (Gibco 2S200-056) added for 3 minutes at 37 ◦ C. The Trypsin-cell mixture was then neutralized adding excess DMEM. When passaging HEK293 cells, a dilution factor of 1:2 is calculated for

every 24 hours of incubation based on the doubling factor of the cells every 24 hours. A maximum of 1:16 dilution for 4 days of incubation time is used when passaging for long periods. When counting cells, the Cell Countess II (Invitrogen AMQAX1000) was used, and cells counted twice with the average used as the final count.

Cell Transfection

HEK293 cells were seeded into a 24-well plate at a density of 1.6e+5 cells per well in 0.5 ml. Cells were incubated in 37 ◦ C for 24 hours. After 24 hours, each well was then transfected with a total of 0.5 ug of DNA using Lipofectamine 3000 (L3000001) and Opti-MEM (ThermoFisher 31985062), following the manufacturer protocol. If doing co-transfection and using two different plasmids (reporter plasmid + transcription factor plasmid), then 0.25 ug of each plasmid was used. Lipofectamine and DNA mixture was added to the cells. They were incubated for 24 hours at 37 ◦C before being processed for flow cytometry or Western Blot.

Lentivirus Packaging

The lentiviral vector is packaged using reverse transfection of HEK293 with third generation lentiviral packaging. Packaging plasmids and reporter/transcription factor constructs were mixed with TransitIT 293 (Mirus Bio MIR2700) following manufacturer protocol. Virus production was done in a small scale using T25 tissue culture flasks. A total of 2.67e+6 HEK293 cells were added to the lentivirus packaging mixture and media added with HEPES (ThermoFisher 15630080) into a T25 flask. Cells were incubated at 27◦ C for 48 hours before cell media was collected into 5 ml Eppendorf tubes. Cell media was centrifuged at 400xg for 4 minutes and supernatant transferred to a new tube without

disturbing the cell pellet. This was repeated for a total of two centrifuge spins. Supernatant containing virus was aliquoted, labelled, and stored in a -80 ◦ C freezer before titering.

Lentivirus Titer and Transduction

Titration: HEK293 cells were plated in a 96-well plate at 5e4 cells per well. Cell supernatant containing virus that had been previously frozen were thawed and then added to the cells in a serial dilution of 1:2 in 6 sequential columns from 50 uL of virus to 1.5625 uL virus. 10 ng/uL of Polybrene was also added to each well. The cell culture plate was incubated for 24 hours in an incubator at 37 oC. After 24 hours, the cells were removed from the plate, washed with PBS twice, and then stained with antibodies against Thy 1.1 (Biolegend 140312) or Thy 1.2 (Biolegend 202526). The stained cells were processed through flow cytometry (iQue) and fluorescence measured. To calculate the titer, only the samples that were > 30% and < 5% marker positive were used. These values were normalized based on how much virus was added and averaged to provide an average of titer for each virus construct. This titer value was then used for calculations in further transductions.

Transduction: Using the titer value, HEK293 cells were treated with virus and then plated on a tissue culture plate. Depending on the experiment, drug TMP or DMSO was added at a concentration of 10 ng/uL. Cells were incubated for 24 hours and then processed through flow cytometry for a fluorescence readout.

Flow Cytometry and Cell Sorting

The machine, iQue was used for flow cytometry. The cells were first treated with Trypsin and washed with PBS twice. The cell pellets were then incubated with antibodies anti-Thy 1.1 (Biolegend 140312) or Thy 1.2 (Biolegend 202526) antibody at 4 ◦ C for 30 minutes. Following the instrument manual, the cells were processed through the iQue and the results analyzed through the FlowJo program.

For cell sorting, the machine Sony Cell Sorter was used. The process for staining the cells is similar to staining for the iQue. However, in this case the cells were collected and saved into a 15 mL Eppendorf tube, into which they were cultured into a T25 or T75 flask depending on volume.

Western Blot

Cell pellets after virus transduction were collected. They were lysed in T-Per Buffer (Thermofisher 78510) and centrifuged. The supernatant was collected and saved while the resulting pellet was discarded. 2.5 uL of this supernatant was then used for running the Pierce BCA Protein Assay Kit (Thermofisher 23225) to measure protein concentration. The manufacturer protocol for the kit was followed, and readout was measured in a plate reader. The concentrations were calculated from the readout values using a standard curve.

A total of 30 ug of the cell lysate was denatured using DTT and loading buffer by incubating at 96 ◦ C for 6 minutes with 300 rpm agitation. A NuPage Bis-Tris gel was used to load the protein. The gel was run through a voltage of 100-160 V for 20-30 minutes. The iBlot2 system was used to transfer the gel onto a membrane, following

manufacturer protocols. Afterward, the iBind system was used to stain the membrane overnight (24 hours). The LiCOR system was used to view and image the membrane.

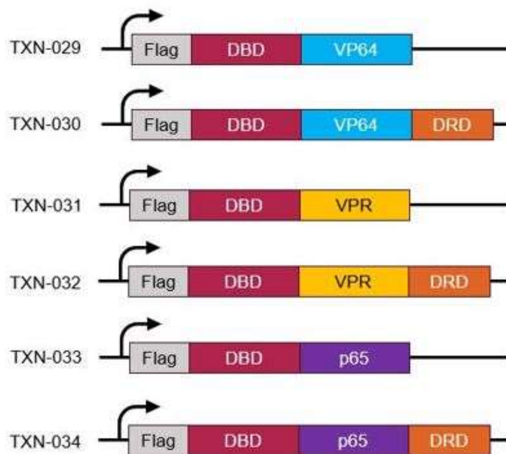
Chapter III.

Results

The purpose of this study was to determine the effects of different activation domains involved in a drug-dependent artificial transcription factor system. Three different activation domains were used: VPR, VP64, and p65. Each domain was attached to a transcription factor construct that expresses GFP mRNA. When transiently transfected or transduced with a separate construct that contains the GFP gene, HEK293 cells can be induced to express GFP with or without the presence of the drug, TMP depending on if the transcription factor construct contains a constitutive or drug-responsive regulated domain. The level of highest activation and lowest basal expression is different depending on which activation domain is used. The most successful activation domain is considered one that has the lowest basal expression level and the highest expression level when activated.

Construct Expression Testing

One constitutive construct was designed for each activation domain. TXN-029, TXN-031, and TXN-033 contain VP64, VPR, and p65 respectively (Fig 1). These constructs were transiently transfected into HEK293 along with one effector construct containing minCMV promoter or another containing ybTATA promoter. The expression levels of GFP were measured (see Fig 2). This test was done to confirm that the effector and transcription factor constructs were designed properly and work as expected. The construct pELDS-001 is used as a negative control because it is an empty backbone vector.



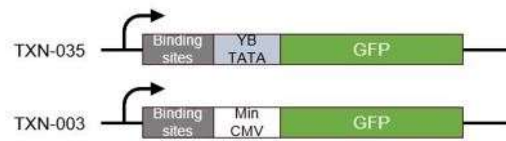


Figure 1. Plasmid Map of Constructs

Plasmid maps of all constructs used. A total of 6 different transcription factor constructs and two reporter constructs were designed and made. Flag tag is used for Western Blots to detect with Flag antibody. DBD is DNA binding domain responsible for binding to the gene of interest, in this case GFP, on the reporter construct. DRD means drug responsive domain and is the regulatory portion of the construct that recognizes and binds to the drug Trimethoprim (TMP).

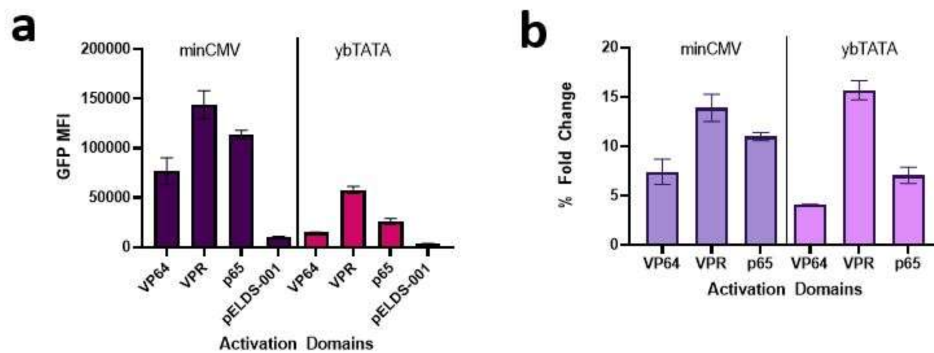


Figure 2. Constitutive Construct Co-Transfection

Measurement of GFP expression in co-transfection experiments with constitutive constructs. (a) MFI of GFP comparing co-transfection with minCMV vs ybTATA promoters. Activation domain VPR shows the highest MFI. pELDS-001 construct is used as an empty vector for negative control. (b) Same data is graphed is using GFP fold change calculation, with results showing similar patterns.

The expression of Thy 1.1 was also looked at among the constitutive constructs (see Fig 3). This metric shows how effective the transcription factor construct was able to integrate and express into the cells. The transfection efficiency of the three constitutive constructs were not equal, with TXN-031 (VPR) having the lowest. However, this did not notably affect the GFP expression level of the transcription factor system (Fig 3b).

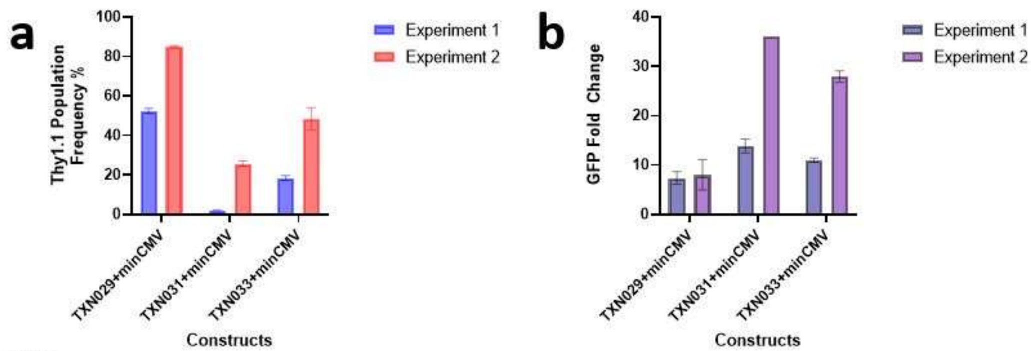


Figure 3. Transfection Efficiencies: Constitutive Constructs

Measuring transfection efficiencies of constitutive constructs (TXN029/VP64, TXN031/VPR, TXN033/p65) by co-transfection with minCMV promoter (TXN003). Experiment was repeated twice and results for both experiments compared. (a) The population frequency, measured through flow cytometry and looking at Thy 1.1 expression, shows that TXN031 has a lower expression value out of the three constructs, and this trend is consistent with both experiments. However, the low expression of TXN031 does not seem to affect the GFP fold change when measuring GFP expression (b) and is the highest out of the three constitutive constructs.

The purpose of the constitutive constructs is to provide a positive control for each activation domain in terms of drug response since the constitutive constructs should theoretically show the highest level of GFP expression because they do not have drug-responsive domains.

The regulated constructs were tested using transient transfection and checking their Thy 1.1 expression levels (Fig 4). All three constructs showed relatively similar expression levels.

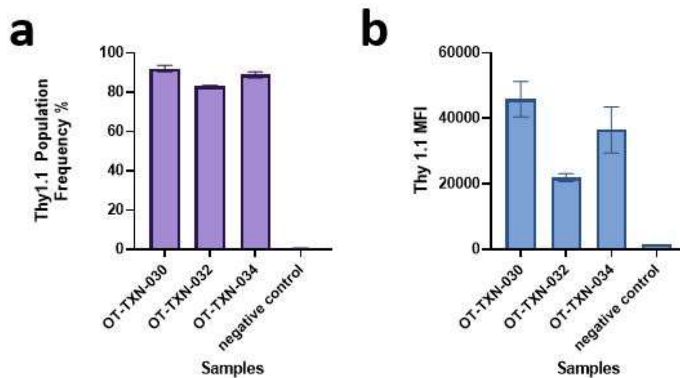


Figure 4. Transfection Efficiencies: Regulated Constructs

Measuring transfection efficiencies of regulated constructs (TXN030/VP64, TXN033/VPR, TXN034) by single plasmid transfection. (a) Thy 1.1 expression by population frequency of single cells was measured using flow cytometry and shows similar values for all three constructs. (b) Thy 1.1 MFI was also measured.

Transient Transfection Drug Response

Co-transfection in HEK293 was performed using combinations of the two effector constructs and six transcription factor constructs. The drug, TMP, was added for the

positive drug samples and DMSO was used as a negative control for the drug. The cells were processed through flow cytometry after 48 hours for checking Thy 1.1, Thy 1.2, and GFP expression levels. The frequency of double positive percentage (Fig 5a) shows the metrics of the efficiency of the co-transfection and the samples show similar levels. Comparing GFP MFI and GFP fold change between the two different effector constructs used showed that the minCMV promoter elevates the GFP expression levels of all the constructs. This is expected, since minCMV is a 'leaky' promoter and has a very high basal rate as compared to ybTATA. As such, for many of the constitutive constructs and some of the regulated constructs, the GFP MFI is so high that it exceeds the flow cytometry readings and, as seen ins Fig 5b, where the bar graphs are cut off at the top at max level. Meanwhile, the regulation levels of GFP can be seen easier using the effector construct with ybTATA (Fig 6). Constitutive VPR (TXN-031) shows the highest GFP MFI levels. Similarly, regulated VPR (TXN-032) shows the highest GFP levels among the regulated constructs when in the presence of drug. This is expected as VPR is a very strong activation domain. VP64 and p65 constructs also show good regulation. From these results, it can be concluded that ybTATA is a better reporter construct to use in this experiment because it provides a better lower basal level and inducibility than minCMV. Therefore, it is decided to proceed forward only with the ybTATA reporter construct in the final experiment.

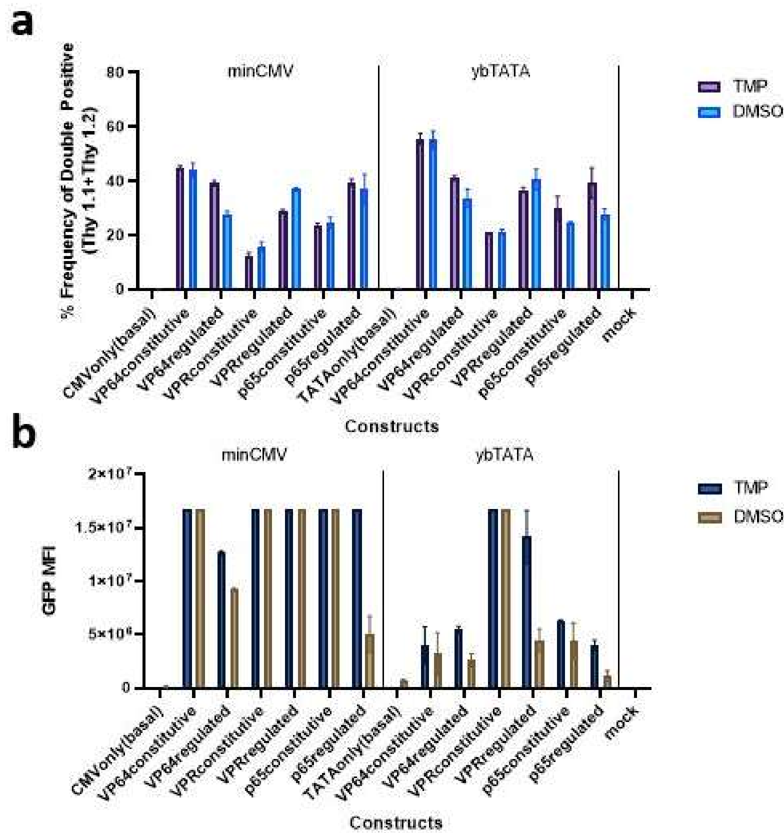


Figure 5. Co-Transfection with Drug Dosage: Double Positive and GFP MFI

Co-transfection of transcription factor construct, and reporter construct in the presence or absence of drug Trimethoprim (TMP) or DMSO as negative control. (a) shows the frequency of double positive cells, which means each cell is successfully transfected with both plasmids. The values of frequency between TMP and DMSO remain similar for each plasmid combination. In (b), the GFP MFI values are measured for each combination. The minCMV promoter construct results in most of the combination exceeding the limit of detection of GFP due to the high expression. The ybTATA combinations show a better variation of ON/OFF drug regulation with more GFP fluorescence in presence of TMP for the regulated constructs.

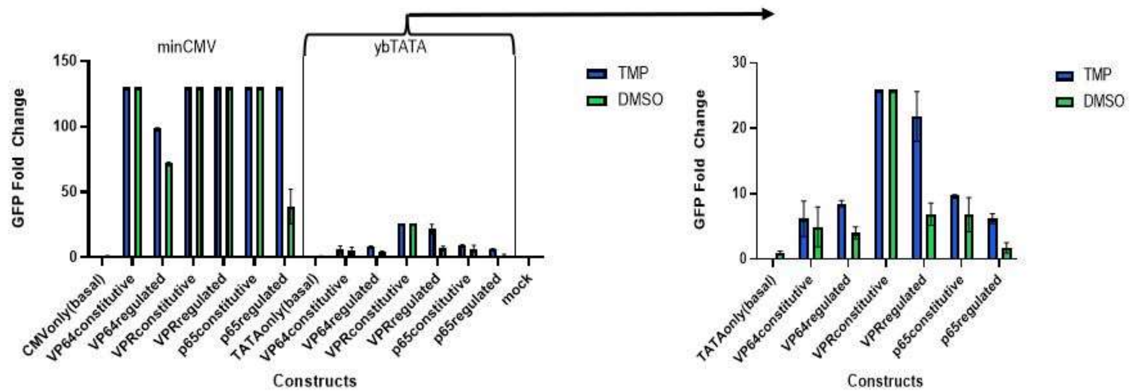


Figure 6. Co-Transfection with Drug Dosage: GFP Fold Change

GFP Fold Change is measured by subtracting the background GFP MFI and dividing by the basal value of the reporter-only transfection. For the minCMV samples, the fold change for many are indistinguishable between drug on and drug off due to exceeding the instrument fluorescence reading. In this experiment (a) for minCMV we can only see regulation in VP64 and p65 constructs. The ybTATA values are magnified in a separate graph (b) to highlight the differences by changing the scale. VPR shows the highest GFP MFI values for both constitutive and regulated while VP64 and p65 constructs show varying degrees of regulation.

Lentivirus Production and Titer

Each construct was packaged into lentivirus in HEK293 and the final lentivirus product supernatant tested in a series of dilution to calculate virus titer. Interestingly, the VPR-based constructs (TXN-031 and 032) had very low titers, meaning very little virus was able to be produced from the cells (Fig 7a). Virus production was repeated for these two constructs, but virus titer levels were the same (not shown). A theory explaining this could be that the VPR sequence may have had an effect with lentivirus production in the cells. Because there was a small amount of VPR-based construct lentivirus able to be produced, the VPR-based constructs were not used in subsequent experiments. This was an acceptable decision, also because it can already be seen in the transient transfection

data that the VPR constructs have extremely high basal expression levels, which makes detecting regulation difficult. The comparison between VP64 and p65 is expected to show better regulation levels.

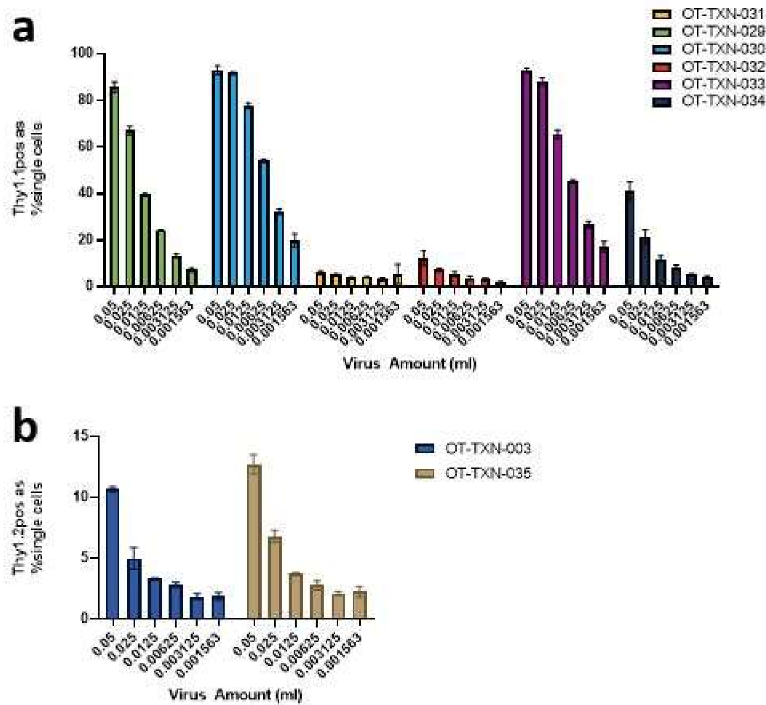


Figure 7. Construct Virus Titers

Results of virus titers for the reporter constructs (b) and transcription factor constructs (a). Virus was added to cells in serial dilution and expression of either Thy 1.1 or Thy 1.2 were measured. For most of the samples, the graphs trend similarly based on amount of virus added. TXN-031 (constitutive, yellow bar) and TXN-032 (regulated, red bar), which are both VPR-based constructs, show the lowest virus titers. These VPR constructs were remade, and virus titer repeated (not-shown) with similar results. The presence of VPR sequence may have had an effect with lentivirus production.

Reporter Cell Line Sorting

The next steps for this experiment entailed moving away from transient transfection and entirely into lentivirus transduction. To accomplish this, a cell line containing the reporter construct (which will be constant throughout all the different transcription factor testing) was first made. First, HEK293 cells were transduced with ybTATA or minCMV reporter construct virus and allowed to expand for a couple of days. The cells were then processed through the Sony Cell Sorter, where only the Thy 1.2 positive cells containing the integrated construct were collected (Fig 8). Two sorts were required since the expression levels of the cell lines after the first sort only showed around 50% Thy 1.2 positive cells (8c).

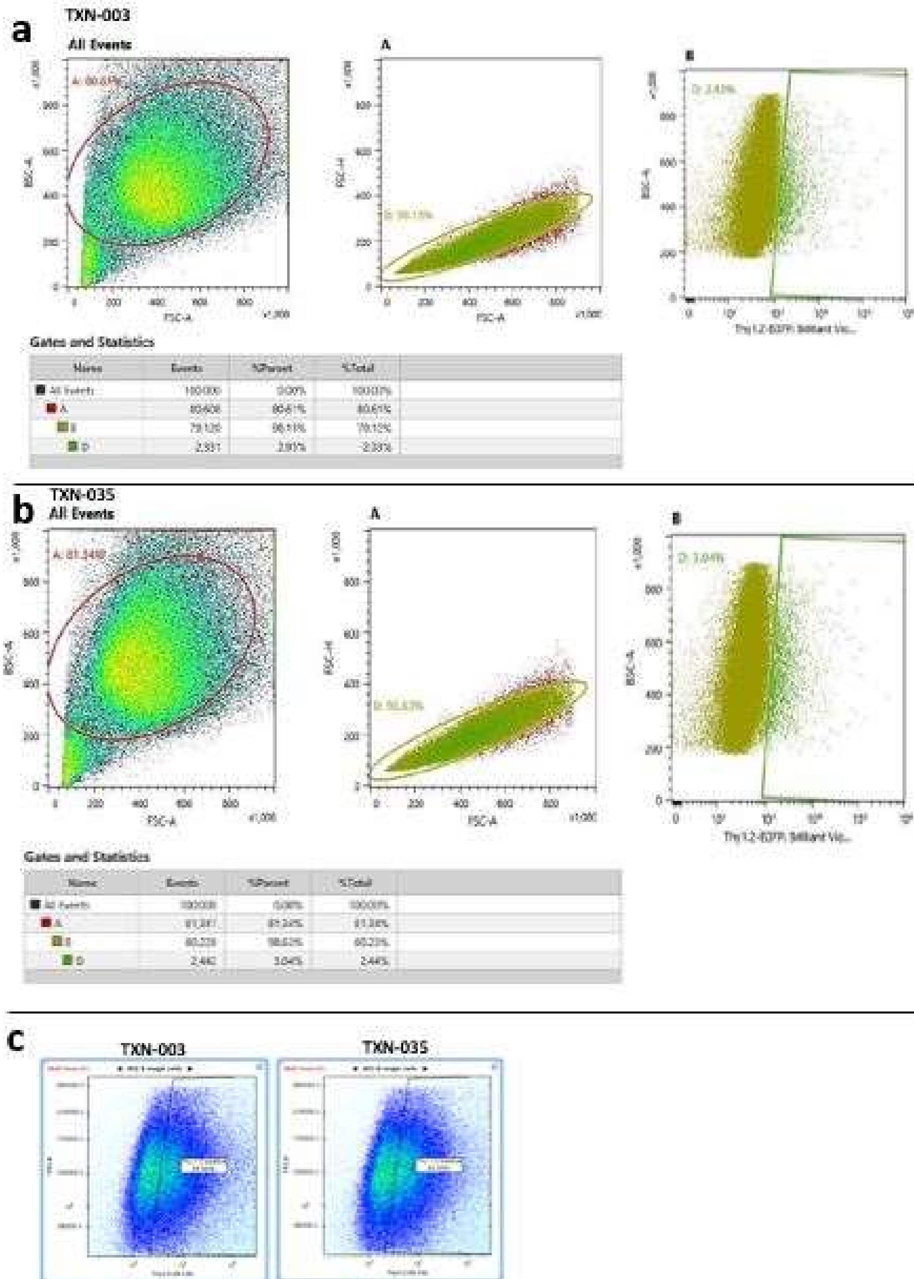


Figure 8. Reporter Cell Line Sort 1

Dot-plot graphs from the Sony Cell Sorter during cell sorting creating cell lines TXN-003 (a) and TXN-035 (b). For TXN-003, 2.95% of cells were Thy 1.2 positive and a total of 211,828 cells were sorted and cultured. For TXN-035, 3.04% of cells were Thy 1.2 positive and a total of 224,525 cells were sorted and cultured. After a week, a subset of the cultured cells were processed through the iQue flow cytometer and checked for

positive percentage. TXN-003 showed 55.66% and TXN-035 showed 54.38% (c), based on these results, the cell lines were sorted a second time (See Figure 9.)

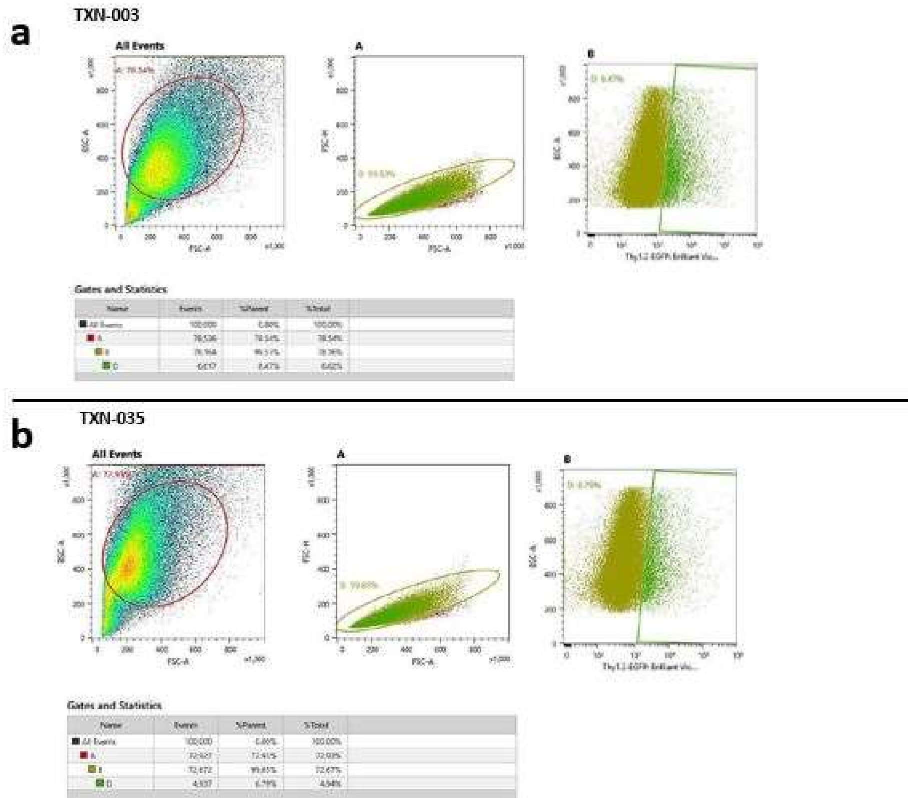


Figure 9. Reporter Cell Line Sort 2

Dot-plot graphs from the Sony Cell Sorter for the second cell line sort. (a) TXN-003 showed 8.47% positive population (increased from 2.95% in first sort) and a total of 389,550 cells were sorted and cultured. (b) TXN-035 showed 6.79% positive population (increased from 3.04% in first sort) and a total of 228,511 cells were sorted and cultured. After a week, these cells were transfected with transcription factor constructs and tested for double positive expression.

After the second sort, the Thy 1.2 expression levels were measured and shown to be around 80% (Fig 10a). A quick test with the new cell lines was done by transducing with TXN-029 (VP64 constitutive) and looking at the GFP fold change. Here, the high

basal level of minCMV can be seen once again (10b). From this point forward, only the TXN-035 (ybTATA) cell line was used for further experiments.

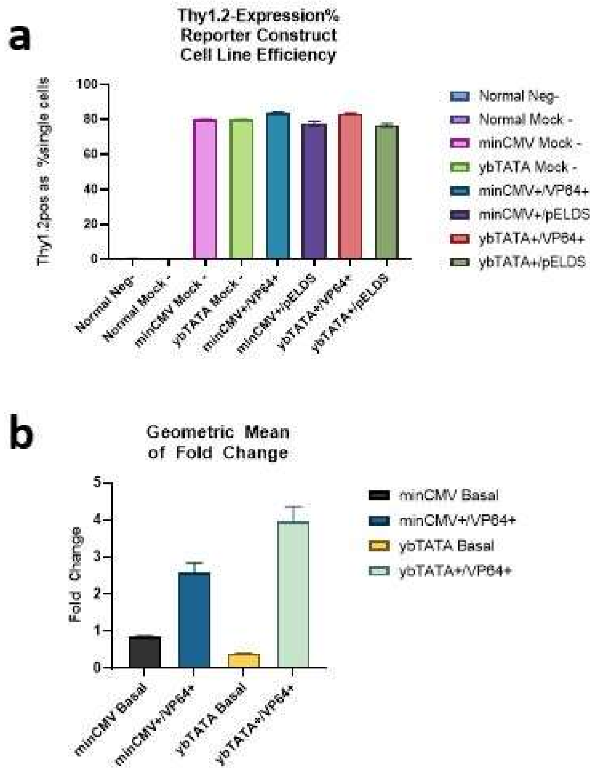


Figure 10. Reporter Construct Efficiency and Fold Change

Cell lines of minCMV and ybTATA reporters were transduced with construct TXN-029 (VP64+ constitutive). The expression of Thy 1.2 was measured (a) and shows that both the minCMV and ybTATA cell lines have around 80% positive expression of the reporter construct, making them a viable cell line to use for further experiments. In the same experiment, the fold change of GFP was measured (b) and the results are shown. The fold change of ybTATA with the VP64 construct shows the highest fold change with the ybTATA basal having the lowest value. This means that ybTATA reporter construct shows the best lowest basal and highest change difference. From this point forward, only the ybTATA cell line (TXN-035) was used for further experiments.

Drug Responsive Regulation in Lentivirus System

The levels of GFP fold change and MFI in a lentivirus transduction system is like the transient transfection system. The constitutive constructs had similar expression regardless of presence of drug, and the regulated constructs had elevated expression in the presence of drug. Comparing between VP64 and p65, the range of expression between 'on' and 'off' is larger in VP64 than p65 in two different virus MOIs tested (Fig 11). This can also be seen on the FACS dot plot (Fig 12) where the population of ON cells (GFP+) with the regulated constructs shifts twice as much as in the constitutive.

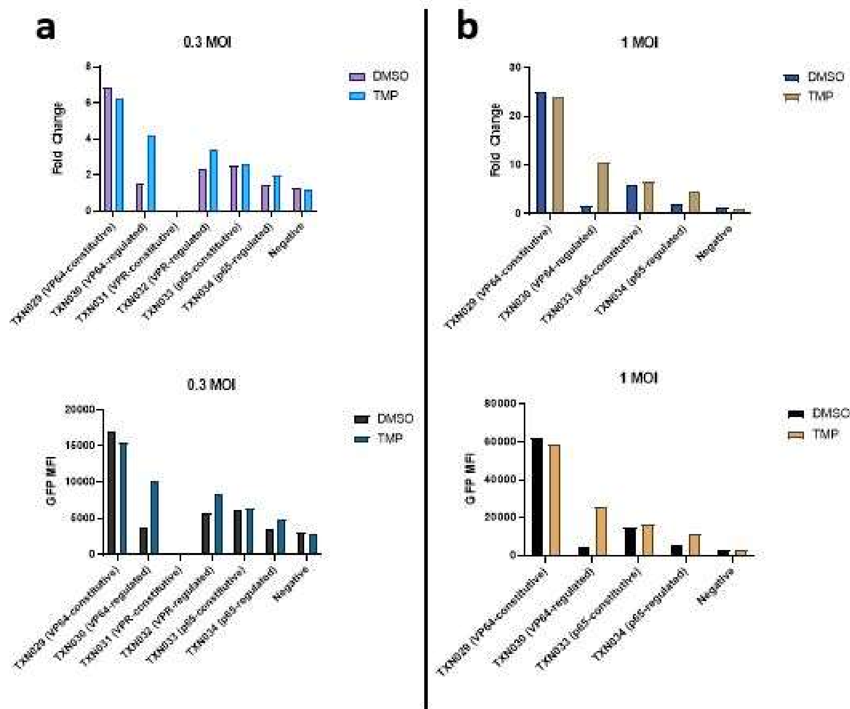


Figure 11. ybTATA Cell Line Transduction

The ybTATA cell line was transduced with the various transcription factor constructs in two different MOIs, 0.3 and 1.0. The VPR constructs had very low virus titers (see Figure 7), which meant that there was not enough virus to do the necessary transductions. As

such, in the 0.3 MOI experiment (a), the TXN031 (VPR constitutive) sample is missing because there was not enough virus. Likewise, in the 1 MOI experiment (b), both VPR constructs were omitted entirely because there was not enough virus. From this point forward, both VPR constructs were not used in future experiments and the focus is comparing between VP64 and p65. The patterns between constructs and for both MOIs are similar, with VP64 showing higher fold change and GFP MFI compared to p65.

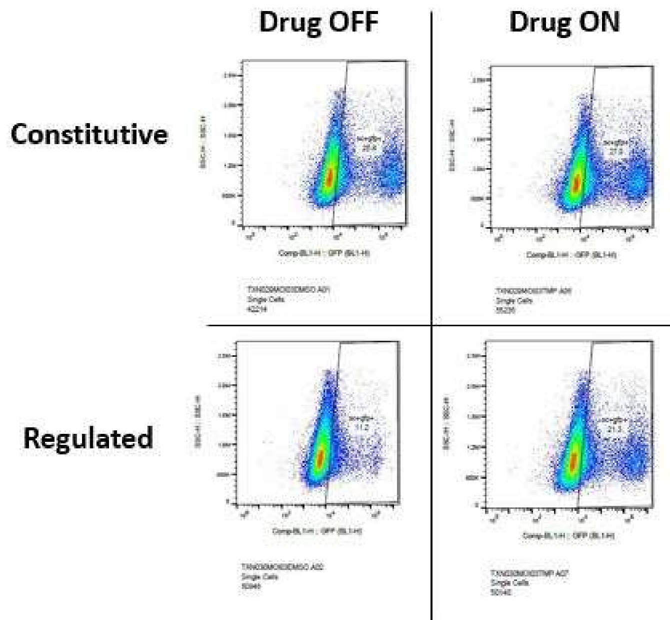


Figure 12. Example of Drug Off and On Dot Plot

Dot-plot graphs comparing the two VP64 constructs (constitutive and regulated) in the presence of absence of drug. This experiment was done using virus MOI of 0.3 and GFP was measured. For the constitutive construct, the presence or absence of drug has minimal effect on GFP positive cells (25.8% GFP positive without drug and 27.0% GFP positive with drug). For the regulated construct, without the drug, the amount of GFP positive cells is 11.2%. However, when the drug is present, the number of positive cells increases to 21.3%, showing that there is a drug-responsive regulated effect in the construct.

The first run with the lentivirus system was performed with only one replicate, and so this experiment was repeated in triplicates to provide statistically satisfactory

results. This triplicate experiment can be seen in Fig 12, with the visible error bars. The triplicate experiment matches the results of the previous experiment with one replicate, providing the same results.

VP64 is the best activation domain out of the three tested, having a wider range between low basal and high activation. It is comprised of four VP16 activation domains, and VP16 is widely used in regulatory protein interactions.

To further confirm the results, a Western Blot was done using protein extracted from the transduced cells. The Flag-antibody was used to stain the protein to measure the expression level of the transcription factor. The stained band of the sample with the regulated VP64 construct is slightly brighter with TMP present, than with DMSO, as seen on Fig 16. Western Blot is not a suitable method for quantitative comparison, but the result of the Western Blot shows that regulation is also seen in protein level through a different method compared to flow cytometry.

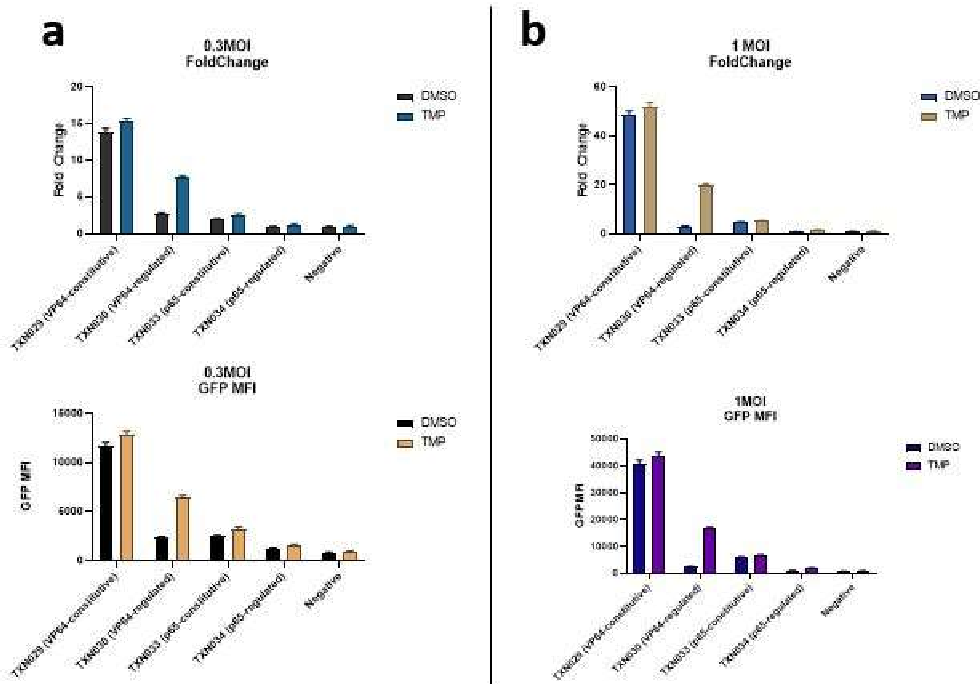


Figure 13. Fold Change and GFP MFI of MOI Comparison

Results of GFP MFI and Fold Change for virus MOI of 0.3 (a) and 1.0 (b). Cell line TXN-035 (ybTATA reporter) were transduced with constitutive or regulated VPR or p65 transcription factors and processed through flow cytometry after 48 hours. The differences between the MOI are the increase in fluorescence MFI and fold change. However, between the two MOIs, the patterns are comparable. VP64 shows an increase in fold change with a wider range of basal and activation, as compared to p65. Thus, VP64 appears to be the activation domain that shows the best regulation between the two.

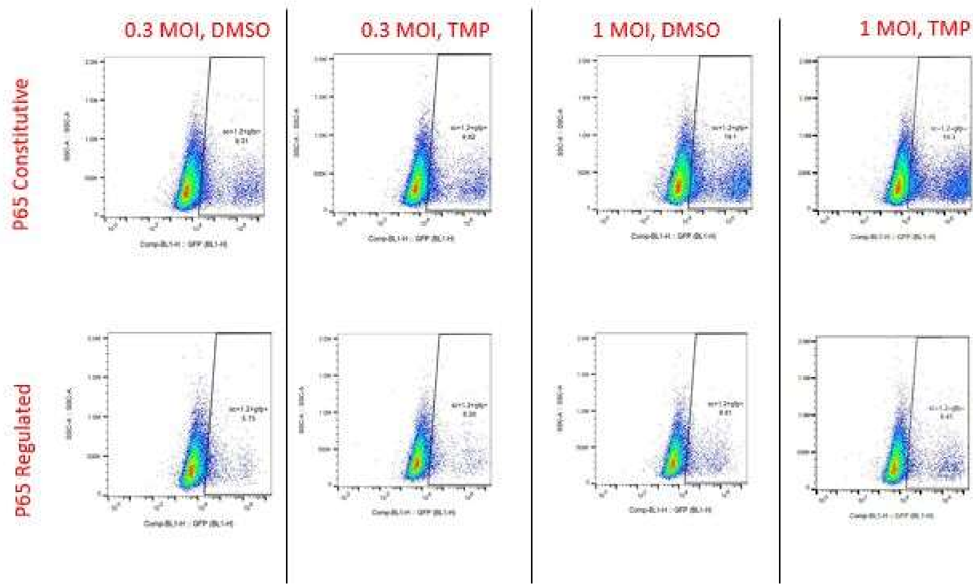


Figure 14. Dot Plot Comparisons of Different MOIs: p65 Activation Domain

Flow cytometry dot-plots comparing P65 constitutive and regulated constructs with and without the presence of drug TMP using virus MOI of 0.3 and 1. The population of Thy 1.2+ and GFP+ cells remain the same with the P65 constitutive construct with and without drug, increasing only when the MOI is increased due to higher background. With the regulated P65 construct, the positive population increases with drug from 5.75% to 6.20% with MOI of 0.3 and 6.81% to 9.41% with MOI of 1, showing some regulation.

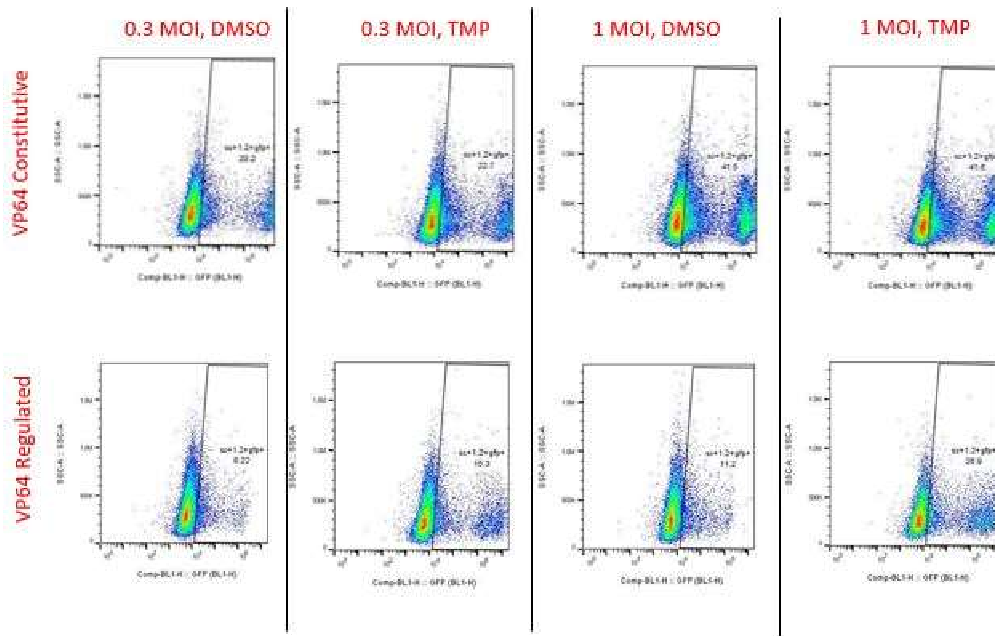


Figure 15. Dot Plot Comparisons of Different MOIs: VP64 Activation Domains

Flow cytometry dot-plots comparing VP64 constitutive and regulated constructs with and without the presence of drug TMP using virus MOI of 0.3 and 1. The population of Thy 1.2+ and GFP+ cells remain the same with the P65 constitutive construct with and without drug, increasing only when the MOI is increased due to higher background. With the regulated VP64 construct, the positive population increases with drug from 5.75% to 6.20% with MOI of 0.3 and 6.81% to 9.41% with MOI of 1, showing good regulation. As compared to P65 (Figure 14), VP64 regulated construct shows increased activation and a lower basal.

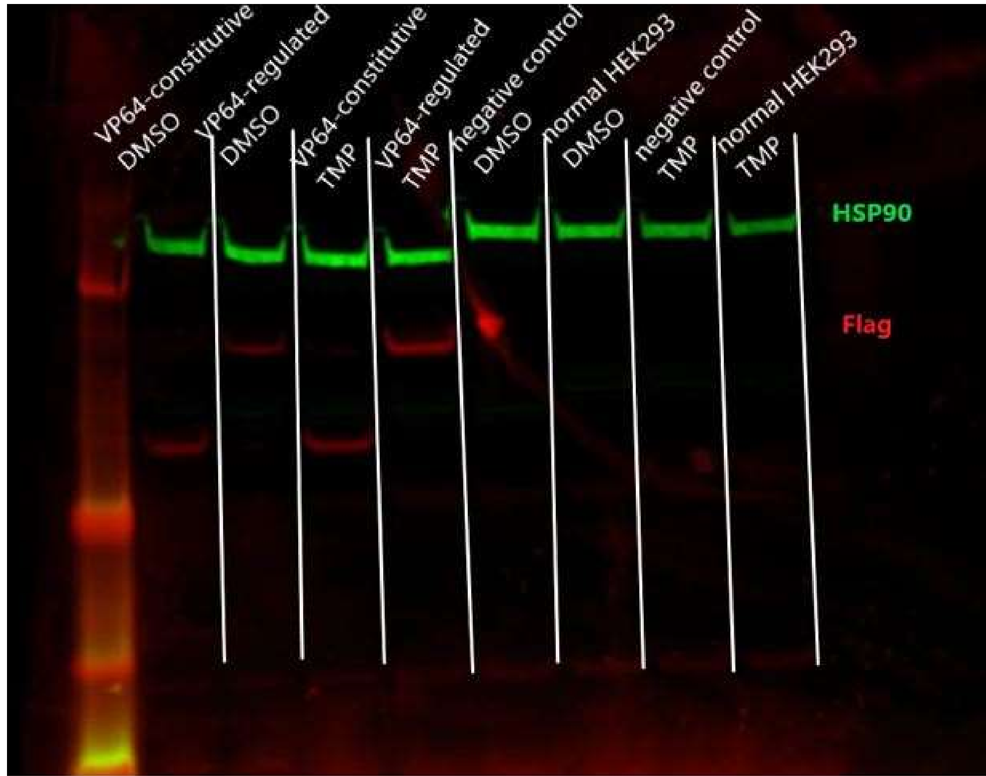


Figure 16. Western Blot of Gene Regulation

Cell lysate from transduction of ybTATA cells with transcription factor constructs VP64 were isolated and protein extracted. HSP90 (green) was used as a control and Flag-tag (red) was detected for the presence of the expression of the construct. For the VP64 regulated constructs, the molecular weight of the plasmids are heavier due to the added regulation domain, as compared to their constitutive counterparts. We are expecting to see an increase in expression for the constitutive constructs when drug is added by looking at the brighter band of detection.

Chapter IV.

Discussion

A synthetic, engineered transcription factor system consists of many different components that work together to regulate gene expression. Using a drug-responsive destabilizing domain fused to the transcription factor allows for tightly controlled regulation of transcription and a means to turn on or off gene expression. In this study, the focus of the effects of different activation domains in regulating the expression of a GFP gene in a lentiviral transduction model is investigated.

To test the different activation domains in a synthetic transcription factor system, plasmid constructs were designed and tested in a transfection-based method using mammalian HEK293 cells. At first, the constitutive constructs were tested—constitutive meaning that they did not have the drug-responsive regulatory domains attached so presence of drug was not needed for activating the transcription factor. The reporter construct, where the GFP gene is located, is on a separate plasmid and co-transfected with the transcription factor plasmid. Presence of GFP was measured in flow cytometry to confirm that the entire system worked. Once the constitutive constructs were confirmed to work, the regulated constructs were tested by dosing the cells with drug. This allowed a preliminary preview of how the constructs were regulating GFP expression. It was at this stage where it was noticed that the reporter construct containing the minCMV reporter showed very high basal expression and because the criteria of success in this study was to find the broadest range of expression, with low basal, the decision was made to drop the minCMV reporter construct for future experiments and focus on only the YB_TATA reporter construct.

Next, lentivirus containing the constructs were produced, by transfecting HEK293 cells with each construct and collecting the supernatant containing the virus. These viruses were then titrated to measure the multiplicity of infection (MOI) of each virus to ensure each transduction would have similar copy number of virus in each cell. It was here where it was noticed that the transcription factor constructs containing the VPR activation domain had very low virus production. It was theorized that perhaps the VPR domain itself interfered with the virus packaging mechanism. More optimizations would need to be done to improve the VPR yield. For this study, the VPR constructs were dropped because it was also known based on the preliminary transfection results that VPR showed extremely high basal and very strong activation, which did not meet the criteria for the biggest range of regulation. Therefore, the VP64 and p65 constructs were moved forward in the study.

A HEK293 cell line was produced containing the YB_TATA reporter construct by transducing cells with the construct and sorting cells using the cell marker Thy 1.2 as a readout. By transducing this cell line with the different transcription factor construct and dosing with or without drug, the results for measuring regulation could be determined. Measuring with flow cytometry, VP64 and p65 activation domains could be measured and compared for regulation.

A Western Blot staining for the Flag-tag in the transcription factor constructs was also done. This provided a different readout than the flow cytometry by looking at protein regulation instead. It was difficult to see the difference between drug on/off regulation through the Western Blot gel, but it did seem possible that there was regulation by way of more protein being produced when drug was present than when it was not. However,

more optimization for the Western Blot would be needed to confirm this. It would be interesting in the future to look at the protein regulation of transcription factor to compare the ability of the destabilizing domain to regulate protein level of the artificial transcription factor.

Based on the results, out of the activation domains VPR, VP64, and p65, it appears to that VP64 shows the best range of expression, with the lowest ‘off’ state and a high ‘on’ state. Based on various studies done with activation domains, this result is not unusual. While VPR does indeed have the strongest activation power out of the three domains, it has very high basal expression. The domain p65 is the only human-based activation domain tested in this study, and it is the weakest out of the three. This is to be expected, as the virus-derived domains of VPR and VP64 tend to have more robust activation than their human-counterpart. While the VPR domain had extremely strong activity, this seemed to result in poor ability to regulate it with the destabilizing domains.

Synthetic genetic circuits are challenging to implement in clinically relevant cell types (*in vivo*) and present translational incompatibilities. They also can be challenging to reprogram for new regulatory specificities. The non-mammalian origins of VP64 and VPR may provide additional challenges by way of eliciting immune responses in humans. To use this system in a clinically robust setting, more work needs to be done in finding ways to develop activation domains that are non-immunogenic but still provide the same capabilities as virus-based domains. However, in knowing that VP64 is an optimal candidate this can be used to further investigate possibilities of engineering a unique activation domain with components of VP64 but with minimal immunogenic potential.

Broader Implications

Finding ways to regulate gene expression is vital to understanding cellular pathways. Having a good dynamic range to minimize off-target and high basal activity is important as a therapeutic agent. Finding which activation domain works best to achieve this will help with gene regulation therapy in the future.

A single ligand domain system using FDA approved small molecules will make it easier and simpler to transfer into clinical trials. This system ensures specificity, and the small molecule control confers speed, reversibility, and dose-dependence. It enables conditional perturbation of specific proteins with unprecedented control by regulating protein stability. The ability to manipulate the expression of mammalian genes using synthetic transcription factors is highly desirable in both fields of basic research and industry for diverse applications, including stem cell reprograming and differentiation, tissue engineering, and drug discovery (Martella 2021).

Improving Cas9 technology using DRDs would immensely enhance its capabilities. One can imagine that rapid disabling of Cas9 activity (through the addition or removal of a drug that binds to a DRD fused to Cas9) after a desired genomic modification is valuable to prevent off-target activity. Dose-based control of transcript expression is essential to permit induction of physiologically relevant levels of mRNA transcripts. The chemical control of Cas9 endowed by DRD fusion would enable robust control of genome-interrogating activities across multiple dimensions, including dose, time, gene targets, and specificity (Maji 2016).

Next Steps

The transcription factor system is incredibly complex; endogenous transcription factors normally control entire gene networks and signaling pathways or multiple cellular processes at once. It is important to note that previous reports observed an inverse relationship between basal expression levels of the genes of interest and the relative upregulation (Martella 2021). This makes it potentially difficult to translate the system from different cell lines and genes. Improving DRDs is also important by varying their designs, such as changing the proteins' orientation, structure, and variations in cell penetrability of the ligands. Destabilizing domains are not as portable as library-derived small molecule or RNAi (Banaszynski 2006). Finding a way to streamline the process of the mammalian design cycle is critical for synthetic biology to better integrate with complex human applications such as stem cell therapy and tissue engineering (Burrill 2012). This technology's precision and control in regulating protein function are likely to be invaluable for validating potential therapeutic targets. Additionally, its application holds promise for advancing our comprehension of basic biological processes and pathways, showcasing its multifaceted impact on both therapeutic development and fundamental scientific exploration.

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