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An Estradiol-Inducible Promoter Enables Fast, Graduated Control of Gene Expression in Fission Yeast

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Abstract

The fission yeast *Schizosaccharomyces pombe* lacks a diverse toolkit of inducible promoters for experimental manipulation. Available inducible promoters suffer from slow induction kinetics, limited control of expression levels and/or a requirement for defined growth medium. In particular, no *S. pombe* inducible promoter systems exhibit a linear dose response, which would allow expression to be tuned to specific levels. We have adapted a fast, orthogonal promoter system with a large dynamic range and a linear dose response, based on β -estradiol-regulated function of the human estrogen receptor, for use in *S. pombe*. We show that this promoter system, termed Z₃EV, turns on quickly, can reach a maximal induction of 20 fold, and exhibits a linear dose response over its entire induction range, with few off target effects. We demonstrate the utility of this system by regulating the mitotic inhibitor Wee1 to create a strain in which cell size is regulated by β -estradiol concentration. This promoter system will be of great utility for experimentally regulating gene expression in fission yeast.

INTRODUCTION

Cell biological studies often take advantage of inducible promoters, ideally ones that are orthogonal to the system of interest. However, the inducible promoters available for the fission yeast *Schizosaccharomyces pombe* all have significant drawbacks ((Forsburg 1993) and Table S1). The inducible promoter most frequently used in *S. pombe* research is the endogenous *nmt1* promoter and its variants (Maundrell 1993). The *nmt1* gene has a strong promoter, which is regulated by thiamine, and has been engineered into a series of variants with low to high expression levels (Basi et al. 1993). The disadvantages of the *nmt1* promoter include a high constitutive activity, even in its weakest version, pleiotropic effects of induction by thiamine removal, and a long, 16-hour induction time. A synthetic *nmt1*-based promoter repressed by lacO improves the kinetic performance (Kjærulff & Nielsen 2015). Another synthetic promoter strategy combined tetO with the CaMV 35S promoter

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AUTHOR CONTRIBUTIONS

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(Faryar & Gatz 1992; Erler et al. 2006). However, tetO/CaMV expression is comparable to only the weakest *nmt1* promoter variant and also takes between 9 to 12 hours to induce (Forsburg 1993; Erler et al. 2006). A more recent version of the tetO system has improved its expression kinetics (Zilio et al. 2012). Nonetheless, mitochondrial toxicity of tetracycline remains a concern (Moullan et al. 2015). The endogenous *urg1* promoter has been shown to have favorable induction and repression kinetics (Watt et al. 2008), but it suffers from the disadvantages of being regulated by uracil (which requires growth in defined media and has other transcriptional effects), working best only at its endogenous locus (Watson et al. 2013), and not having a well-characterized dose response.

McIsaac *et al.* recently designed and implemented a synthetic promoter system, named Z₃EV, in *Saccharomyces cerevisiae*. This system is characterized by rapid and tunable induction of a desired target gene with the exogenous mammalian hormone β -estradiol. In *S. cerevisiae*, this system was shown to have a large dynamic range with minimal off-target activity (McIsaac et al. 2013; McIsaac et al. 2014). To increase the array of gene expression tools available in fission yeast, we have adapted the Z₃EV system for use in *S. pombe*.

The Z₃EV artificial transcription factor is a fusion of three domains: the Zif268 zinc-finger DNA binding domain containing three zinc fingers, the human estrogen receptor ligand-binding domain, and the VP16 viral activator domain (McIsaac et al. 2013; McIsaac et al. 2014). The estrogen receptor is an effective allosteric switch: binding of β -estradiol to the estrogen receptor disrupts its interaction with Hsp90 and causes rapid nuclear import of the Z₃EV protein. The Z₃EV-responsive promoter (Z₃EVpr) consists of six Zif268 DNA binding sequences inserted into a modified *S. cerevisiae* GAL1 promoter from which the canonical Gal4 binding sites have been removed. Target genes placed immediately downstream of Z₃EVpr in a strain expressing Z₃EV become conditionally expressed upon β -estradiol addition to the culture. A GFP reporter driven by the Z₃EV system in *S. cerevisiae* plateaued at about ~10-fold fluorescence over background when induced with 100 nM β -estradiol (McIsaac et al. 2013). GFP mRNA increased to between 50–100 fold over background approximately 20 minutes after induction with 1 μ M of β -estradiol (McIsaac et al. 2013; McIsaac et al. 2014). Though *S. cerevisiae* and *S. pombe* diverged ~420 million years ago (Sipiczki 2000), the conservation of Hsp90 (which is present in all organisms except Archaea (Gupta 1995)) suggested the Z₃EV system may also function in *S. pombe*.

In this manuscript, we describe the successful adaptation of the Z₃EV system for use in *S. pombe* and a set of constructs to make its implementation straightforward. We find that the Z₃EV system enables rapid, titratable induction of target gene expression in *S. pombe*. Increasing β -estradiol concentration results in a proportional increase of target gene expression. We use RNA-seq to show that the system has few off-target effects and thus can be used to regulate genes of interest with minor perturbations to cell physiology. Finally, we demonstrate that this system enables the study of native gene function: induction of the cell cycle regulator Wee1 by Z₃EV results in dose-dependent increase in cell size. Collectively, these results highlight the utility of Z₃EV to flexibly control gene expression in *S. pombe* without the significant drawbacks of previous synthetic systems.

MATERIALS AND METHODS

Media and growth conditions

We used standard fission yeast culture and growth conditions (YES medium, 30°C) as previously described (Forsburg & Rhind 2006). β -estradiol (E2758, Sigma Aldrich) was dissolved in ethanol at 10mM and stored at -20°C. β -estradiol was added to media from this 10mM stock to the final concentration. An equivalent volume of ethanol was added to untreated control cultures.

Strain and Plasmid construction

Strains and plasmids used in this study are listed in Tables S2 and S3. To create the Z₃EV protein integration plasmid (pFS461), we used Gibson assembly (NEB) to join two fragments: the Z₃EV CDS was amplified from DBY19011 (McIsaac et al. 2014) using primers MO198 and MO199 (Table S4), the *leu1* integrating vector (pFS181) (Sivakumar et al. 2004), which contains a strong constitutive *adh1* promoter, was amplified using primers MO197 and MO200. pFS461 was cleaved uniquely within the *leu1* ORF using XhoI and transformed into parent strain yFS110 to yield the transformant yFS949.

The plasmid containing GFP-NLS expressed from the Z₃EV promoter (pFS462) was created by Gibson assembly (NEB) of four fragments: the Z₃EV promoter sequence was amplified from pMN9 (McIsaac et al. 2013) using primers MO219 and MO220, the *his7* integration vector was amplified from pBSSK-His7(F) (Apolinario et al. 1993) using MO218 and MO225, the GFP-NLS CDS was amplified from pSO745 (courtesy of Snezhana Oliferenko) using MO221 and MO222 and inserted downstream of the Z₃EV promoter; the *leu1* C-terminal sequence was amplified from *S. pombe* genomic DNA using MO223 and MO224, and appended downstream of the GFP-NLS sequence. pFS462 was cleaved uniquely within the *his7* ORF using AflIII (NEB) and transformed into yFS949, to yield transformant strain yFS951. The *S. pombe* genome contains no potential Zif268 binding sites when searched with NCBI BLASTN under normal or nearest match specificity.

The plasmid containing beetle luciferase expressed from the Z₃EV promoter (pFS465) was created by Gibson assembly (NEB) to replace GFP with luciferase in pFS462. The beetle luciferase ORF was amplified from pFS470 using primers MO236 and MO237; the pFS462 vector was amplified with MO235 and MO238. pFS465 was cleaved uniquely at the *his7* ORF using AflIII and transformed into the Z₃EV protein-expressing strain yFS948. This intermediate strain was crossed with a strain constitutively expressing renilla luciferase as a translational fusion with *ade4* (yFS871) to yield our dual-luciferase strain (yFS954).

The plasmid containing the Z₃EV promoter with a KanMX integration cassette (pFS478) was created by Gibson assembly (NEB) of two fragments: the Z₃EV promoter sequence was amplified from pFS462 using primers MO240 and MO241, and the KanMX cassette and plasmid backbone were amplified from pFS156 using primers MO239 and MO242. The Z₃EV-KanMX cassette was then amplified from pFS478 using primers MO286 and MO287 and the resulting amplicon replaced the promoter region upstream of *wee1* in the Z₃EV-expressing strain yFS945, yielding our inducible *wee1* strain (yFS970).

Flow cytometry

GFP fluorescence was quantified using a Becton Dickinson FACScan flow cytometer. Cells were harvested at specified time points after induction with β -estradiol, spun down at 1 kG for 5 minutes at 4°C, washed with water, quickly spun down at RT, and resuspended in PBS. For each sample, we recorded the green fluorescence signal in the FL1 channel from 10,000 cells and normalized the mean intensity to the 0 timepoint or 0 dose sample.

RNA extraction and RT-qPCR

10 ODs of cells were harvested at specified time points after induction, spun down at 1 kG for 5 minutes at 4°C, washed with water, briefly spun down at RT, flash frozen in LN₂, and stored at -20°C. RNA was extracted from the cell pellet by bead beating with Trizol (Ambion) according to the manufacturer's instructions, followed by isopropyl alcohol precipitation, ethanol wash, and stored at -20°C. The RNA was treated at a ratio of 1 µg of RNA per unit of Amp-grade DNase I (Invitrogen) per the manufacturer's instructions. Gene-specific cDNA was obtained using Superscript III (Invitrogen) per the manufacturer's instructions and the following primers: MO233 for GFP, MO191 for *cdc2*, and LD8 for *ade4*. qPCR primers MO233 and MO221 yielded an amplicon length of 290nt within the GFP CDS. Primers MO192 and MO193 yielded an amplicon of 152nt within the *cdc2* CDS. Primers LD9 and LD10 yielded amplicon size 163nt within the *ade5* CDS. BLASTing these RT and qPCR primers against the *S. pombe* genome showed that they were either unique to the target gene, or in the case of GFP had no match within the genome and were unique to the GFP CDS. Specificity of the primers was shown via melt curve derivative, and standard curves were generated for all primer sets. qPCR was performed in 20 µl reactions containing 5 µl of cDNA, 10 µl 2X SYBR FAST qPCR Master Mix Universal (KK4601, KAPA Biosystems), 0.4 µl of each 10 µM primer and 4 µl water. Reactions were run in technical triplicates on a Bio-Rad CFX system, and Cq values were obtained via the Bio-Rad CFX Manager.

Dual luciferase assay

Dual luciferase assay (Promega) was performed according to manufacturer's instructions. Ten ODs of cells were suspended in 200 µl 1x Passive Lysis Buffer (PLB) and approximately 200 µl of zirconia/silica beads (Biospec). The cells were lysed by vortexing for 10 minutes at maximum RPM at 4°C. The lysate was spun at 16 kG at 4°C for 5 minutes. Triplicate 10 µl samples of cleared lysate were loaded into separate wells of a 96 well plate, each with 50 µl of pre-dispensed Luciferase Assay Reagent II. The luminometer was programmed for a 2 second rest and a 10 second measurement. 50 µl of Stop and Glow Reagent was dispensed, followed by the same rest and measurement.

Curve fitting

Dose-response data was fit with

$$y = \text{base} + ((\text{max} - \text{base}) * (x^{\text{slope}} / (x^{\text{slope}} + \text{EC}_{50}^{\text{slope}})))$$

where base is the reported level at 0 dose, max is the reporter level at saturating dose, EC_{50} is the dose (effector concentration) at 50% reporter expression, and slope is the ratio of change in reporter expression relative to dose (DeLean et al. 1978). Fold increase is calculated as the max/base.

Response-kinetics data was fit with

$$y = \text{base} + ((\text{max} - \text{base}) * (1 - e^{-(t - \text{lag}) / \text{half-life}}))$$

where base is the reporter level at $t = 0$, which is set to 1, max is the reporter level at the end of the time course, lag is the time until the reported level begins to increase, and half-life is the half-life of the reporter. Note that this observed half-life combines both the degradation of the reporter and its dilution due to cell growth during the time course.

Fits were calculated in Igor Pro 6.37 (Wavemetrics).

RNA-seq

10 ODs of cells were harvested before and after 3 hours of induction with 1 μM β -estradiol in three biologically independent replicates. RNA was extracted as described above. RNA quality was confirmed by denaturing gel and Bioanalyzer (Agilent) analysis.

Ribosomal RNA was depleted from 1 μg of total RNA using Ribo-Zero™ Yeast Magnetic Gold (Illumina), followed by a cleanup using Agencourt RNAClean XP beads (Beckman Coulter) and elution with 19.5 μl of Elute, Prime, Finish mix from the TruSeq stranded mRNA Sample Preparation Kit (Illumina). We performed library construction per the vendor's instructions starting with cDNA synthesis. We pooled the resulting barcoded cDNA libraries and subjected them to 70 base pair paired-end sequencing on an Illumina NextSeq.

Sequencing reads were aligned to the Ensembl EF2 *S. pombe* genome assembly accessed from Illumina's iGenome database <http://support.illumina.com/sequencing/sequencing_software/igenome.html> (modified to include GFP) and to the EF2 transcriptome (also modified to include GFP) using Tophat2 with the options -I 10000 -i 10 to constrain splice junction searching to < 10,000 bp and > 10 bp. We estimated transcript abundance and called differentially expressed genes using Cuffdiff2 with a false discovery rate of 5% (Trapnell et al. 2009). We filtered out genes with no counts or noisy signal (coefficient of variation > 0.5) in either the untreated and treated samples. Sequence data available at NIH GEO with accession number GSE92246.

Gene enrichment analysis was conducted on the AnGeLi server (Bitton et al. 2015).

RESULTS

Z₃EV Functions in *S. pombe*

To adapt the Z₃EV system to *S. pombe*, we expressed the Z₃EV protein from the strong constitutive *adh1* promoter on a plasmid (pFS461) integrated at the *leu1* locus and expressed

GFP-NLS from Z₃EVpr on another plasmid (pFS462) integrated at the *his7* locus (Figure 1A). Upon addition of β -estradiol, Z₃EV translocates into the nucleus and drives GFP expression (Figures 1B and 1C). This Z₃EV-driven GFP system enabled us to confirm the function of the Z₃EV system in *S. pombe* and quantitatively characterize its expression kinetics. Upon addition of 1 μ M β -estradiol, we observed an approximately 4-fold increase in fluorescence signal over 5 hours of induction (Figure 1D). Fitting an induction-kinetics model to the data indicates that GFP takes about 1 hour to express, consistent with the slow maturation of GFP fluorescence (Heim et al. 1994). The fit also allows us to infer that the half-life of GFP in cells is about 2 hours. However, this observed half-life combines both the protein degradation rate and its dilution rate due to cell growth. The fact that the yeast doubling time of about 2 hours suffices to explain the observed GFP half-life suggests that the rate of GFP degradation is significantly slower than its rate of dilution. The range of GFP expression from Z₃EVpr is within the range of endogenous gene expression and between the strengths of the wild type and medium strength *nmt1* promoters (Basi et al. 1993) (Figure 1E).

Z₃EV Expression is Linearly Dependent on β -Estradiol

To characterize the dose response of Z₃EV to β -estradiol in *S. pombe*, we assayed Z₃EV-dependent expression over 4 logs of β -estradiol concentration. We observed only background fluorescence below 1 nM, and an upper plateau of approximately 4-fold induction at or above 100 nM (Figure 1F). The background fluorescence below 1 nM was similar in un-induced cells, cells expressing Z₃EV protein without a reporter, and wild-type cells (Figures 1C and 1F). Between 1 nM and 100 nM β -estradiol, we observed a linear dose-response of GFP expression to β -estradiol concentration (Figure 1F). Fitting with a sigmoidal dose-response curve shows a low nanomolar EC₅₀ (effector concentration at 50% response) and a slope (the ratio of change in response relative to dose) in the neighborhood of 1.

Although the GFP reporter allows rapid validation of the system and single-cell analysis of expression, the background auto-fluorescence of cells obscures the full dynamic range of Z₃EV-dependent expression. To more accurately measure the dynamic range of the Z₃EV system, we used a beetle luciferase reporter. Luciferase has excellent signal-to-noise characteristics and by using a Z₃EV:beetle luciferase reporter strain constitutively expressing renilla luciferase (Voon et al. 2005), we have an internal control for dual-luciferase quantification experiments. Luciferase expression showed similar kinetic and dose-response profiles to the GFP reporter (Figures 2A,B). However, the dynamic range of the luciferase luminescence was much larger, with maximal expression over 20-fold greater than repressed levels (Figure 2B).

mRNA Expression is Rapidly Induced by Z₃EV Activation

To directly assay the transcriptional activity of Z₃EVpr in response to Z₃EV activation, we measured changes in reporter mRNA levels. A response-kinetics assay using RT-qPCR is similar to the luciferase assay with a 20-fold dynamic range peaking at 2 hours (Figure 3A). However, induction at the mRNA level is much faster than either of the reporter proteins. Induction of GFP transcripts was evident by 5 minutes after β -estradiol addition (Figure

3A). Fitting an induction-kinetics model to the data indicates that there is no measurable lag in mRNA expression and that the time taken to reach maximum expression is limited solely by the relatively long, ~40 minute, half-life of the GFP mRNA. Thus, the RT-qPCR-based assay provides a more sensitive measure of promoter activity than the fluorescence- or luminescence-based assays, and demonstrates the rapid transcriptional response of the Z₃EV system. Nonetheless, the dose-response parameters of all three assays are similar, with low nanomolar EC₅₀s and slopes near 1 (Figures 1F, 2B, 3B).

Growth effects of Z₃EV Induction

There is some evidence that the VP16 domain can be toxic in yeast (Berger et al. 1992; Remacle et al. 1997; Silverman et al. 1994; McIsaac et al. 2011). To test the effect of the Z₃EV protein (which includes a VP16 domain) on fitness, we compared the growth of wild-type cells to cells expressing the Z₃EV protein and to cells expressing both Z₃EV protein and Z₃EVpr-GFP under varying levels of β -estradiol induction.

Growth rates at various β -estradiol doses were measured and culture doubling times were calculated (Figure 4A). Wild-type cells are unaffected by addition of β -estradiol. In contrast, both the Z₃EV protein-only expressing strain and the strain containing both Z₃EV and a GFP reporter, showed a similar modest increase of doubling time with increasing β -estradiol (Figure 4A). However, the decrease in growth rate is less than 10% at 100 nM β -estradiol and even at 10 μ M, where the reduction in growth rate is about 25%, the cells appear healthy by DIC microscopy. In particular, after six hours in at 1 μ M β -estradiol, wild-type cells (yFS101) show no significant alteration in length at division (13.4 ± 0.16 v. 13.6 ± 0.18) and the Z₃EV protein-only expressing cells (yFS949) show a modest 8% increase in length at division (13.4 ± 0.27 v. 14.5 ± 0.20). Interestingly, cells containing both Z₃EV and a GFP reporter (yFS951) show a more significant 22% increase (15.1 ± 0.28 v. 18.3 ± 0.26), suggesting that over-expression of even apparently benign proteins can have subtle phenotypes. Since the EC₅₀ for the reduction in growth rate is 10- to 30-fold higher than that for target-gene expression and the reduction only becomes substantial at concentrations above those required for maximal target-gene expression, the Z₃EV system can be used over its full dynamic range with only a slight effect on growth rate.

Z₃EV has Few Off-Target Effects

To investigate the genome-wide effect of the β -estradiol induced Z₃EV system in *S. pombe*, we used RNA-seq to profile the transcriptomes of our Z₃EV strain under uninduced and induced conditions. Most genes remained unaffected by the induction of the Z₃EV system with 1 μ M of β -estradiol. Only 265 out of 5992 expressed loci showed greater than 1.32-fold change in expression after induction, a cutoff set so that genes reported as differentially expressed have a q value (FDR-corrected p value) of less than 0.05 (Table S5). Approximately equal numbers of genes showed an increase versus a decrease in expression. The largest increase was GFP mRNA, which showed a 4.9-fold increase (Figure 4B). The gene with the largest decrease in expression was *pho84*, at 3.2 fold.

Gene ontology enrichment analysis showed no significant enrichment within the set of genes that increased with Z₃EV induction. The set of genes that decreased with Z₃EV induction

showed between 4–7 fold enrichment for various cytoplasmic translation or amino acid metabolic factors. It is unclear if the decrease in expression of genes associated with translation and amino acid biosynthesis is a cause or consequence of the reduced growth rate observed at 1 μ M β -estradiol.

Z₃EV Regulation of *wee1* Expression Creates β -Estradiol-Dependent Cell Size Control

To demonstrate the experimental utility of the Z₃EV promoter, we constructed a strain in which expression of the mitotic inhibitor Wee1 is under control of the Z₃EV promoter. Wee1 is a dose-sensitive regulator of mitotic entry and thus cell size (Russell & Nurse 1987). We therefore expected that by regulating Wee1 expression, we would be able to regulate the size of cells at division. To do so, we replaced the endogenous *wee1* promoter with the Z₃EV promoter in a strain expressing the Z₃EV protein. In response to increasing doses of β -estradiol, cell size at division increases from 13 μ m to 28 μ m with a dose-response similar to those of the Z₃EV-responsive fluorescent and luminescent reporters (Figure 5).

DISCUSSION

Research using the fission yeast *S. pombe* has been hampered by the lack of convenient systems for regulation of gene expression. An ideal system would have a large dynamic range over which expression could be controlled by varying inducer concentration, would be rapidly induced in response to addition of inducer, would be based on an exogenous regulatory system, so as to have minimal incidental transcriptional effects, and would function in rich media, for cost efficiency. To establish such a system, we implemented a β -estradiol-regulated transcription system based on the Z₃EV synthetic transcription factor, which was originally developed for use in *S. cerevisiae*, and showed that it operates successfully in *S. pombe*.

Maximal Z₃EV activation by saturating levels of β -estradiol (> 100 nM) induces about a 20-fold increase in transcription from genes driven by Z₃EVpr, the Z₃EV responsive promoter (Figures 2A,B and 3A,B). This dynamic range compares favorably with other inducible promoters in *S. pombe*, which mostly show between 10- and 25-fold induction (Forsburg 1993; Watt et al. 2008), with the exception of the full-strength *nmt1* promoter which can induce up to 300-fold, often resulting in pathological overexpression.

The level of expression from Z₃EVpr is within the range of normal *S. pombe* gene expression, with its uninduced level being just less than the *wee1* promoter and its maximal induced level being about 30% of maximal *nmt1* expression (Figures 1E and 5). However, the absolute level of expression from any promoter will depend on the stability of the target gene's message and protein product. For applications that require lower basal expression levels, gene expression could be modified by adding mRNA or protein destabilizing elements (Voon et al. 2005), as has been done with the *urg1* promoter (Watson et al. 2013).

One of the major drawbacks of the *nmt1* promoter—the most commonly used promoter in *S. pombe* experiments—is its slow activation kinetics, requiring 16 hours to induce. Z₃EV induces quickly, with mRNA expression evident at 5 minutes after induction (Figure 3A). Modeling of its induction kinetics suggests that there is no measurable lag in mRNA

expression. The lag seen in the expression the GFP and luciferase reporter proteins are presumably due to their slow maturation kinetics. The modeling further suggests that the time to reach maximum mRNA expression is determined solely by the half-life of the target mRNA.

A major advantage of the Z₃EV system over other expression systems available in *S. pombe* is that it has a linear dose response over the full range of its induction (Figures 1F, 2B, 3B and 5B). By titrating the concentration of β -estradiol, it is possible to obtain any expression level within its range, making the system convenient for controlling expression levels and allowing levels to be changed at will, affording tremendous experimental flexibility. Other *S. pombe* promoter systems have been modified to produce series of promoters of varying strengths (Basi et al. 1993; Watson et al. 2013). However, this approach allows access to only a fixed number of expression levels and does not allow flexible control of expression within one strain. The linear dose response of Z₃EV, in combination with the use of mRNA and protein destabilization elements to modify the absolute range of Z₃EVpr-driven expression for any particular target gene, will afford a wide range of expression levels for any target protein (Voon et al. 2005; Watson et al. 2013).

Another significant advantage of the Z₃EV system is that, being entirely foreign to *S. pombe*, it has very few off-target transcriptional effects. Only 265 genes show statistically significant changes in expression at the $q < 0.05$ level, which corresponds to a change of greater than 1.32 fold, and only 33 genes had a greater than 2-fold change in expression (Table S5). The statistically significantly up regulated genes did not show significant enrichment in any particular classes of genes. The down regulated genes showed enrichment for various cytoplasmic translation or amino acid metabolic factors. Since these genes are not enriched for even weak matches to the Z₃EV binding site, it seems unlikely that they are direct targets. Instead their down regulation may reflect the slight reduction in growth rate at 1 μ M β -estradiol (Figure 4A).

Even though activation causes only slight off-target changes in gene expression, it does have modest effects on cellular growth rates (Figure 4A). At 100 nM β -estradiol, the lowest concentration that induces maximal expression from Z₃EVpr, cells double 10% slower than untreated cells; at 10 μ M, they grow 25% slower, although they appear healthy. The presence of only minor off-target effects, along with the fact that growth rate continues to decline at doses above those required for maximal specific transcription induction, suggests that the mild growth defect induced by β -estradiol is not due to specific transcriptional effects but may be due to non-specific disruption of chromatin structure, possibly related to the known toxicity of ectopic expression of the VP16 transcriptional activator (Berger et al. 1992; Remacle et al. 1997; Silverman et al. 1994; McIsaac et al. 2011), which may dysregulate endogenous chromatin remodeling complexes.

A final advantage of the Z₃EV system is that it does not require specific media and works in both rich and synthetic medium (Figure 1E). This fact allows for increased experimental flexibility and is economically advantageous relative to promoters that require synthetic media.

Balancing the advantages of the Z₃EV system is the fact that it requires introduction of an exogenous transcription factor. In the current configuration, it requires crossing in the integrated Z₃EV expression cassette or integrating it in a *leu1-32* background.

The utility of the Z₃EV system in *S. pombe* is demonstrated by its ability to regulate cell size when used to drive expression of the mitotic inhibitor Wee1. Wee1 is a protein kinase that negatively regulates Cdc2, the master regulator of mitosis (Nurse 1990). Wee1 is a dose dependent regulator of entry into mitosis and thus the level of Wee1 expression controls the size of cells at division. (Russell & Nurse 1987). We replaced the *wee1* promoter with Z₃EVpr and showed that we could regulate cell size at division from 13 µm to 28 µm by titrating β-estradiol concentration (Figure 5). Although a number of strategies have been previously used to elongate cells, these approaches use disruptive treatments, such as temperature shift or checkpoint activation, and generally result in acute G2 arrest, so arbitrary intermediate cell sizes have not been experimentally accessible. Thus, beyond being a simple demonstration of the utility of the Z₃EV system in *S. pombe*, this strain will be a useful tool for the study for cell size biology.

Given the limited existing repertoire of promoters available for the *S. pombe*, and the multiple advantages of the Z₃EV system, we believe that it will prove to be an invaluable tool for future functional studies in this important model organism.

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Figure 1. Z3EV Regulates Dose-Dependent Expression in *S. pombe*

(A) Schematic representation of the β -estradiol-induced promoter constructs.

(B) Schematic representation of the β -estradiol-induced promoter system. The system was designed such that (1) added β -estradiol (2) binds to Z3EV protein leading to (3) translocation into the nucleus, (4) transcriptional activation of the GFP reporter gene, (5) GFP expression, and (6) translocation of GFP back into the nucleus.

(C) Z3EV-dependent expression of GFP. Cells expressing Z3EV and containing the Z3EVpr:GFP expression cassette (yFS951) and wild-type cells (yFS110) were grown with or without 1 μ M β -estradiol for 4 hours and imaged for green fluorescence. The outlines of the cells, taken from bright-field images, are also shown.

(D) Z3EVpr:GFP induction kinetics. *adh1*:Z3EV Z3EVpr:GFP cells (yFS951) were induced with 1 μ M β -estradiol and sampled every 20 minutes. Mean fluorescence of cells in the culture relative to uninduced cells was measured by flow cytometry. $n=3$; the mean and standard error bars are shown. The curve is an induction-kinetics model fit to the data from which the induction parameters were extracted.

(E) Z3EVpr:GFP expression levels relative to other promoters. wt (yFS105), *rpa1*-GFP (yFS831), *adh1*:Z3EV Z3EVpr:GFP (yFS951), *nmt41*:tub1-GFP (yFS386), *nmt1*:tub1-GFP (yFS387) and *adh1*-GFP (yFS313) cell were grown in the rich medium (YES) or synthetic medium (EMM). The ZEV promoter was induced with 1 μ M β -estradiol. The *nmt1* promoter, or a moderate-strength mutant thereof (*nmt4I*), was repressed with 15 μ M

thiamine (B1). Cells were assayed for mean fluorescence relative to wild-type background fluorescence by flow cytometry. $n=3$ for all strains; the mean and standard error bars are shown.

(F) Dose response of Z₃EVpr:GFP expression. *adh1::Z₃EV Z₃EVpr:GFP* cells (yFS951) were induced with various concentrations of β -estradiol, sampled after 5 hours and assayed for mean cellular fluorescence relative to uninduced cells by flow cytometry. $n=3$; the mean and standard error bars are shown. The curve is a sigmoidal dose-response model fit to the data from which the dose-response parameters were extracted.

Figure 2. Z₃EV Regulates Expression Over a Large Dynamic Range

(A) Z₃EVpr:bLuc induction kinetics. Cells expressing Z₃EV, containing the Z₃EVpr:bLuc expression cassette and expressing a control Ade4-rLuc fusion (yFS954) were induced with 100 nM β -estradiol and sampled as indicated. Mean beetle luciferase activity of cells relative to renilla luciferase activity was measured by luminometry and normalized to uninduced cells. $n=3$ for all points except 720 minutes, for which $n=1$; mean and standard error bars are shown. The curve is an induction-kinetics model fit to the data from which the induction parameters were extracted.

(B) Dose response of Z₃EVpr:bLuc expression. Z₃EV Z₃EVpr:bLuc Ade4-rLuc cells (yFS954) were induced with various concentrations of β -estradiol, sampled after 4 hours and assayed for mean beetle luciferase activity of cells relative to renilla luciferase activity by luminometry. Data was normalized to the uninduced sample. $n=3$; mean and standard error bars are shown. The curve is a sigmoidal dose-response model fit to the data from which the dose-response parameters were extracted.

Figure 3. Z₃EV Exhibits Rapid Induction Kinetics

(A) Z₃EVpr:GFP mRNA induction kinetics. Z₃EV Z₃EVpr:GFP cells (yFS951) were induced with 1 M μ M β -estradiol and sampled as indicated. GFP mRNA levels relative to *cdc2* mRNA were measured by qPCR and normalized to uninduced cells. Early time points are shown in the inset graph. $n=3$; the mean and standard error bars are shown. The curve is an induction-kinetics model fit to the data from which the induction parameters were extracted.

(B) Dose response of Z₃EVpr:GFP mRNA expression. Z₃EV Z₃EVpr:GFP cells (yFS951) were induced with various concentrations of β -estradiol, sampled after 5 hours and assayed

for GFP mRNA levels relative to *ade4* mRNA levels by qPCR. Data was normalized to the uninduced sample. n=3; the mean and standard error bars are shown. The curve is a sigmoidal dose-response model fit to the data from which the dose-response parameters were extracted.

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Figure 4. Global Effects of Z₃EV Activity on Cell Growth and Gene Expression

(A) Activation of Z₃EV causes a modest reduction in growth rate. Growth curves of wild-type (yFS110), Z₃EV (yFS949) and Z₃EV Z₃EVpr:GFP (yFS951) strains at varying concentrations of β -estradiol. Growth was measured by optical density over 11 hours. The curve is a sigmoidal dose-response model fit to the data from which the dose-response parameters were extracted. The longer doubling time for the wild-type control at the lowest doses of β -estradiol may be due to the fact that our wild-type strain is auxotrophic for leucine, whereas both Z₃Ez strains are leucine prototrophs.

(B) Activation of Z₃EV causes few off-target effects on mRNA expression. RNA-seq FPKM values of induced vs. un-induced Z₃EV Z₃EVpr:GFP cells (yFS951). GFP mRNA is highlighted in green.

Figure 5. $Z_3\text{EV}$ -Dependent Regulation of Cell Size

(A) $Z_3\text{EVpr}:\text{wee1}$ regulates cell size in response to β -estradiol. Wild-type cells (yFS105) and cells expressing $Z_3\text{EV}$ and $Z_3\text{EVpr}:\text{wee1}$ (yFS970) were grown for 6 hours in varying concentrations of β -estradiol.

(B) Quantitation of the length of cells at septation of cultures shown in 5A. The curve is a sigmoidal dose-response model fit to the data from which the dose-response parameters were extracted.