Supplementary Material 1

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Yeast strains, plasmids and oligonucleotides.

Contents:

Details of yeast strain and plasmid constructions

1) Background information:

Tetracycline repressor (tetR) fusion proteins bind at promoters containing tetO DNA sequences (derived from the bacterial Tn10 transposon) only in the absence of tetracycline or doxycyline, and are displaced by drug addition (Belli et al. 1998a); (Belli et al. 1998b); (Gari et al. 1997). Expression can be regulated, with the level of expression dependant on the number of tetO sequences present in the promoter. Originally developed for mammalian cells (Gossen and Bujard 1992); (Gossen et al. 1993), the tet system allows tight regulation of gene expression. Furthermore, a mutated tetR' protein which binds to tetO only in the presence of tetracycline or doxycyclin was used in the development of a tet-regulated dual activator/repressor system (Gossen et al. 1995); (Belli et al. 1998b), in which tet-responsive transcriptional repressor and activator proteins, that react to doxycycline in opposing ways, are co-expressed, in order to produce tighter regulation. In the "tetON" system, a tetR-repressor protein (e.g. tetR-SSN6 in which tetR is fused to the yeast Ssn6 repressor protein) represses transcription in the absence of effector and tTA' hybrid transactivator protein (tetR'-VP16, containing the activator domain of VP16 from herpes simplex virus) activates transcription in the presence of effector. Conversely, in the "tetOFF" system, tetR-VP16 protein (tTA) activates transcription in the absence of effector and tetR'-SSN6 represses transcription in the presence of effector (Belli et al. 1998b); (Yen et al. 2003). This provides a greater dynamic range of expression (Maya et al. 2008).

2) Overview of the yeast tet strain development:

To create tetOFF/ON strains, tTA/tTA' activator (fusion of tetR/tetR' with VP16 from pCM225; Belli et al., 1998b) was integrated into the *LYS2* locus deleting the whole *LYS2* gene, and tetR'/R repressor (fusion of tetR'/tetR with SSN6 from pCM242; Belli et al., 1998b) was integrated at either *ura3* or *leu2* (because of unknown problems integrating at *ura3* in BY4741). To avoid the possibility of mutations due to PCR amplification of these very long constructs we cloned *LYS2* and *URA3* flanking sequences upstream and downstream of the integration cassettes and excise the cassettes from such plasmids using restriction enzymes. We introduced the tetracycline dual activator/repressor system into three different haploid MATa strains, W303, BY4741 and CEN-PK2-1C (Table S1 below). For initial tests the *tet07-CYC1* hybrid promoter (see Supplementary material 2 for sequence) was inserted in front of the non-essential *STE3* gene (retaining its 5'UTR). *STE3* was chosen on account of the short half-life (4.5 min) of its transcripts (Heaton et al. 1992), which facilitates testing the speed and efficiency of repression or activation. Transcription was repressed or induced by the addition of doxycycline (dox) at a concentration of 4µg/ml and RNA was analysed by northern blotting. In all the "tet-OFF" strains, *STE3* expression was efficiently repressed approximately 10 min after dox addition (Supplementary Fig. S1). Strains YMK118 (derived from CEN-PK2-1C) and YMK120 (W303) gave better levels of expression than YMK119 (BY4741), and YMK120 and its *ura3* derivative. YIK120, were chosen for further study (see Table S1 for genotypes).

In contrast, none of the corresponding tet-ON strains showed induction even after 15 h in the presence of dox (data not shown). We tested integration of tTA' at different chromosomal locations including *HIS3* and *URA3*, correcting a few sequence differences discovered when comparing the promoters and 5'UTRs of the tTA and tTA' constructs in pCM175 and pCM176 (Belli et al. 1998b), and we tried an improved rtTA-M2 version of the transactivator (Resch et al. 2008), integrated at *LYS2* in W303 but these as well as other integrated tTA' constructs produced only low level or no induction (data not shown). We are currently unable to explain the failure to obtain dox induction with transactivator genes integrated in the genome. Finally, a functional tetON strain, YIK91, was produced with tTA' expressed under the control of the strong *S. cerevisiae ADH1* promoter (P_{ADH1}tTA') on the pRS414-ADH1 vector (Mumberg et al., 1950, and with the tetR-SSN6 repressor gene expressed from the *Schizosaccharomyces pombe adh1* promoter (P_{adh1}tetR-SSN6 from pCM247; (Belli et al. 1998b) integrated along with *LEU2* as marker at the *leu2* locus of W303.

3) Detailed gene manipulations for the tet-regulated strains:

To create plasmids pMK119-Lys and pMK120-Lys for integration of tTA/tTA', *LYS2* 3' flanking sequence was amplified from W303 genomic DNA using forward 5'-AACTCGAGTGCTGGTGCTCGTGGAAGCT-3' and reverse 5'-AA**GATATC**GACCTGTGAAGATCACAAGGT-3' primers (site XhoI used for cloning is underlined, EcoRV used both for cloning and excision of the cassette for transformation is in bold). Similarly, the 5' flank of *LYS2* was amplified using a forward primer 5'-AAGCATGC**GATATC**AATCGTTCGGACTCGACGAA-3' (recognition site SphI is underlined, EcoRV site used for excision of the cassette in bold) and reverse primer 5'-AAGCATGCTTCTCCCAGCGGGTCTTCA-3' (SphI site underlined). The tTA and tTA' were

excised from pCM225 and pCM252 using EcoRI and XhoI sites. All the fragments were combined in pBluescriptII(SK-) backbone to obtain plasmids pMK119-Lys and pMK120-Lys carrying an integration cassette containing 5'flank*LYS2*-tTA/tTA'-3'flank*LYS2* with EcoRV site on both sides. The resulting plasmids were digested by EcoRV and transformed into haploid *MAT***a** strains W303, BY4741 and CEN-PK2-1C (EUROSCARF). Transformants were identified, using α-amino adipate to counter-select *LYS2*.

Into these strains the tetR'/R repressor was integrated as follows. *URA3* gene flanking sequences were amplified using 5'flank primers 5'-TCACGTA**GTTTAAAC**ACAGGAAGACCAGAAAATCCT'3- and 5'-CGGGCCCACATTAACCTTCTTTGATGGTCAA-3' (cloning sites BsaAI and ApaI underlined, excision site PmeI bold) and 3' flank primers 5'-AGCGGCCGCCGTAATGATTTCTATAATGACGAA-3' and 5'-ACCGCG**GTTTAAAC**GCTGCCCTACACGTTCGCT-3' (cloning sites NotI and SacII underlined, excision site PmeI in bold). The tetR and tetR' including the P_{CMV} promoter were excised from pCM242 and pCM244 respectively using SphI and XhoI. A *URA3*-K.l. gene was excised from the vector pBS1539 (Rigaut et al. 1999) using restriction sites XbaI and XhoI. All four fragments were again combined in the pBluescriptII(SK-) backbone to obtain plasmids pMK135 and pMK136 carrying the following integration cassette PmeI-5'flankURA3-tetR/R'-URA3-K.l.-3'flankURA3-PmeI. The plasmids were digested by PmeI and transformed into the yeast strains carrying tTA/tTA' transactivators and transformants selected on plates lacking uracil.

To generate the YIK120 tetOFF *ura3* strain, the *URA3* marker in YMK120 was replaced, using the PCR-based strategy, by the *hgh* gene from *Klebsiella pneumoniae* encoding resistance to hygromycin B. The *Hgh* cassette was amplified using pAG32 (Goldstein and McCusker 1999); (Goldstein and McCusker 1999) as a template and 5' flank primer **CCGATGAGCATATGTAATAATATTGGGAATTAAGGTGCATTTTCGTATCC**GAAGCTTCGTACGCTGCAGG and 3' flank primer **GAGGTATTGGATAGTTCCTTTTTATAAAGGCCATGAAGCTTTTTCTTTCC**CATCGATGAATTCGAGCTCG (underlined nucleotides correspond to the *hgh* cassette, nucleotides in bold are sequences upstream or downstream respectively of the *URA3* gene in the YMK120 strain).

To create tetON strains expressing the improved rtTA-M2 version of transactivator (Urlinger et al. 2000) the tTA in pMK119-Lys was replaced by a synthesized gene encoding the rtTA-M2 using restriction sites NcoI and XmaI. The resulting plasmid pMK154 was digested with EcoRV and transformed into W303 wild type strain yielding strain YMK156, however, the level of induction was poor.

To construct the successful tetON strain YIK91, the gene encoding tetR hybrid repressor (fusion of tetR with SSN6 expressed from the *S. pombe adh1* promoter, from pCM247 (Belli et al. 1998b)), was integrated along with *LEU2* as marker at the *leu2* locus, and the gene encoding the tTA' hybrid trans-activator (fusion of tetR' with VP16 from pCM252; (Belli et al. 1998b)) was amplified using a 5' flank primer CGCGGATCCATGTCTAGATTAGATAAAAG and a 3' flank primer CGCGGATCCCTACCCACCGTACTCGTCAATTC, both containing BamH1 sites (underlined), and cloned in the BamH1 site between the *S. cerevisiae ADH1* promoter and *CYC1* terminator in the pRS414-ADH1 vector (Mumberg et al. 1995) to produce p414ADH1-tTA'. The correct sequence and orientation of the amplified tTA' were verified by sequencing.

To create an integrative tetO7 cassette, the tetO7 promoter region (excised from pCM252) was cloned together with either the Nourseouthricin (Nat) resistance gene (excised from pFA6a-NATMX6) or Kanamycin (Kan) resistence (excised from pFA6a-KanMX6) in the pBluescriptII. The 5' and 3' flanking regions of the *HIS3* gene were amplified from W303 genomic DNA, using primers adding PmeI restriction site to each region and cloned upstream and downstream of the above described cassette. The resulting plasmids pMK121 or pMK123 can be digested with PmeI to create cassette with ends homologous to the *HIS3* flanking regions. The Ribosys reporter gene was cloned downstream of the tetO7 promoter using EcoRI and NotI restriction sites. For pMK121 and pMK123 see Figure 1 and fully annotated sequences (GenBank format) in Supplementary material.

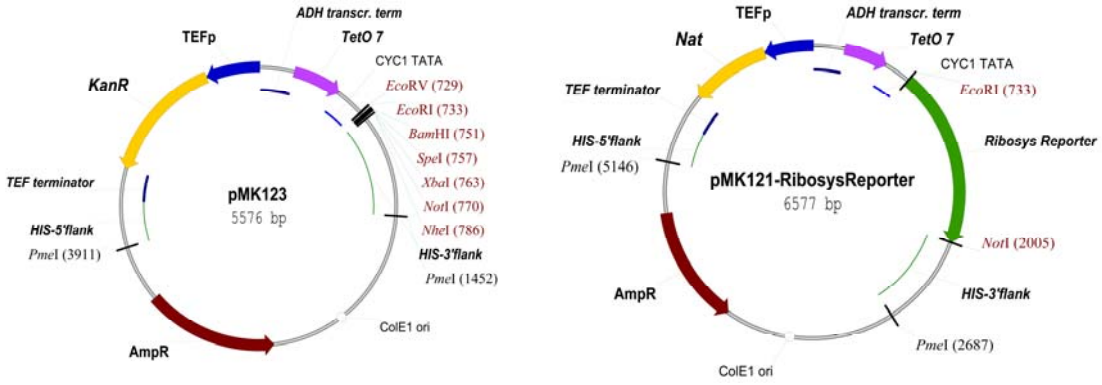

Figure 1. Plasmids pMK123 and pMK121 with integrated Ribosys reporter.
The gene of interest can be cloned into the multiple cloning site downstream of the Tet07-CYC1 promoter, as shown here for pMK121. The plasmid is then digested with PmeI and the relevant DNA fragment is introduced into budding yeast cells, selecting for KanR (pMK123) or Nat (pMK121), where it should integrate at the *his3* locus by homologous recombination. The complete sequences of pMK121 and pMK123 are provided as GenBank files.

Table S1: Yeast Strains.

Strain	Genotype	Comment
CEN-PK2	*MAT*a, *his3Δ1, leu2-3,-112, trp1-289, ura3-52, MAL2-8C, SUC2*	parent strain
BY4741	*MAT*a, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	parent strain
W303	*MAT*a, *his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	parent strain
YMK118	lys2*:: tTA, ura3::*P_{CMV}*tetR'-SSN6 URA3-K.l.*otherwise as CEN-PK2	tetOFF derived from CEN-PK2
YMK119	lys2*:: tTA, leu2::*P_{CMV}*tetR'-SSN6 URA3-K.l.*otherwise as BY4741,	tetOFF derived from BY4741
YMK120	lys2*:: tTA, ura3::tetR'-SSN6 URA3-K.l.*otherwise as W303	tetOFF derived from W303
YIK120	*Mat***a***, ade 2-1, trp1-1, leu2-3,-112, his3-11,-15, can1-100* *lys2:: tTA, ura3-1::tetR'-SSN6, Hph* W303 plus tTA in *LYS2*, *tetR'-SSN6, Hph* in *ura3*	tetOFF *ura3,* derived from YMK120 by replacing *URA3* with *Hph*
YMK156	W303, rtTA-M2 in lys2	
YRA1	*his3::Ribo1-Nat* otherwise as YIK120	tetOFF Ribo1 *
YRA2	*his3::ILRibo1-Nat* otherwise as YIK120	tetOFF ILRibo1*
YRA3	*his3::5'SSRibo1-Nat* otherwise as YIK120	tetOFF 5'SSRibo1*
YRA4	*his3::3'SSRibo1-Nat* otherwise as YIK120	tetOFF 3'SSRibo1*
YIK91	W303, *leu2::*P_{adh1}*-tetR-SSN6-LEU2*, p414(*CEN,TRP1*, P_{ADH1}*-tTA'*)	tetON derived from W303
YIK91/Ribo1	*his3::Ribo1-Nat* otherwise as YW561	tetON Ribo1*
YIK91/ILRibo1	*his3::ILRibo1-Nat* otherwise as YW561	tetON ILRibo1*
YIK91/5'SSRibo1	*his3::5'SSRibo1-Nat* otherwise as YW561	tetON 5'SSRIbo1*
YIK91/3'SSRibo1	*his3::3'SSRibo1-Nat* otherwise as YW561	tetON 3'SSRibo1*

[a] These were derived from YIK120 or YIK91 by integration of the corresponding Pme1-linearized pMK121 plasmid containing the Ribo1 reporters under control of $tetO_7$/*CYC1-UAS* promoter into the *his3* locus (*his3:: RiboReporter-Nat*).

Table S2: Plasmid used in this work.

Plasmid	Features	Source
pAG32	AmpR, *hgh* gene from *Klebsiella pneumoniae*	Euroscarf
pBS1539	*URA3 K. lactis*	(Rigaut et al. 1999)
pCM173	CEN TRP1, tetR'-VP16	Belli 1998; obtained from http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/tet_plas.html
pCM225	*CEN, TRP1,* P_{CMV} *tTA(tetR-VP16), tetO7-CYC1*	
pCM242	*CEN,* P_{CMV1}*(tetR-SSN6):LEU2*	
pCM244	*CEN,* P_{CMV1}*(tetR'-SSN6):LEU2*	
pCM247	*CEN,* P_{adh1}*(tetR-SSN6):LEU2*	
pCM252	*CEN TRP1,* P_{CMV1}*tTA'(tetR'-VP16), tetO7-CYC1*	
pFA6a-NATMX6		(Van Driessche B. et al. 2005)
pFA6a-KanMx6		(Wach et al. 1994)
pMK119-Lys	pBluescriptII(SK-) EcoRV- 5'flank*LYS2*-tTA-3'flank*LYS2* -EcoRV	This work
pMK120-Lys	pBluescriptII(SK-) EcoRV- 5'flank*LYS2*-tTA'-3'flank*LYS2* -EcoRV	This work
pMK121	pBluescriptII(SK-) PmeI-5'flankHIS3-KanR-$P_{tetO7\text{-}CYC1}$-MCS-3'flankHIS3-PmeI.	This work (see Fig. S3)
pMK123	pBluescriptII(SK-) PmeI-5'flankHIS3-NAT-$P_{tetO7\text{-}CYC1}$-MCS-3'flankHIS3-PmeI.	This work (see Fig. S3)
pMK135	pBluescriptII(SK-) PmeI-5'flankURA3-P_{CMV}*(tetR-SSN6)-URA3*-K.l.-3'flankURA3-PmeI.	This work
pMK136	pBluescriptII(SK-) PmeI-5'flankURA3-P_{CMV}*(tetR'-SSN6)-URA3*-K.l.-3'flankURA3-PmeI.	This work
pMK154	pBluescriptII(SK-) EcoRV- 5'flank*LYS2*-rtTA-M2-3'flank*LYS2* -EcoRV	This work
p414ADH1	pRS414(*CEN,TRP1*, P_{ADH1}')	(Mumberg et al. 1995)

Table S3. Sequences of oligos used as primers and probes to quantify RNAs.

Oligos	Sequence 5’ to 3’
3HA probe	CATAGTCCGGGACGTCATAG
ACT1 probe	TCTTGGTCTACCGACGATAGATGGGAAGACAGCA
Exon2F	CCCATGTCTCTACTGGTGGTG
Exon2R	CATAGTCCGGGACGTCATAGG
m2_F	TTCGGGGGATCGACGGTAT
m2-R	CCAAAGAAGCACCACCACCA
p2_F	AATTCGGGGGATCGACGGTA
p2_R	GGTGCAAGCGCTAGAACATACCA
L1-F	AGAAGGGCCCTAATCTCGATGG
L1-R	GCAAGCGCTAGAACATACATAGTACA
3’-F	CGATTGCTTCATTCTTTTTGTTGC
3’-R	CCTGGCAATTCCTTACCTTCCA
E2-F	TGGTGGTGGTGCTTCTTTGG
E1-R*	CCCGCATAGTCAGGAACATCG
At Lsm-F	CTCGCTTCCCTCTCCACCTTTT
At Lsm-R	CGGTTCTAGAAGGTGCGTGTGA
Prem-T7	CTAATACGACTCACTATAGGGAGAATTCGGGGGATCGACGGTA
Lar-T7	CTAATACGACTCACTATAGGGAGAAGGGCCCTAATCTCGATGG
At Lsm-T7	CTAATACGACTCACTATAGGGAGACTCGCTTCCCTCTCCACCTTTT
3’cleavage# assay primer	TTAAACTTAAAATACGCTGACCCGACA

*E1-R was also used as primer for cDNA synthesis in most experiments.
#The 3’ cleavage assay primer maps downstream of the most distal polyA site.

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