Synthetic Toolkit for Complex Genetic Circuit Engineering in *Saccharomyces cerevisiae*

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Supplementary figures:

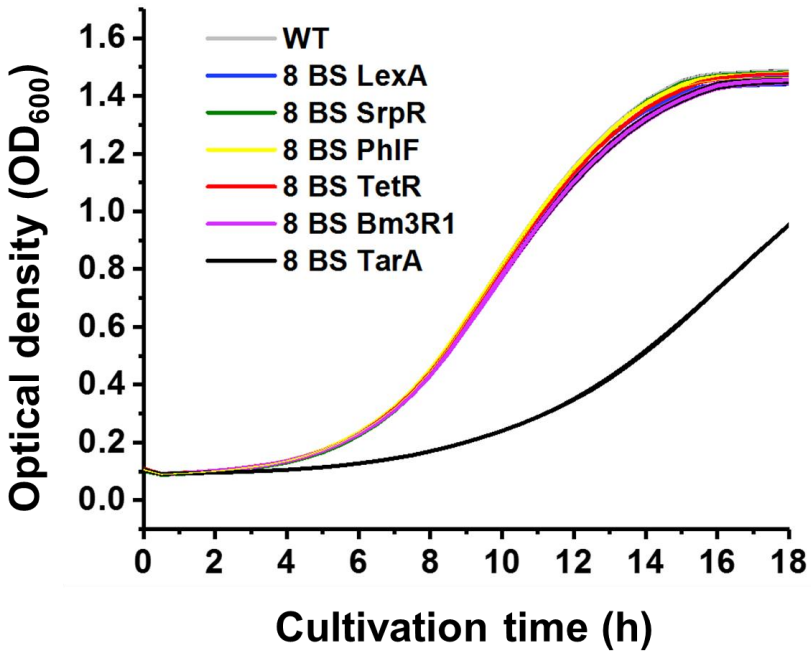
Figure S1: Orthogonality matrix values, growth curves of the sTF strains, and sequence alignments of selected binding sites

A

Venus fluorescence (AU) – numerical values

			sTF					
		Wo sTF	LexA	SrpR	PhlF	TetR	BM3R1	TarA
Binding sites (8 BS)	TarA	4.9 ± 0.1	4.8 ± 0.2	9.7 ± 0.5	5.4 ± 0.1	5.1 ± 0.2	4.7 ± 0.2	5514.0 ± 268.5
	BM3R1	10.6 ± 0.3	11.1 ± 0.8	11.6 ± 0.2	11.8 ± 0.0	10.7 ± 0.9	7079.3 ± 293.5	12.0 ± 0.9
	TetR	7.1 ± 0.3	7.2 ± 0.2	7.0 ± 0.4	7.2 ± 0.1	5953.7 ± 136.7	7.3 ± 0.2	5.4 ± 0.3
	PhlF	4.8 ± 0.3	4.6 ± 0.1	2.9 ± 0.1	3745.3 ± 139.7	4.9 ± 0.2	4.9 ± 0.1	3.4 ± 0.0
	SrpR	10.1 ± 1.0	46.4 ± 2.4	3424.7 ± 186.7	12.9 ± 0.3	24.5 ± 2.7	10.8 ± 0.1	79.4 ± 8.1
	LexA	9.0 ± 0.4	3517.5 ± 103.1	8.1 ± 0.7	8.3 ± 0.2	8.5 ± 0.6	8.4 ± 0.2	6.3 ± 0.3

B



C

Sequence alignment of SrpR- and TarA-binding sites

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SrpR-BS       1 GTTTACAAACAAACAAGCATGTATGTATAT
TarA-BS3      1 ------AAACATACCTCACGGTATGTTC--
TarA-BS1      1 ------AAACATACCGTGTGGTATGTTC--
TarA-BS2      1 ------CAACATACCGAGTGGTATGTTC--
consensus     1       .****.**.....******..
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Sequence alignment of SrpR- and LexA-binding sites

```
SrpR-BS*      1 CTAGTATATACATACATGCTTGTTTGTTTGTAAAC
LexA-BS3      1 -CTGTATGCGCATACAG-----------------
LexA-BS1      1 -CTGTATGTACATACAG-----------------
LexA-BS2      1 -CTGTATATATATACAG-----------------
consensus     1   ..****....*****.
```

Figure S1. Orthogonality matrix values, growth curves of the sTF strains, and sequence alignments of selected binding sites

(A) Numerical values of the sTF - BS pairs orthogonality matrix presented in the **Figure 1B**. The values represent the mean Venus fluorescence of three biological replicates ±SD. **(B)** Growth curves of strains containing the sTF- and the corresponding (8 BS) reporter- cassettes. The cultivation was performed in SCD medium. Each growth curve represents the mean of five biological replicates and the line width is ±SD. **(C)** Sequence alignments of SrpR-, TarA-, and LexA-binding sites. The observed mild cross-reactivity of some sTFs and binding sites (A) was further analyzed by inspecting similarities in the sequences of the binding sites. The SrpR-BS contains partial sequence identity with the TarA-BS, which may explain the activation of the SrpR-BS-containing expression cassette by the TarA-sTF. As the SrpR-sTF require longer BS compared to the TarA-sTF, only minimal activation was detected in case of SrpR-sTF in combination with the TarA-BS-containing expression cassette. There is also partial sequences identity between the SrpR-BS extended by few adjacent base-pairs existing in the expression cassette (SprR-BS*) and the LexA-binding sites, which may explain the activation of the SrpR-BS-containing expression cassette by the LexA-sTF. The LexA-BS is however not able to recruit the SrpR-sTF. There is no sequence similarity between the TetR- and the SrpR-binding sites. The sequence alignments were done with the Multiple Sequence Alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Visualization of the alignments was done with the BoxShade online tool (https://embnet.vital-it.ch/software/BOX_form.html).

Figure S2: Tuning of the sTF expression

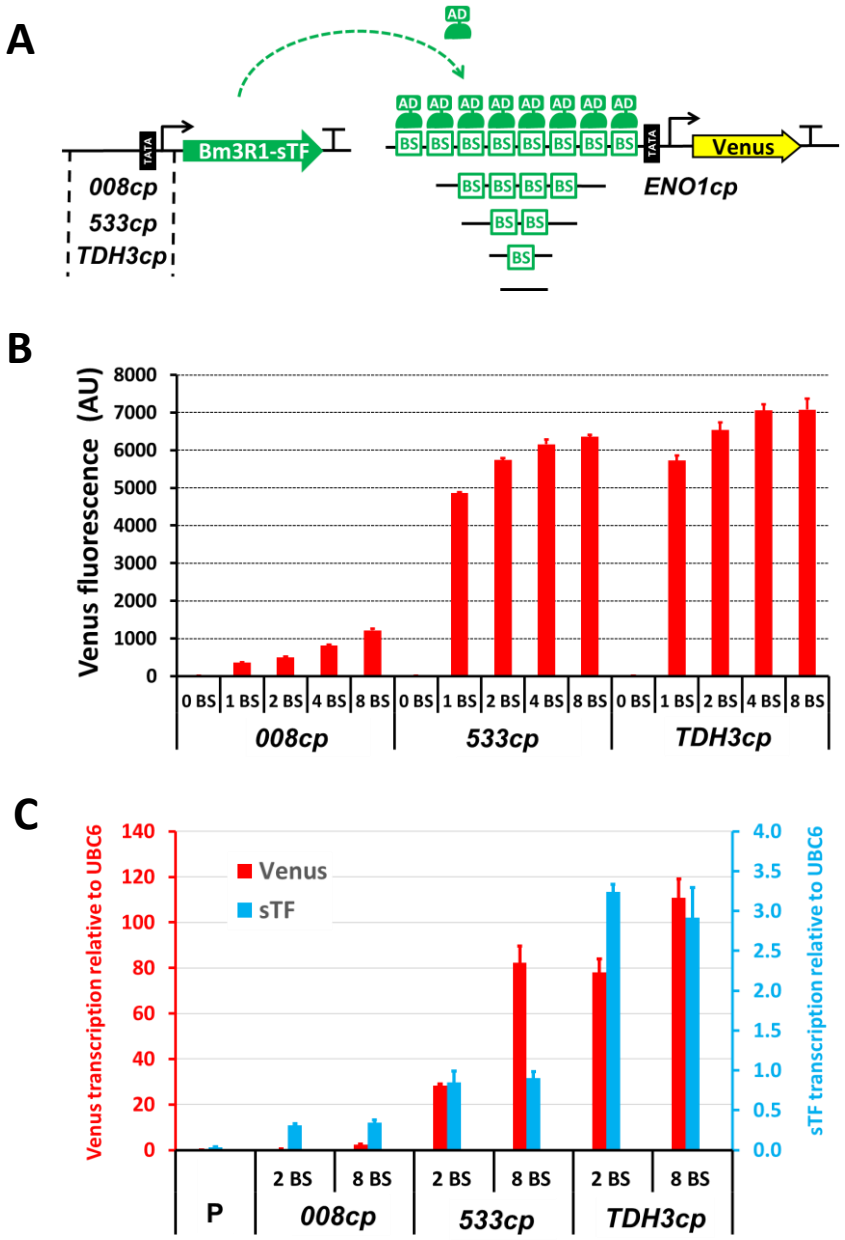


Figure S2. Tuning of the sTF expression

(A) Scheme of the expression system for testing the influence of the sTF level for transcription tuning. The system is composed of two genome-integrated DNA cassettes, where the Bm3R1-sTF is under the control of three differently strong core promoters - *008*cp, *533*cp, or *TDH3*cp. The number of the Bm3R1-specific binding sites in the promoter of the reporter gene was varied from 0 to 8. **(B)** Fluorescence analysis of the strains expressing Venus under the Bm3R1-sTF dependent promoters with varying number of sTF BSs. The values represent the mean of three biological replicates ±SD. **(C)** Transcription analysis of the selected strains (from B) containing either 2 or 8 BSs in the sTF-dependent promoters of the reporter cassettes. "P" represents a parental strain. The values represent the mean of two biological and two technical replicates ±SD.

Figure S3: Additional analysis of the DNA parts

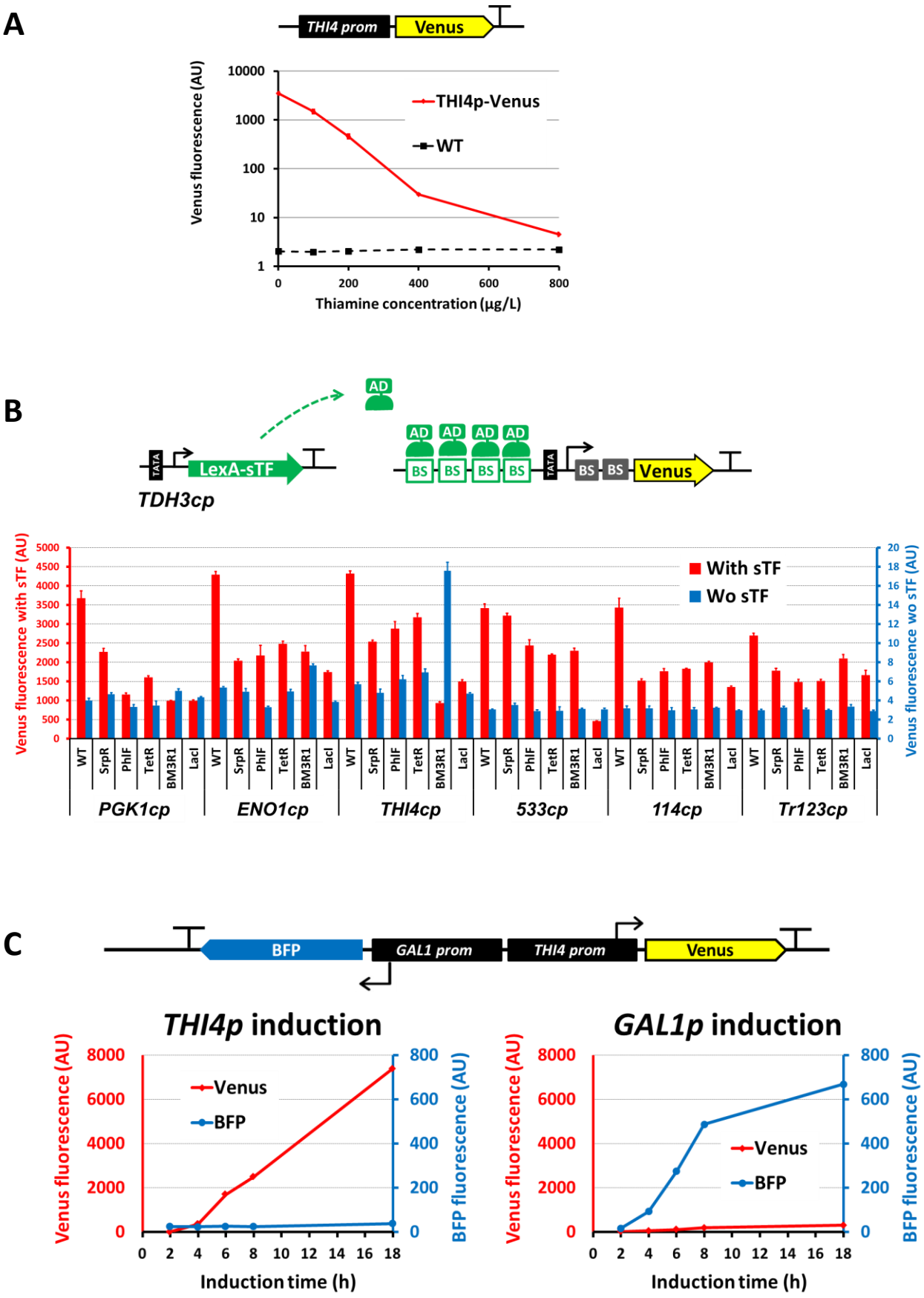


Figure S3. Additional analysis of the DNA parts

(A) Analysis of the *THI4* promoter in various concentrations of thiamine. The Venus expressed under the native *S. cerevisiae THI4*p and the resulting fluorescence measured after 18 h cultivation in SCD medium containing the indicated thiamine concentration. The WT represents the background Venus fluorescence of the parental strain. **(B)** Analysis of the repressible core promoter contribution to the Venus fluorescence level achieved by the expression systems used in the sRep development experiment (**Figure 2**). The sRep systems tested here (shown in the scheme) did not contain the sRep-cassette to assess the non-repressed activity of the system in presence of the sTF (red bars with values plotted on the left primary y-axis). In addition, the basal transcription activity of the tested core promoters was tested in the absence of sTF (blue bars with values plotted on the right secondary y-axis). WT represents the wild type core promoter without BSs for the sReps (grey boxes in the scheme). The values represent the mean of three biological replicates ±SD. **(C)** The dynamics of the bidirectional *THI4* - *GAL1* promoter driven expressions was studied in cultivations where the cells were transferred from repressing conditions (SCD with high concentration of thiamine and glucose) to inducing conditions (absence of thiamine or presence of galactose, respectively). The reporter Venus was under the *THI4*p and BFP under the *GAL1*p (bidirectional cassette).

Figure S4: Bistable switch circuit version B

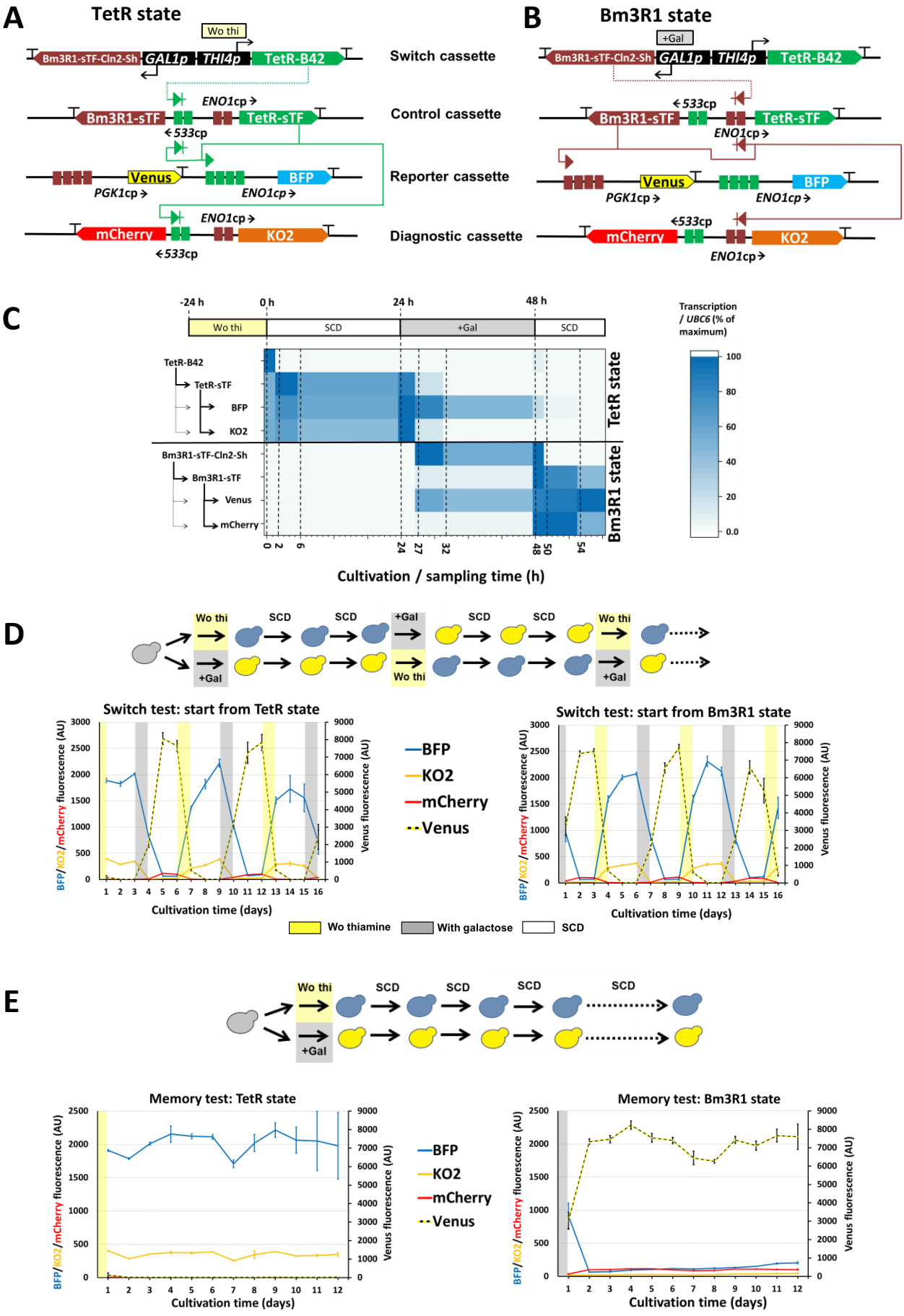


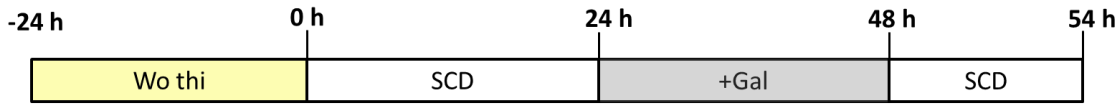
Figure S4. Bistable switch circuit version B

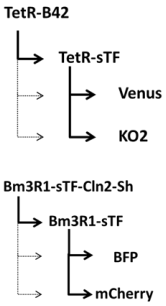
Schemes of the version B of the bistable switch circuit in the TetR state **(A)** and the Bm3R1 state **(B)**. The system is identical to the bistable switch version A shown in **Figure 3A-B**, with two main exceptions: First, the core promoters in the reporter cassette are non-repressible and instead the wild type versions of *PGK1*cp and *ENO1*cp are used. Second, the expression of Venus is activated in the Bm3R1 state and not in the TetR-state. Transcriptional activation is indicated by a filled arrow. The vertical line in front of the arrow indicates simultaneous repression to other direction. An open arrow next to core promoters (CPs) indicates the direction of transcription activation facilitated by the core promoter. **(C)** Time course transcription analysis of the bistable switch during the transition between the states. The cells were cultivated as shown in **Figure 3C**. The version B showed similar expression profile dynamics as the version A with a notable difference in the behaviour of the reporter genes (BFP and Venus). During the switch from the TetR- into the Bm3R1-state in the presence of galactose, the lack of active repression in the reporter cassette results in slow, prolonged deactivation of the BFP expression (reporter of the TetR state), likely caused by the residual amount of the TetR-sTF in the cells. In addition, an instant upregulation of the Venus expression (reporter of the Bm3R1 state) is seen as a consequence of no repression by the residual TetR-sTF. In the heat map, the values are relative to the maximum transcript level of each gene (in percent). The value 100 % corresponds the highest measured signal in the time course. The *UBC6* transcription was used as a reference value in the analysis. The shown values are the mean values of two biological and two technical replicates. The absolute (numerical) values from this analysis are listed in the **Supplementary Figure S5B**. **(D)** The system was switched several times between the TetR- and Bm3R1-states by cultivating cells for 24 hours in the inducing conditions, in the absence of thiamine (yellow shading) or in the presence of galactose (grey shading), followed by a 48 h cultivation in non-inducing conditions (SCD). Each cultivation was diluted every day to a fresh medium. The colour of the yeast cells in the scheme represents the main fluorescent protein expressed in each state, BFP (blue) or Venus (yellow). The fluorescence values in the graphs represent the mean of four biological replicates ±SD. **(E)** A memory test of the bistable switch. Either TetR- or Bm3R1-state was switched on, followed by cultivation in non-inducing SCD medium for 11 days (culture diluted in fresh medium every day). The fluorescence values represent the mean of four biological replicates ±SD.

Figure S5: Bistable switch time-course transcription values

Bistable switch version A

A



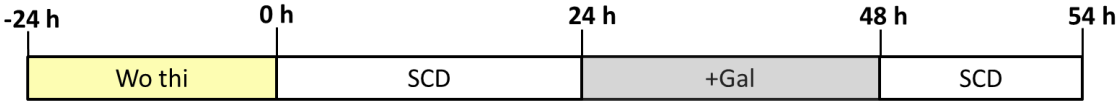


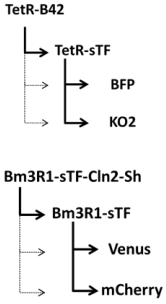
Ver A transcription relative to *UBC6* (avg ± SD)

Gene	Cultivation time (h)								
	0	2	6	24	27	32	48	50	54
TetR-B42	49.86 ± 11.17	0.49 ± 0.06	0.07 ± 0.02	0.22 ± 0.09	0.32 ± 0.06	0.34 ± 0.01	3.36 ± 0.66	0.19 ± 0.04	0.04 ± 0.01
TetR-sTF	5.32 ± 0.93	7.07 ± 0.88	4.27 ± 0.77	7.78 ± 2.19	1.86 ± 0.3	0.12 ± 0.04	0.03 ± 0.01	0.06 ± 0.02	0.06 ± 0.01
Venus	39.7 ± 5.57	60.81 ± 5.54	50.86 ± 5.29	38.57 ± 5.38	24.69 ± 5.51	2.00 ± 0.12	7.59 ± 1.85	3.18 ± 1.03	4.3 ± 1.43
KO2	8.82 ± 1.3	10.23 ± 2.18	5.43 ± 0.62	13.41 ± 3.19	2.5 ± 0.34	0.34 ± 0.05	0.14 ± 0.03	0.07 ± 0.03	0.08 ± 0.01
Bm3R1-sTF-Cln2-Sh	0.52 ± 0.04	0.05 ± 0.02	0.04 ± 0.01	0.17 ± 0.05	31.39 ± 2.12	25.96 ± 7.37	21.33 ± 3.61	0.66 ± 0.03	0.08 ± 0.03
Bm3R1-sTF	0.01 ± 0	0 ± 0	0 ± 0	0.01 ± 0	0.5 ± 0.06	0.38 ± 0.03	5.71 ± 0.63	3.55 ± 0.48	2.2 ± 0.57
BFP	3.33 ± 0.75	4.06 ± 0.34	4.8 ± 0.93	4.24 ± 1.04	4.4 ± 0.74	5.07 ± 0.73	70.71 ± 3.21	91.3 ± 16.9	61.15 ± 29.68
mCherry	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.17 ± 0.07	0.15 ± 0.02	3.55 ± 0.65	3.07 ± 0.35	1.79 ± 0.26

Bistable switch version B

B





Ver B transcription relative to *UBC6* (avg ± SD)

	Cultivation time (h)								
	0	2	6	24	27	32	48	50	54
TetR-B42	40.26 ± 6.66	0.75 ± 0.23	0.05 ± 0.01	0.13 ± 0.01	0.5 ± 0.04	0.39 ± 0.04	3.7 ± 1.2	0.21 ± 0.06	0.03 ± 0.01
TetR-sTF	4.08 ± 0.24	7.87 ± 0.61	4.93 ± 0.17	6.63 ± 2.67	1.54 ± 0.27	0.11 ± 0.06	0.03 ± 0.01	0.09 ± 0.03	0.1 ± 0.04
BFP	82.2 ± 14.09	120.05 ± 33.52	91.97 ± 20.96	157.85 ± 30.02	127.83 ± 16.12	75.98 ± 6.38	35.82 ± 6.9	9.99 ± 3.06	2.47 ± 0.13
KO2	7.37 ± 0.63	8.93 ± 1	6.31 ± 0.89	13.8 ± 3.09	1.9 ± 0.13	0.31 ± 0.02	0.14 ± 0.05	0.12 ± 0.03	0.11 ± 0
Bm3R1-sTF-Cln2-Sh	0.67 ± 0.13	0.05 ± 0.01	0.03 ± 0.01	0.26 ± 0.03	31.02 ± 4.22	17.3 ± 1.8	30.74 ± 9.34	0.6 ± 0.09	0.08 ± 0.02
Bm3R1-sTF	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0	0.55 ± 0.22	0.47 ± 0.08	5.03 ± 0.68	3.91 ± 0.51	2.24 ± 1.05
Venus	1.13 ± 0.13	0.72 ± 0.19	0.5 ± 0.04	1.62 ± 0.03	70.85 ± 4.01	54.34 ± 7.15	99.7 ± 30.19	103.86 ± 24.11	119.4 ± 42.64
mCherry	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.21 ± 0.05	0.15 ± 0.02	3.28 ± 0.11	3.29 ± 0.39	1.72 ± 0.75

Figure S5. Bistable switch time-course transcription values

Numerical values of the transcription analysis of the bistable switch version A **(A)**, or version B **(B)**. These values were used for calculating the heat-map visualizations of the experiments shown on the **Figure 3C** and **Supplementary Figure S4C**. The values represent the mean values of two biological and two technical replicates ±SD. *UBC6* was used as a reference gene.

Figure S6: Flow cytometry analysis of the bistable switch

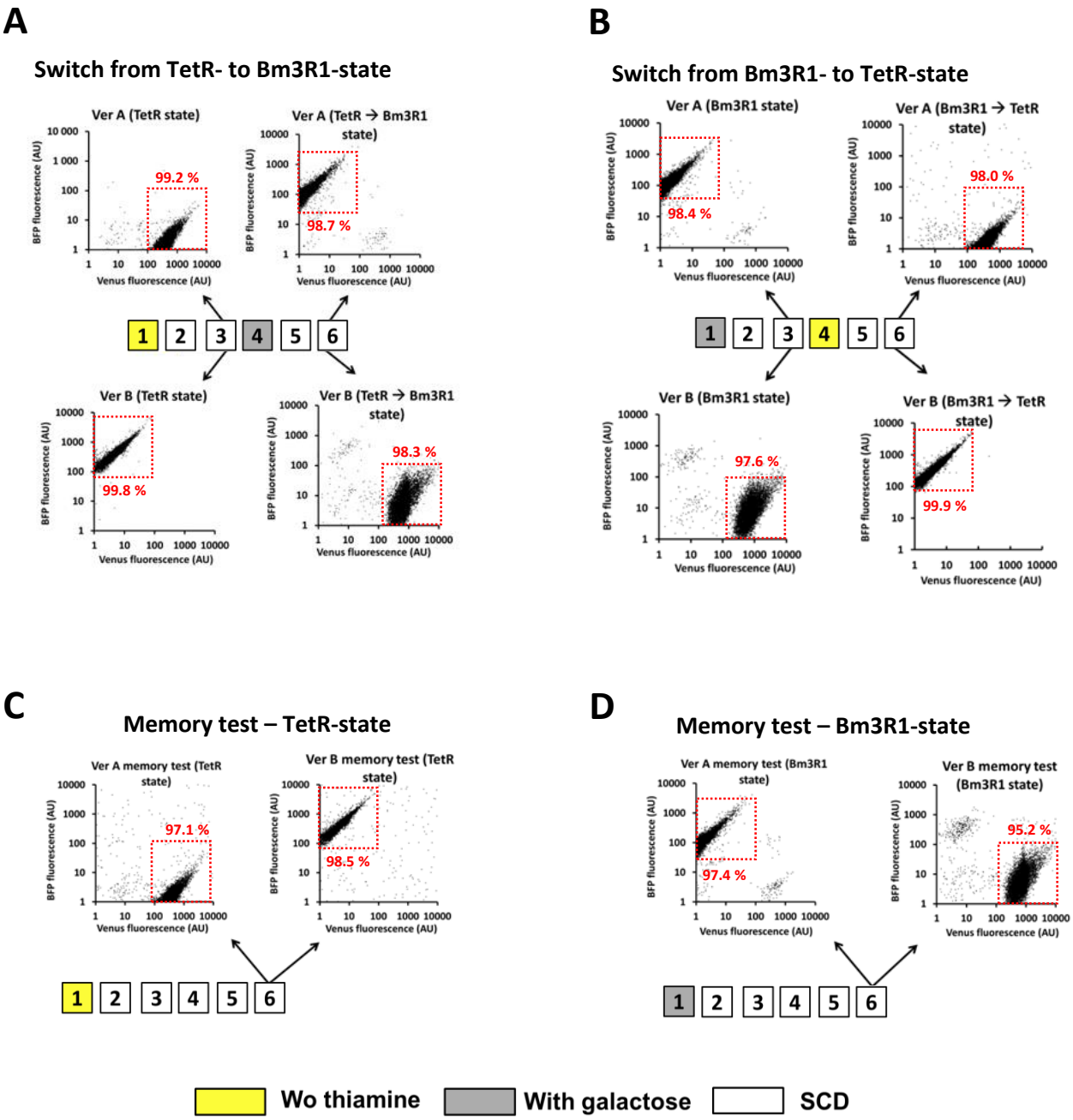


Figure S6. Flow cytometry analysis of the bistable switch

Flow cytometry analysis of the reporter fluorescence was performed at selected time-points for strains containing the bistable switch version A (Ver A) or version B (Ver B). In each plot, a population of 10,000 cells is shown from the cultures indicated in the schemes. The red rectangles represent gated areas containing cells with a fluorescence pattern corresponding to the expected bistable switch state, and the fraction of the cells conferring the correct behaviour is shown as a percent of whole population. **(A, B)** The analysis from testing of serial transitions between the states is shown in the **Figure 3D** (for version A) and **Supplementary Figure S4D** (for version B). **(A)** The cultures were initially synchronized in the TetR-state (yellow box) followed by a 2 day cultivation in the non-inducing medium (white boxes), after which the switch to the Bm3R1-state (grey box) was performed, again followed by a 2 day cultivation in the non-inducing medium. Flow cytometry was performed at days 3 and 6. **(B)** The cultures were initially synchronized in the Bm3R1-state (grey box) followed by a 2 day cultivation in the non-inducing medium (white boxes), after which the switch to the TetR-state (yellow box) was performed, again followed by a 2 day cultivation in the non-inducing medium. The flow cytometry was performed at days 3 and 6. **(C, D)** The analysis of samples from the memory test are shown in the **Figure 3E** (for version A) and **Supplementary Figure S4E** (for version B). The induction either to the TetR-state **(C),** or the Bm3R1-state **(D)** was performed in the absence of thiamine (yellow box) or the presence of galactose (grey box), respectively. The cultivations were continued in the non-inducing medium (white boxes), and the flow cytometry analysis was carried out at day 6 of the cultivation.

Figure S7: Bistable switch with violacein pathway – additional data

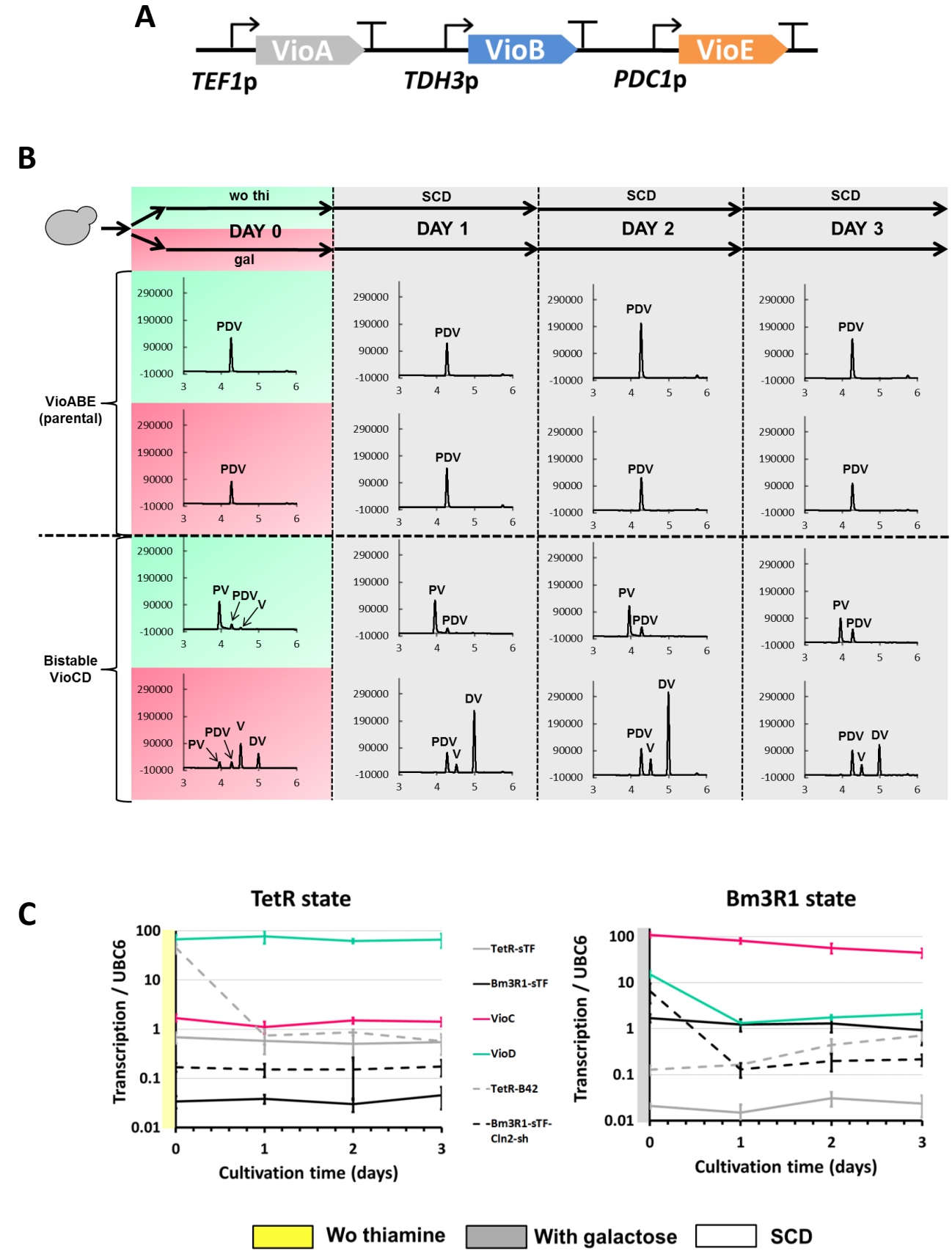


Figure S7. Bistable switch with violacein pathway – additional data

(A) A scheme of the DNA cassette encoding the first three steps of the violacein pathway (VioA, VioB, and VioE enzymes). This cassette was integrated into the genome of the *S. cerevisiae* strain forming the VioABE background strain. **(B)** The UPLC analysis of the intracellular violacein pathway metabolites in the VioABE background strain and in the strain containing, in addition, the bistable switch to control *vioC* and *vioD* genes (Bistable VioCD) shown in the **Figure 4B**. Both of the strains were cultivated in the absence of thiamine (wo thi, green box) or in the presence of galactose (gal, pink box), followed by a 3 day cultivation in the non-inducing SCD medium (days 1-3, grey boxes). All cultivations were diluted to a fresh medium every 24 h. The intracellular violacein pathway metabolites were analysed after 24, 48 and 72 h cultivation in non-inducing conditions. Prodeoxyviolacein (PDV), proviolacein (PV), deoxyviolacein (DV) and violacein (V) were analyzed. **(C)** Transcription analysis of the yeast strain containing the bistable switch controlling the expression of violacein pathways genes *vioC* and *vioD* during the cultivation described in (B). The values represent the mean of two biological and two technical replicates ±SD.