Supplementary Information:

Structure of the mathematical model. To validate the design, to modify the experimental setup, and to finally confirm hypotheses derived from experimental results, we implemented a semi-quantitative mathematical model of the system based on ordinary differential equations (ODEs). The model takes into account each molecular species actively involved in the network and the basic structure is equivalent to a previously published model (1). Most parameters were found in the literature or extracted from previous work (1) while some others were estimated by minimizing the error with the experimental data within a constrained range of values. We established the model by considering the production and degradation of the two transactivators (tTA and ET1), the fluorescent protein d2EYFP, and the interactions between these components (Fig. 1b). In particular, the model was set up to capture all relevant time delays in the system (for instance, caused by maturation of d2EYFP) because these delays are critical for oscillator performance. Consequently, we considered transcription and translation processes individually, and mechanistically captured all corresponding degradation reactions, including the degradation of TAG_{LUC}-tTA mRNA catalyzed by the RISC/siRNA complexes. In addition, the time delay for forming active d2EYFP by protein folding is modeled explicitly. We derived a semi-quantitative model based on nine ordinary differential equations (ODEs) that represent the concentration dynamics of the following components: TAG_{LUC}-tTA mRNA (T_m), tTA protein (T_p), ET1 mRNA (E_m), ET1 protein (E_p), $siRNA_{LUC}$ (H_m), active RISC/siRNA complex (H_a), d2EYFP mRNA (G_m), d2EYFP unfolded protein (G_p), d2EYFP folded protein (G_a). In addition, we used a short-hand notation, namely P1-P3, for the plasmids that encode TAG_{LUC}-tTA (P1; pND10), E-$siRNA_{LUC}$-VP16 (P2; pDG157), and d2EYFP (P3; pBP283).

The equations for the dynamic system were set up as follows:

$$\frac{dT_m}{dt} = G_1 \cdot r_T - \frac{k_{AD} \cdot T_m}{K_{TH} + T_m} \cdot H_a - k_{DT_m} \cdot T_m \qquad (1)$$

$$\frac{dT_p}{dt} = k_{TL} \cdot T_m - k_{DT_p} \cdot T_p \qquad (2)$$

$$\frac{dE_m}{dt} = G_2 \cdot r_t - k_{DE_m} \cdot E_m \qquad (3)$$

$$\frac{dE_p}{dt} = k_{TL} \cdot E_m - k_{DE_p} \cdot E_p \qquad (4)$$

$$\frac{dH_m}{dt} = G_2 \cdot r_T - \left(k_{DR} + k_{DH_m}\right) \cdot H_m \qquad (5)$$

$$\frac{dH_a}{dt} = k_{DR} \cdot H_m - k_{DH_a} \cdot H_a \qquad (6)$$

$$\frac{dG_m}{dt} = G_3 \cdot r_e - k_{DG_m} \cdot G_m \quad (7)$$

$$\frac{dG_p}{dt} = k_{TL} \cdot G_m - \left(k_{DG_p} + k_f\right) \cdot G_p \quad (8)$$

$$\frac{dG_a}{dt} = k_f \cdot G_p - k_{DG_a} \cdot G_p \quad (9)$$

The model employs parameters G_1 - G_3 to account for different dosages of the three plasmids P1-P3. The kinetics of gene expression are captured by Hill function-based rate laws r_T and r_E for tTA- and ET1-controlled transcription, respectively (see Eqs. (12) and (13) below), and parameters G_1 - G_3 have the dimension of plasmids / cell. The literature presents different ways of modeling siRNA dynamics (2-5). In order to develop a comprehensive but precise model, we introduced two siRNA-related state variables (see Eqs. (5) and (6)): the first state represents the unprocessed siRNAs (H_m), while the second state reflects the concentration of the active forms (H_a) after the siRNAs have been cleaved by a ribonuclease, Dicer, and bound to the RNA-induced silencing complex (RISC) at a rate k_{DR}. The degradation rates of the unprocessed and of the active form (k_{DHm}/k_{DHa}) are different because the active form, after binding to RISC, is more stable. Finally, the active form acts directly on the LUC-tagged mRNA (T_m) by catalyzing the degradation reaction at a rate k_{AD}. To model the effects of antibiotics as the control inputs for the system, one has to distinguish between active and inactive transactivators. Consequently, we denote the concentrations of active transactivators tTA and ET1 by T_a and E_a, respectively. They are proportional to the corresponding total protein concentrations (identified by subscripts p in the ODE system). Michaelis-Menten like inactivation rate laws (see experimental dose-response curves in (6) for tTA and in (7) for ET1) capture the interactions between the regulators and the small molecules tetracycline, Tc, and erythromycin E_c, respectively:

$$T_a = T_p \cdot \left(1 - \frac{T_c}{K_{m_T} + T_c}\right) \quad (10)$$

$$E_a = E_p \cdot \left(1 - \frac{E_c}{K_{m_E} + E_c}\right) \quad (11)$$

To describe gene expression of tTA- and ET1-controlled components, we employed formal reaction rate laws typically used in synthetic biology (8):

$$r_T = k_T \cdot \left[\alpha_T + (1 - \alpha_T) \cdot \frac{{T_a}^{n_T}}{{K_{M_T}}^{n_T} + {T_a}^{n_T}}\right] \cdot f_v \quad (12)$$

$$r_E = k_E \cdot \left[\alpha_E + (1-\alpha_E) \cdot \frac{{E_a}^{n_E}}{{K_M}_E^{n_E} + {E_a}^{n_E}}\right] \cdot f_v \qquad (13)$$

Here, the maximal rate constants k_T and k_E, respectively, have the dimension of number of mRNA molecules transcribed per minute and gene copy. Basal transcriptional activities are accounted for by the parameters α_T and α_E. Controlled gene expression follows Hill-type dose-response curves with promoter-transactivator affinities K_{MT} and K_{ME}, and cooperativity (Hill) coefficients n_T and n_E, respectively. The parameter f_V achieves the conversion of mRNA copy numbers to cellular mRNA concentrations in typical mammalian cells.

Initial Model Parametrization. For model parametrization, we relied on experimentally determined kinetic parameters and on total protein concentrations from the literature as far as possible. However, this approach did not lead to full parametrization of the model. Subsequently, to reduce the number of free parameters, we used the following simplifying assumptions:

i. Transfection efficiencies for all plasmids are equal and, thus, only the plasmid concentrations during transfection determine the cellular gene dosages.
ii. Degradation rates for TAG_{LUC}-tTA, ET1, and d2EYFP mRNAs are similar.
iii. Maximal activation of gene expression by tTA and ET1 is identical because both constructs share the same (VP16) activation domain (9).
iv. Translation rates are identical for all mRNAs. This assumption is already encoded in the model structure and based on the similar lengths of all proteins involved (in the range of 400-500 amino acids).

Parameter estimation. With the above simplifications, the degrees of freedom for parametrization reduced to three siRNA-related parameters. In this low-dimensional parameter space, we could therefore use exhaustive search to estimate the siRNA processing rate (k_{DR}) and the degradation rate of their active form (k_{DHa}/k_{DMa}). The objective function for parameter estimation incorporated error terms for period and amplitude; they are the sums over each different condition (e.g., conditions with different plasmid ratios and amounts, with or without antibiotics) of the quadratic error between the model data ($\widetilde{P}_i$, $\widetilde{A}_i$) and the mean values from the experimental data ($\bar{P}_i$, $\bar{A}_i$). The errors for the period and the amplitude were weighted according to the standard deviation of the experimental data and summed up to form the final error function that had to be minimized. The resulting parameter set is compiled in Supplementary table 1.

$$E_{PA}^2 = \sum_{i \in \{condition\ set\}} \frac{\left(\tilde{P}_i - \bar{P}_i\right)^2}{\sigma_{P_i}} + \sum_{i \in \{condition\ set\}} \frac{\left(\tilde{A}_i - \bar{A}_i\right)^2}{\sigma_{A_i}} \quad (14)$$

Supplementary table 1 : Parameter values

Name	Description	Value	Comments/Reference
G_1	Gene dosage plasmid 1	{50,100,200,1000}	Tunable
G_2	Gene dosage plasmid 2	{50,100,200,1000}	Tunable
G_3	Gene dosage plasmid 3	100	Fixed
K_{mT}	Tetracycline-tTA interaction	9 nM	Calculated from (6)
K_{mE}	Erythromycin-ET1 interaction	136 nM	Calculated from (7)
k_T	Maximal transcription tetracycline-responsive promoter ($P_{hCMV\ *-1}$)	30 min^{-1}	(1)
k_E	Maximal transcription macrolide-responsive promoter (P_{ETR3})	30 min^{-1}	Assumed to be the same as for $P_{hCMV\ *-1}$
α_T	Basal activity the tetracycline-responsive promoter ($P_{hCMV\ *-1}$)	0.090	(9)
α_E	Basal activity of the macrolide-responsive promoter (P_{ETR3})	0.040	(10)
K_{MT}	tTA affinity to $P_{hCMV\ *-1}$	3.0 nM	(1)
K_{ME}	ET1 affinity to P_{ETR3}	100.0 nM	Estimated

K_{TH}	Affinity of target mRNA (TAG_{LUC}-tTA) to RISC/siRNA complex	9 nM	(11)
n_T	Cooperativity ($P_{hCMV*-1}$)	2	(4)
n_E	Cooperativity (P_{ETR3})	2	Estimated
k_{DR}	DICER cleavage and RISC/siRNA complex assembly rate	$6 \cdot 10^{-4}$ min^{-1}	Estimated
k_{AD}	Cleavage of target mRNA by RISC/siRNA complex	0.42 min^{-1}	(11)
k_f	Folding rate of d2EYFP	0.0154 min^{-1}	(1)
k_{DTm}	Degradation rate of tTA mRNA	0.0173 min^{-1}	(1)
k_{DEm}	Degradation rate of ET1 mRNA	0.0173 min^{-1}	(1)
k_{DHm}	Degradation rate of siRNA	0.0540 min^{-1}	(3)
k_{DHa}	Degradation rate RISC/siRNA complex	1.3 10^{-3} min^{-1}	Estimated
k_{DGm}	Degradation rate d2EYFP mRNA	0.0173 min^{-1}	(1)
k_{TL}	General translation rate	0.02 min^{-1}	(1)
k_{DTp}	tTA degradation rate	0.0231 min^{-1}	Half-life of 30min
k_{DEp}	ET1 degradation rate	0.0231 min^{-1}	Half-life of 30min
k_{DGp}	Degradation rate of folded/unfolded d2EYFP	0.0058 min^{-1}	Half-life of 2h

f_v	Concentration scaling factor for copies/cell	$1.85\ 10^{-3}$	(1)

Image analysis and tracking software. Unbiased time-lapse-based quantification of individual oscillating fluorescence dynamics in a population of mammalian cells required a tailored tracking and analysis software which sequentially integrates segmentation, tracking and selection.

Segmentation. Segmentation was performed using fluorescence micrographs and background subtraction. Any efficient and reliable segmentation method has to eliminate background variation. We have achieved this by computing the background of each image separately using two morphological operators (erosion and dilation). The function *imopen* from the MATLAB® image processing toolbox was directly used for this task, including the calculation of the standard deviation of background intensity levels. In order to increase the accuracy for the elimination of small artifacts, we included an additional smoothing operation (MATLAB® func. *medfilt2*). Non-zero pixels were then converted to 1 which yielded binary images. Subsequently, each object was assigned a number (MATLAB® func. *bwlabel*) by increasing its pixel value (first object → 1, second → 2, …) and specific information, such as center position, width and height and the total intensity was recorded and assigned to each object. For manual/visual control of segmentation, we calculated and drew the perimeter of each object (MATLAB® func. *bwperim*).

Tracking. Since proliferation of the mammalian cells was suppressed by cultivation at 32°C, migration was slow and random and could be tracked by a simple greedy algorithm considering the past (time = t-1) and present (time = t) positions of the cell (Supplementary Table 2). To ensure close proximity between the tracked cells, respecting the assumption about their slow motion, we set a maximal threshold on the Euclidean distance between centers at time = t and time = t-1 (max. 20 pixels). Finally, in order to track low intensity cells or cells that shut d2EYFP expression off, the coordinates of the center positions were kept in the memory for a defined number of time steps (memory strength constant).

Supplementary Table 2: Algorithm Outline

```
for t = 2:T

      for each object k
            calculate objectCenter^t_k
            calculate objectFluo^t_k
            distance = Inf;
            m = 0;
            while (distance > distance threshold)
                  m = m + 1;
                  if m > Nb object total
                        Nb object total = Nb object total + 1;
                        distance = -1;
                        m = Nb object total;
                  else
                        distance = euclidean distance between
                        objectCenter^t_k and objectCenter^t-1_m
                  end
            end
            objectFluo^1:t_m = objectFluo^1:t-1_m + objectFluo^t_k
      end

      for k = 1:Nb object total
            if (objectCenter^t-1_k exists and objectCenter^t_k does not
exist)
            disapear_k = t;
      end
      if (disapear_k - t + memory strength > 0 and objectCenter^t_k does not
exist)
            objectCenter^t_k = objectCenter^t-1_k;
      end
end
```

Selection. Since only a fraction of the cells that were successfully segmented and tracked showed the desired expression characteristics, further parameters needed to be defined:

- *touchBorders* : Number of times a cell touches a border – A cell is discarded when the cell touches the border one or more times.
- *disAppearanceNum* : Number of times that the cell disappears – A cell that exhibits signals close to or below the fluorescence detection limit too many times is more likely to have been erroneously tracked. Therefore, the threshold was set considering the expected oscillation period (short oscillation → high

threshold).

- *WidthMax/HeightMax* : Maximum width/height of one cell over time – Patches of cells or other artifacts (eg : debris, residues, air bubbles, …) could thus be eliminated.
- *MaxIntensity* : Maximum intensity of an individual cell over time – Cells exhibiting (i) a maximum intensity exceeding the saturation properties of the digital camera device or (ii) very low intensities, were discarded, by applying two fixed threshold levels.
- *ZeroNum* : Number of times a cell displays zero intensity – Enables the elimination of cells that match any of the following criteria: (i) Very late onset of d2EYFP expression, (ii) apoptosis or necrosis after short observation periods or (iii) dysfunction of the transient segmentation module.
- *JumpUp/Down* : Calculates the number of times that the fluorescence increases/decreases significantly over a specific period of time – Referring to the theoretical model predictions as well as to the obtained experimental data, we expected periods in the range of 26 hours. Therefore, large fluctuations in the fluorescence intensity in a short time period were assumed to originate from dysfunction of the cellular tracking module. This exclusion parameter eliminated the possibility of numbering two neighboring cells with identical numbers. Any huge decrease in fluorescence intensity (*jump down*) was tolerated to enable the selection of cells undergoing apoptosis or necrosis after extended observation periods. In contrast, no huge increase of fluorescent intensity (*jump up*) was permitted, therefore avoiding the risk of false-positive detection of oscillatory signals.

The software produces different files containing the main results:

>> *Output.mat* : Contains the florescence data and the values of the selection criteria for each cell.

>> *fluoPlot.fig* : Graph providing the fluorescence dynamics for individual cells – cell numbering correlates with specific positions in segMovie.

>> *segMovie.avi* : Movie of the segmented and numbered cells that enables the user to confirm correct processing.

Supplementary Figure Legends

Fig. S1. Model prediction for addition of antibiotics. Left panels show tTA (solid line), ET1 (dashed line) and active d2EYFP (dotted line) protein concentrations. Right panels show tTA mRNA (solid line), siRNA (dashed line) and RISC/siRNA complex (dotted line) concentrations. (**a**) pND10 ($P_{hCMV*-1}$→TAG_{LUC}-tTA), pDG157 ($P_{hCMV*-1}$→E-$siRNA_{LUC}$-VP16) and pBP283 (P_{ETR3}→d2EYFP), (1:1:1; 200ng/500μl tetracycline added 10h after transfection, vertical bar), (**b**) pND10 ($P_{hCMV*-1}$→TAG_{LUC}-tTA), pDG157 ($P_{hCMV*-1}$→E-$siRNA_{LUC}$-VP16) and pBP283 (P_{ETR3}→d2EYFP) (1:1:1; 200ng/500μl erythromycin added 10h after transfection, vertical bar).

Fig. S2. Model prediction for variation of plasmid dosages. Left panels show tTA (solid line), ET1 (dashed line) and active d2EYFP (dotted line) protein concentrations. Right panels show tTA mRNA (solid line), siRNA (dashed line) and RISC/siRNA complex (dotted line) concentrations. (**a**) pND10 ($P_{hCMV*-1}$→TAG_{LUC}-tTA), pDG157 ($P_{hCMV*-1}$→E-$siRNA_{LUC}$-VP16) and pBP283 (P_{ETR3}→d2EYFP), (1:1:1, no antibiotics), for experimental data see Fig. 4a, (**b**) pND10 ($P_{hCMV*-1}$→TAG_{LUC}-tTA), pDG157 ($P_{hCMV*-1}$→E-$siRNA_{LUC}$-VP16) and pBP283 (P_{ETR3}→d2EYFP), (0.5:0.5:1, no antibiotics), for experimental data see Fig. 4b, (**c**) pND10 ($P_{hCMV*-1}$→TAG_{LUC}-tTA), pDG157 ($P_{hCMV*-1}$→E-$siRNA_{LUC}$-VP16) and pBP283 (P_{ETR3}→d2EYFP), (2:2:1, no antibiotics), for experimental data see Figure 4c.

Supplementary References

1. Tigges, M., Marquez-Lago, T.T., Stelling, J. and Fussenegger, M. (2009) A tunable synthetic mammalian oscillator. *Nature*, **457**, 309-312.
2. Bartlett, D.W. and Davis, M.E. (2006) Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res*, **34**, 322-333.
3. Bergstrom, C.T., McKittrick, E. and Antia, R. (2003) Mathematical models of RNA silencing: unidirectional amplification limits accidental self-directed reactions. *Proc Natl Acad Sci U S A*, **100**, 11511-11516.
4. Malphettes, L. and Fussenegger, M. (2006) Impact of RNA interference on gene networks. *Metab Eng*, **8**, 672-683.
5. Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O. and Margalit, H. (2007) Regulation of gene expression by small non-coding RNAs: a quantitative view. *Mol Syst Biol*, **3**, 138.
6. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A*, **89**, 5547-5551.

7. Weber, W., Fux, C., Daoud-el Baba, M., Keller, B., Weber, C.C., Kramer, B.P., Heinzen, C., Aubel, D., Bailey, J.E. and Fussenegger, M. (2002) Macrolide-based transgene control in mammalian cells and mice. *Nat Biotechnol*, **20**, 901-907.
8. Kaern, M., Blake, W.J. and Collins, J.J. (2003) The engineering of gene regulatory networks. *Annu Rev Biomed Eng*, **5**, 179-206.
9. Fussenegger, M., Morris, R.P., Fux, C., Rimann, M., von Stockar, B., Thompson, C.J. and Bailey, J.E. (2000) Streptogramin-based gene regulation systems for mammalian cells. *Nat Biotechnol*, **18**, 1203-1208.
10. Weber, W., Marty, R.R., Keller, B., Rimann, M., Kramer, B.P. and Fussenegger, M. (2002) Versatile macrolide-responsive mammalian expression vectors for multiregulated multigene metabolic engineering. *Biotechnol Bioeng*, **80**, 691-705.
11. Haley, B. and Zamore, P.D. (2004) Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol*, **11**, 599-606.