A tunable dual input system for ‘on-demand’ dynamic gene expression regulation

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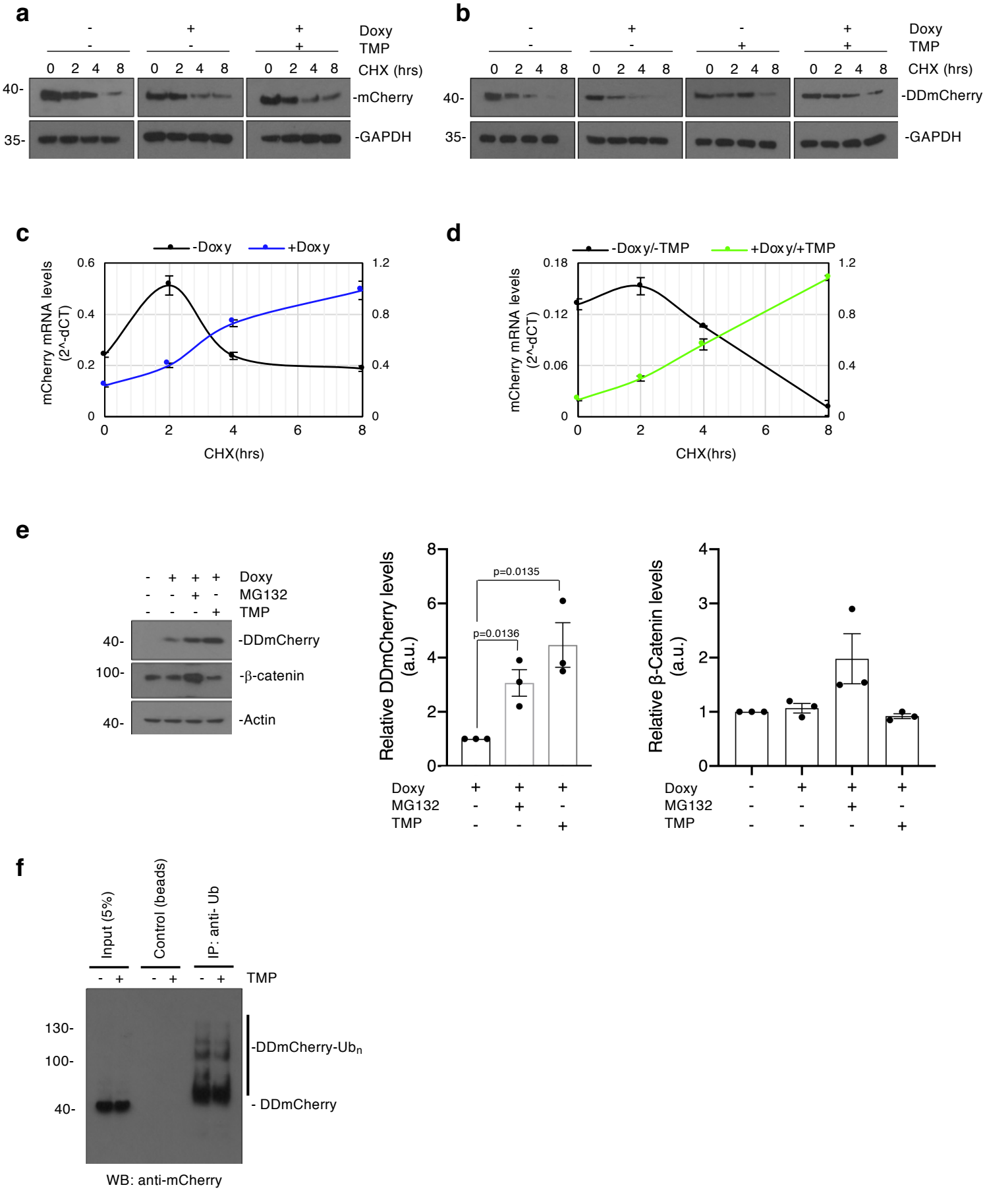


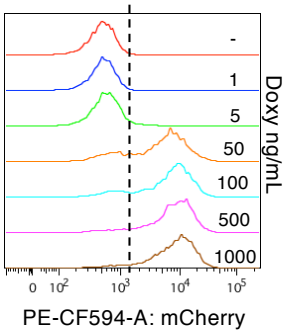
Supplementary Figure 1

Supplementary Figure 1: TMP mechanism of action

(a-d) mCherry and DDmCherry protein (a, b) and mRNA (c, d) levels measured by western-blot and qPCR, respectively, in EF1a-rtTA_TRE3G-mCherry (a, c) and EF1a-rtTA_TRE3G-DDmCherry (b, d) mESCs, treated as indicated in Fig. 1e and relative legend. (e) EF1a-rtTA_TRE3G-DDmCherry mESCs were incubated with Doxy (1000ng/mL) and either TMP (10μM) or MG132 (5μM) for 16hrs. Blocking proteosomal degradation enabled to effectively mimic the stabilising effect of TMP specifically on DDmCherry but not on endogenous β-catenin used as control. Western-blot densitometric quantifications are shown (e, inset). Plotted DDmCherry and β-catenin values are normalised against the housekeeping gene Actin. (f) Ubiquitination status analysis. EF1a-rtTA_TRE3G-DDmCherry mESCs were incubated for 24hrs with Doxy (1000ng/mL) and TMP (100nM), washed and incubated with or without TMP (100nM) for additional 12hrs. Samples were processed as indicated in the Methods, immunoprecipitated with an anti-ubiquitin antibody and blotted with the mCherry antibody to analyse the ubiquitination status of the DDmCherry protein. Note the decrease in poly-ubiquitinated species of DDmCherry when TMP is present. Data are means ± SEM (n=3, e inset). p values from two-tailed unpaired t test are shown. Source data are provided as a Source Data file.

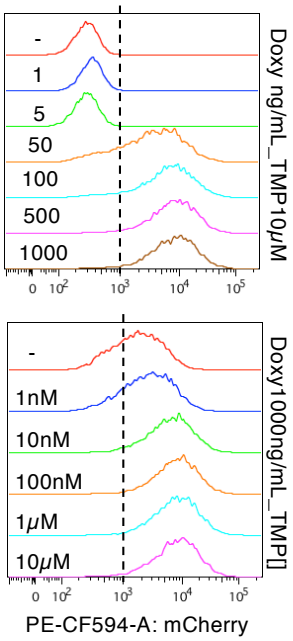
a

EF1a-rtTA TRE3G-mCherry	Doxy Titration	
Doxy	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	515±3.2	0.08±0.031
1ng/mL	539±13	0.175±0.035
5ng/mL	598.67±15.2	3.05±0.63
50ng/mL	5719.7±30.7	75.77±0.5
100ng/mL	7388.7±294.7	81.77±0.67
500ng/mL	8961.7±102.2	89.23±0.088
1000ng/mL	8987.7±76.7	89.83±0.48



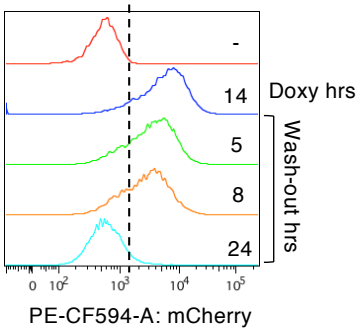
b

EF1a-rtTA TRE3G-DDmCherry		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	286.33±4.7	0.417±0.1
1ng/mL	10μM	310.67±20.2	0.23±0.08
5ng/mL	10μM	292±2.6	0.35±0.036
50ng/mL	10μM	3762.33±33.2	80.1±0.47
100ng/mL	10μM	7478.33±148.2	93±0.38
500ng/mL	10μM	9136.33±283.3	96.5±0.34
1000ng/mL	10μM	9041±224.5	97.17±0.26
1000ng/mL	-	1812±33	69.4±0.7
1000ng/mL	1nM	2829.33±83.5	81.47±0.7
1000ng/mL	10nM	6662.33±58.7	95.1±0.26
1000ng/mL	100nM	8546.7±203.7	97.1±0.15
1000ng/mL	1μM	8541±36.1	96.57±0.17
1000ng/mL	10μM	8650.33±297.4	96.53±0.03



c

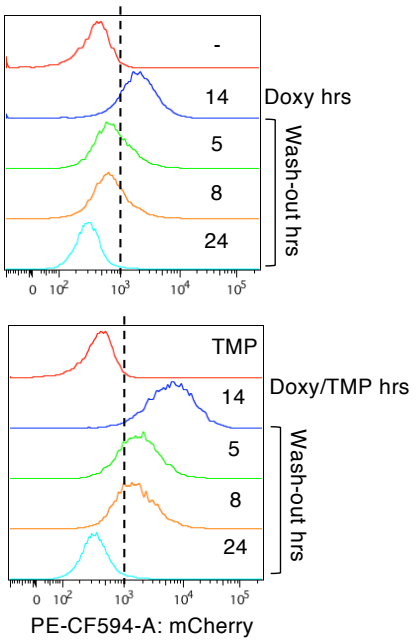
EF1a-rtTA TRE3G-mCherry	Dynamic Time-Course	
Doxy	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	482.5±2.5	0.28±0.09
14hrs 1000ng/mL	6383±328.4	84.2±1.17
5hrs wash-out	3466±204.7	70.3±1.09
8hrs wash-out	3566.5±333	69.47±2.2
24hrs wash-out	562±26.6	3.36±0.85



Supplementary Figure 2

d

EF1a-rtTA TRE3G-DDmCherry		Dynamic Time-Course	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	-	413.5±14.5	0.365±0.055
14hrs 1000ng/mL	-	1821.7±101	45.8±2.9
5hrs wash-out		785.33±36.8	11.22±0.8
8hrs wash-out		737±65.6	10.27±2.6
24hrs wash-out		316±3.6	0.61±0.07
-	100nM	408.5±9.5	0.305±0.095
14hrs 1000ng/mL	100nM	6669±214.5	89.6±0.72
5hrs wash-out		1839.33±62	46.6±1.73
8hrs wash-out		1427±38.9	33.33±1.5
24hrs wash-out		364.67±17.2	1.21±0.047



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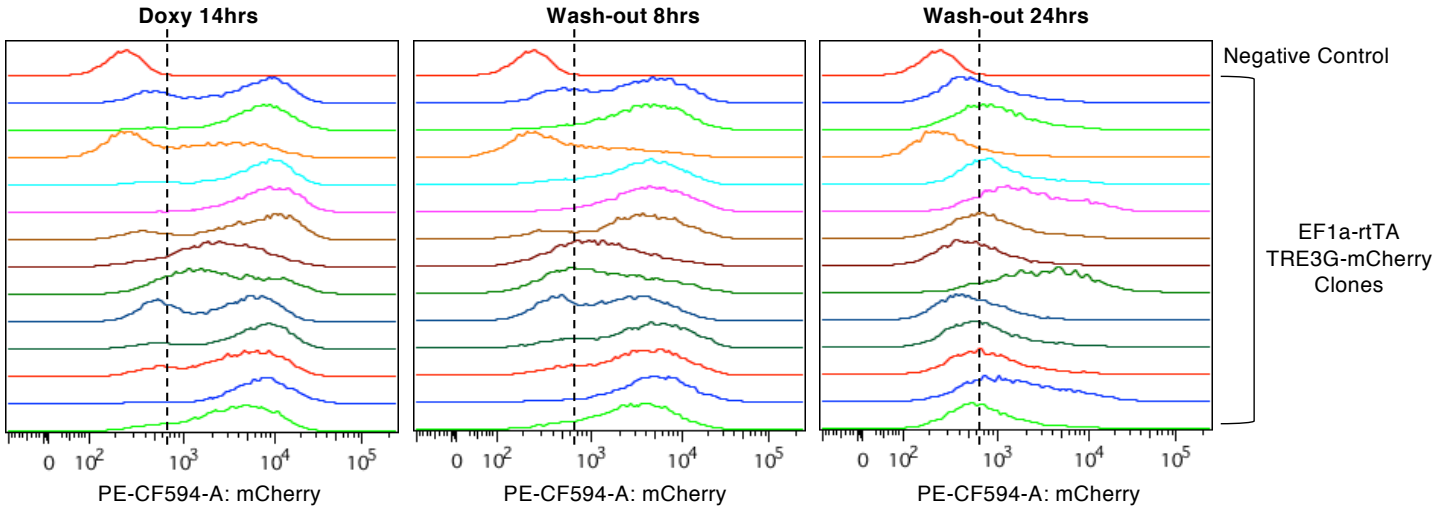

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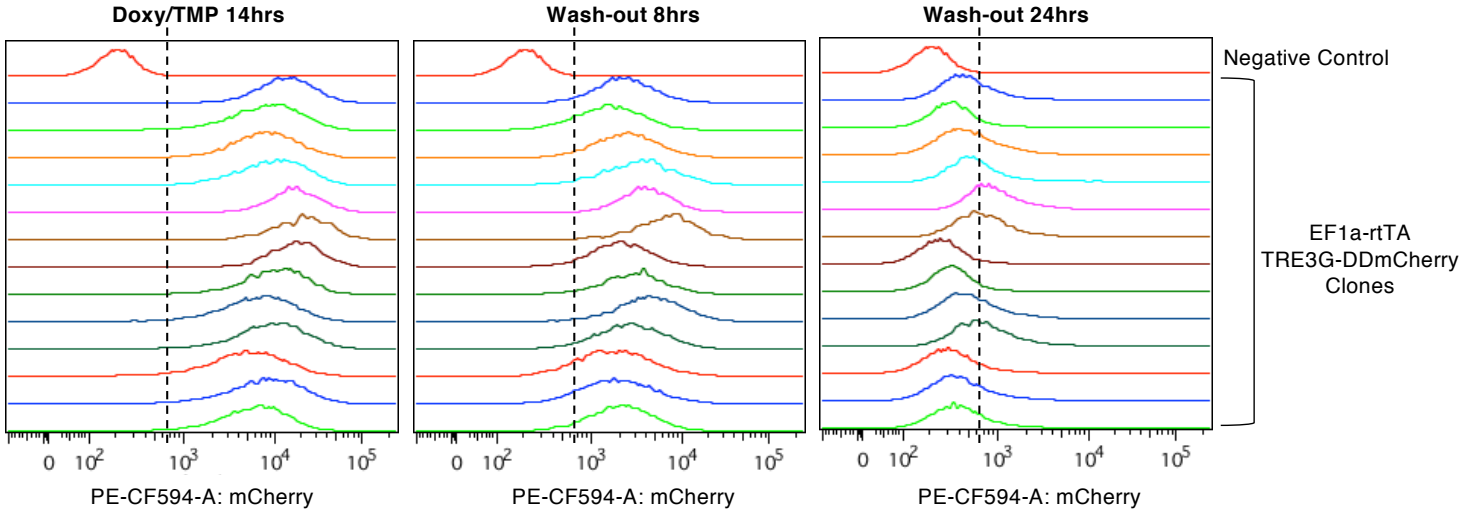
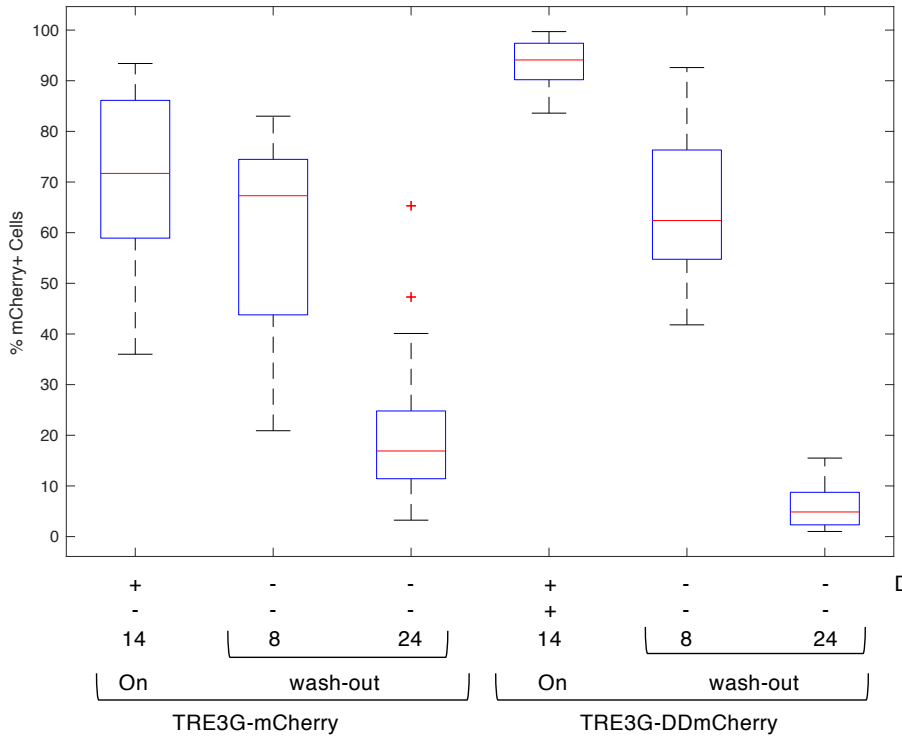


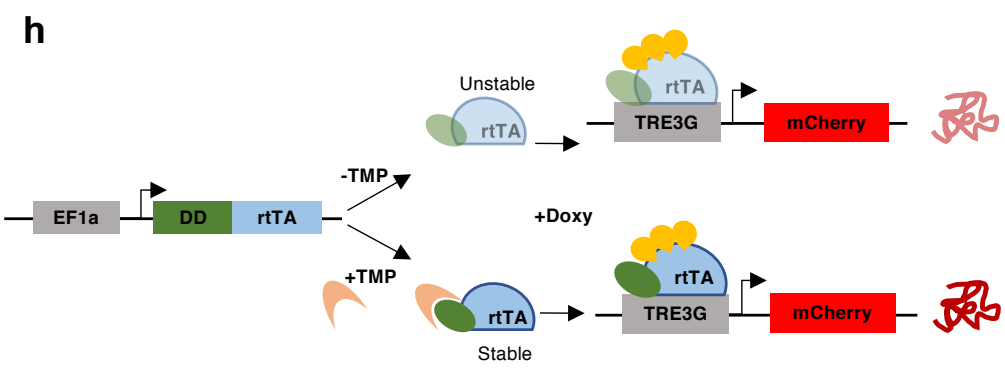
Supplementary Figure 2

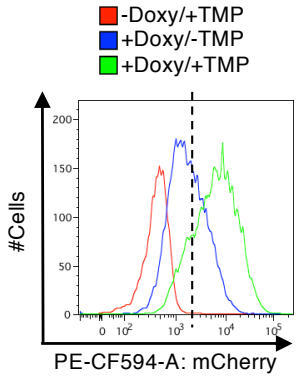
g



EF1a-rtTA TRE3G-mCherry	Coefficient of Variation (CV) of the mCherry+ Cells
14hrs Doxy1000ng/mL	0.2307
8hrs wash-out	0.3217
24hrs wash-out	0.8076
EF1a-rtTA TRE3G-DDmCherry	**Coefficient of Variation (CV) of the mCherry+ Cells**
14hrs Doxy1000ng/mL TMP100nM	0.0498
8hrs wash-out	0.2417
24hrs wash-out	0.7334

h





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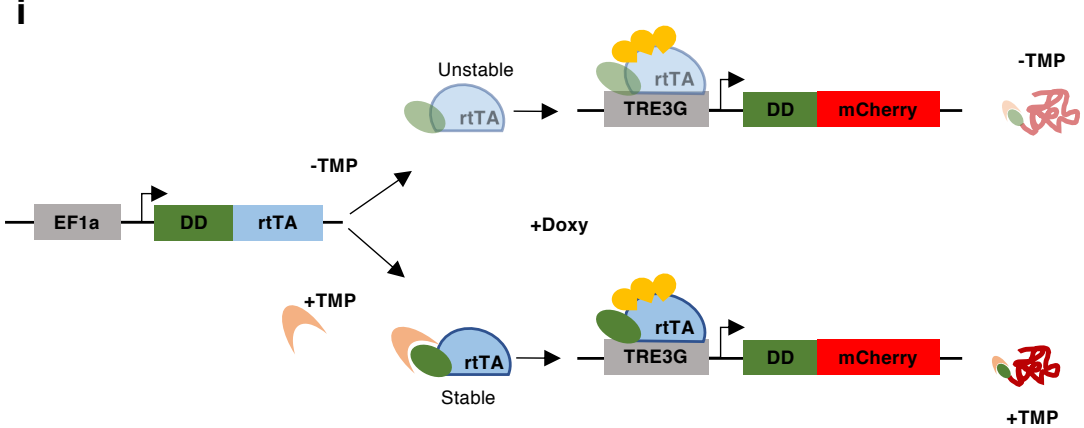
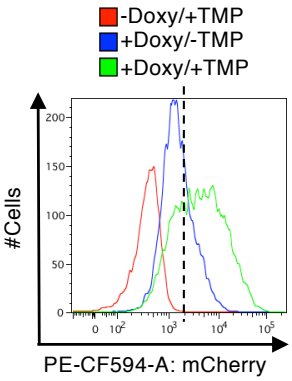
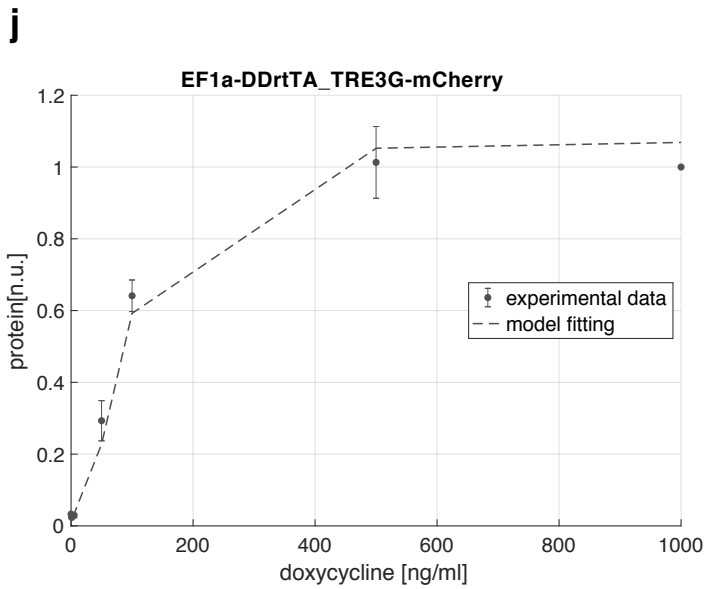
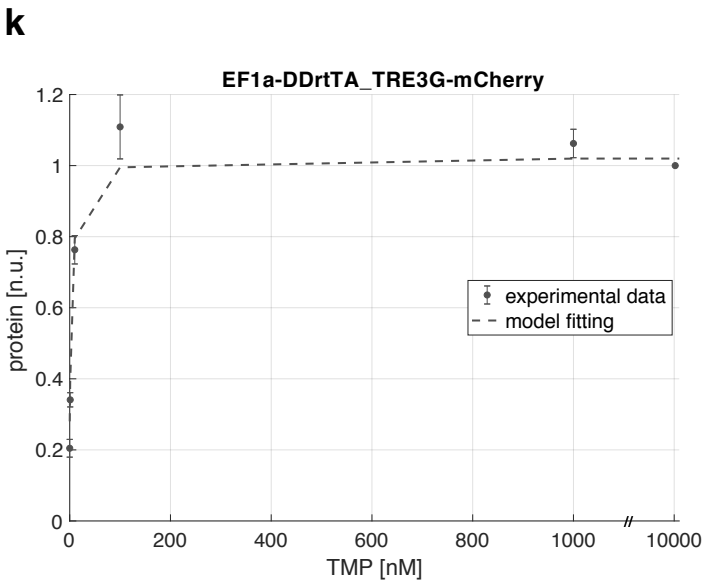
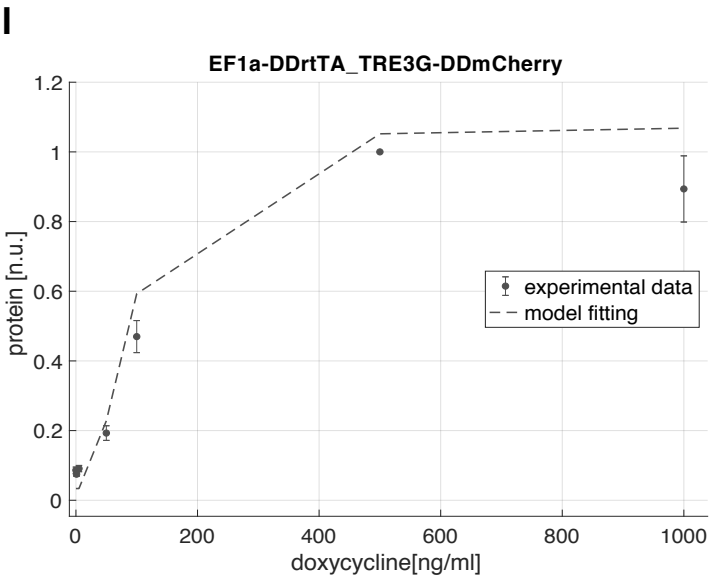


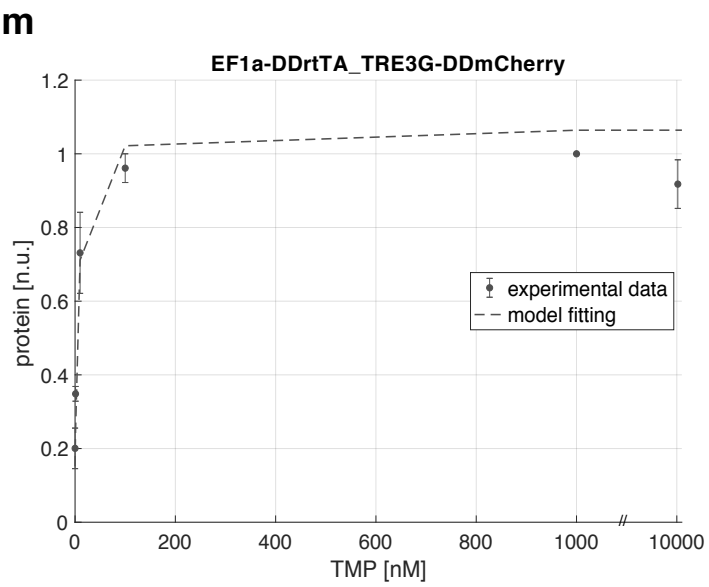


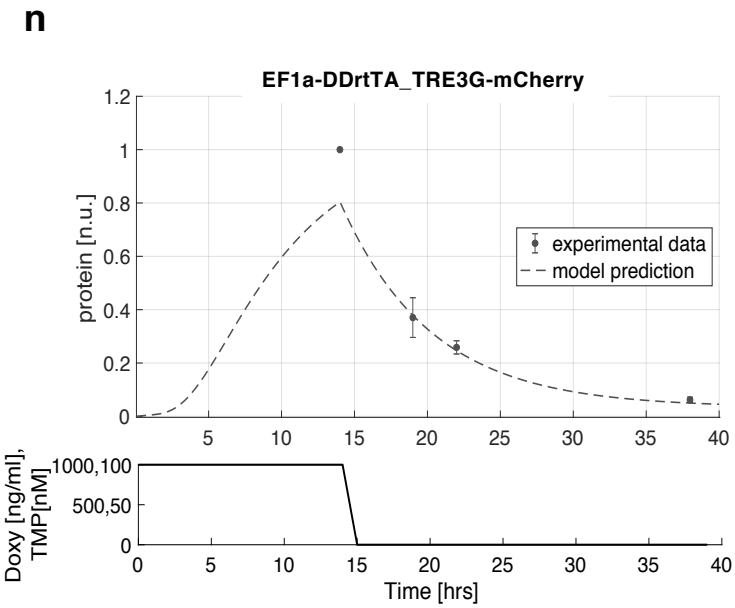
Supplementary Figure 2

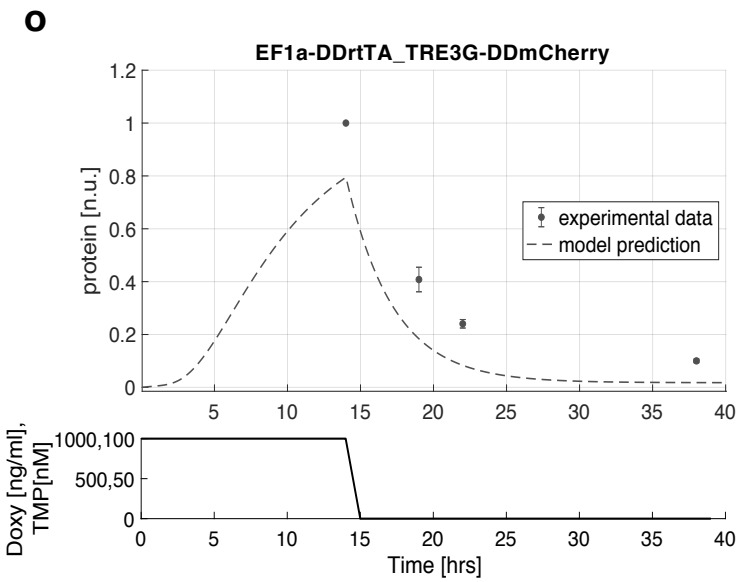








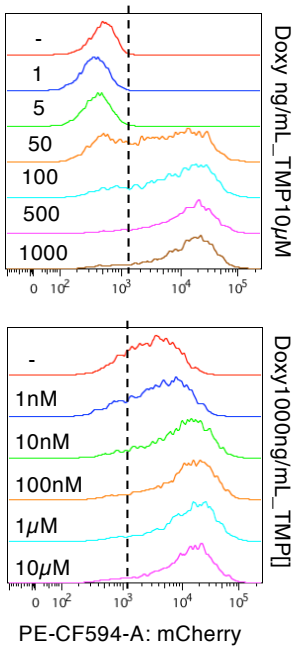




Supplementary Figure 2

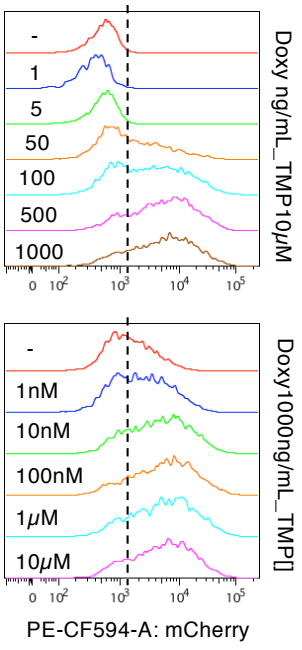
p

EF1a-DDrtTA TRE3G-mCherry		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	546±12.3	0.433±0.085
1ng/mL	10μM	386.7±6.2	0.082±0.02
5ng/mL	10μM	481±36.1	0.42±0.071
50ng/mL	10μM	4848.33±352	66.37±1.3
100ng/mL	10μM	10610.7±292.6	80±0.6
500ng/mL	10μM	16760.7±353	90.3±0.63
1000ng/mL	10μM	16546.33±600	91.3±0.78
1000ng/mL	-	2955±193.5	63±2.2
1000ng/mL	1nM	4924±100	74.2±0.8
1000ng/mL	10nM	11024.7±210	87±0.2
1000ng/mL	100nM	16017.7±754.3	91.6±0.68
1000ng/mL	1μM	15344.7±241	91.3±0.18
1000ng/mL	10μM	14445±146.5	90.6±0.09



q

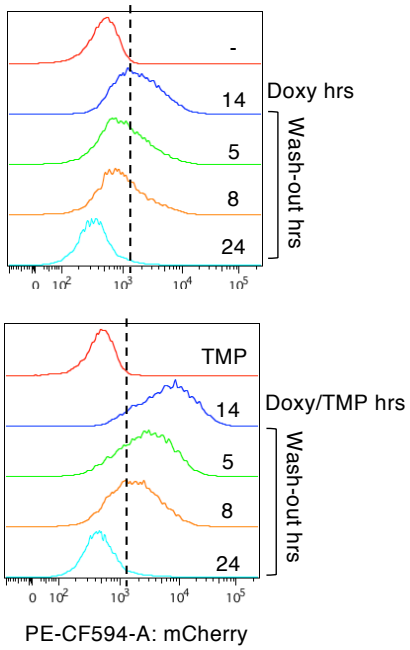
EF1a-DDrtTA TRE3G-DDmCherry		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	553.33±10.7	0.159±0.05
1ng/mL	10μM	479.33±43.4	0.128±0.032
5ng/mL	10μM	583.67±19.8	0.23±0.09
50ng/mL	10μM	1237±88.6	38.7±1.9
100ng/mL	10μM	3013±177.1	59±1.3
500ng/mL	10μM	6413±347	77.3±0.51
1000ng/mL	10μM	5731±116	75.1±0.32
1000ng/mL	-	1221.67±132	33.8±2.5
1000ng/mL	1nM	2123.33±137.6	52.2±2.1
1000ng/mL	10nM	4455±200	71.3±0.43
1000ng/mL	100nM	5856±146.5	76.13±0.75
1000ng/mL	1μM	6093±327.6	79.57±2.67
1000ng/mL	10μM	5592±58	75.8±0.26



Supplementary Figure 2

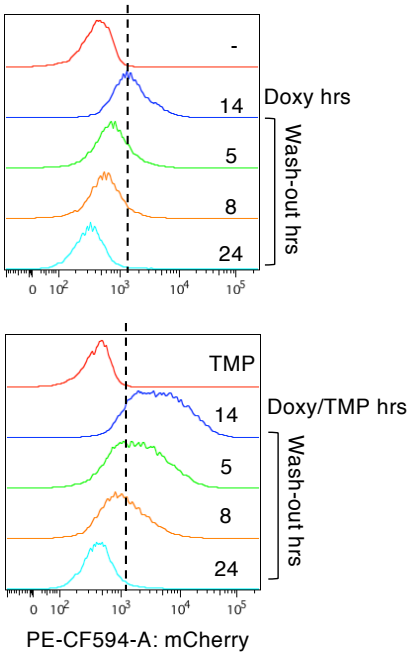
r

EF1a-DDrtTA TRE3G-mCherry		Dynamic Time-Course	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	-	444±14	0.255±0.095
14hrs 1000ng/mL	-	1660±50.7	43.1±1
5hrs wash-out		847.33±38.8	19.33±2
8hrs wash-out		758±45.1	15.8±2
24hrs wash-out		332.67±3.5	1.45±0.18
-	100nM	444.5±20.5	0.715±0.015
14hrs 1000ng/mL	100nM	6386±323	83.1±1.3
5hrs wash-out		2515±138.8	58.77±1.9
8hrs wash-out		1756±139.5	45.5±2.9
24hrs wash-out		421.33±17.3	2.65±0.16

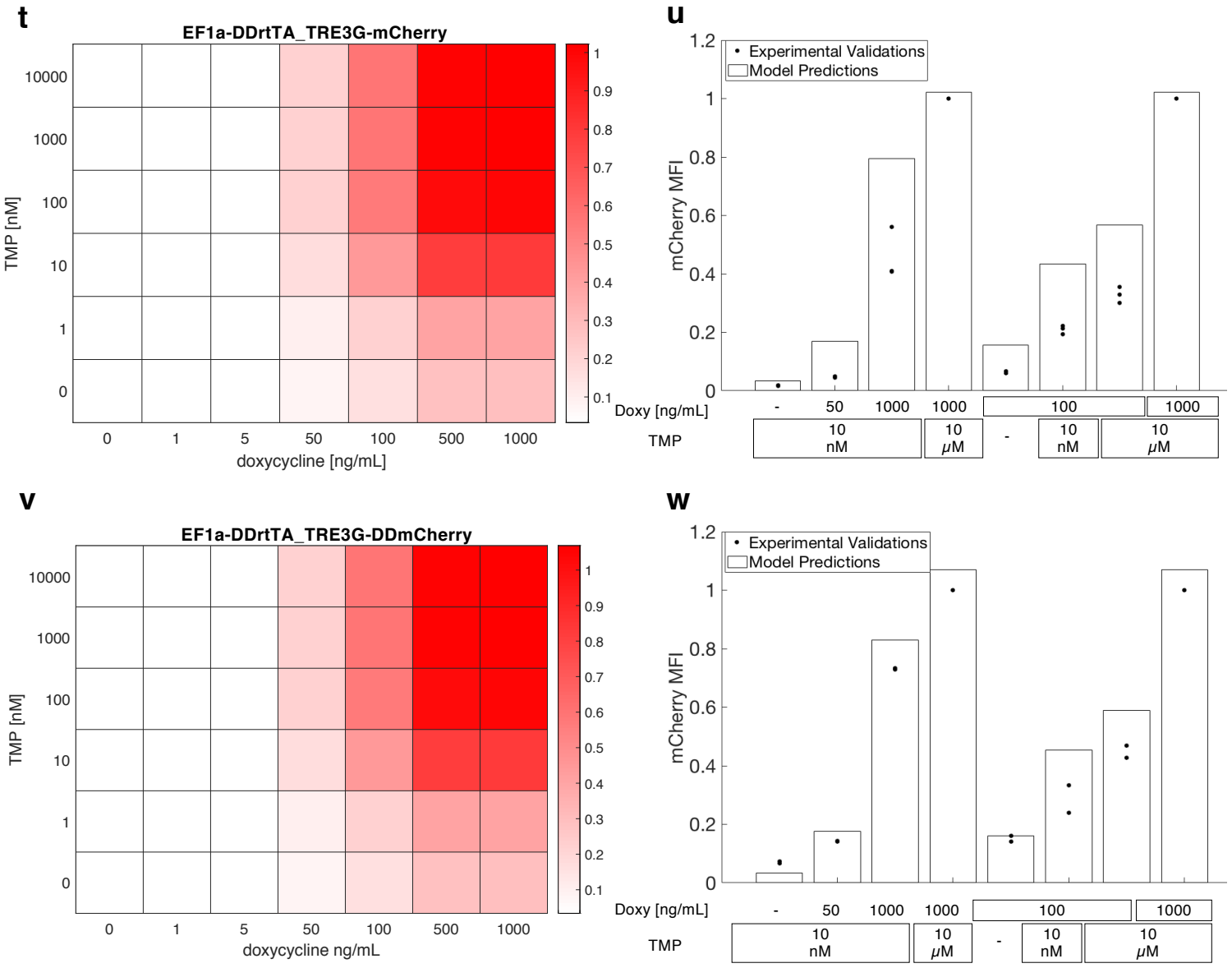


s

EF1a-DDrtTA TRE3G-DDmCherry		Dynamic Time-Course	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	-	428.5±9.5	0.94±0.06
14hrs 1000ng/mL	-	1256±127.5	29.5±2.6
5hrs wash-out		728.33±16.7	9.15±0.13
8hrs wash-out		586.33±3	4.72±0.39
24hrs wash-out		340.67±3.3	0.99±0.1
-	100nM	421±1	0.43±0.16
14hrs 1000ng/mL	100nM	4149±45.6	71.3±1.3
5hrs wash-out		1695±96.5	44.3±2.4
8hrs wash-out		999.67±29.6	23.7±1.16
24hrs wash-out		416±14.3	2.89±0.13

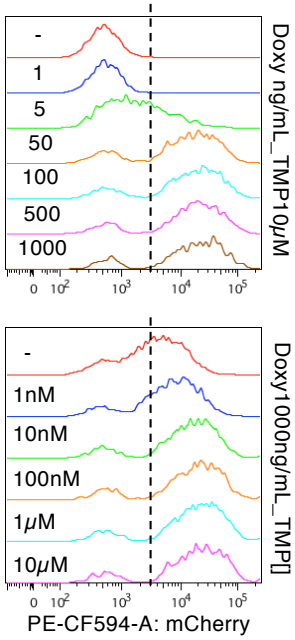


Supplementary Figure 2



x

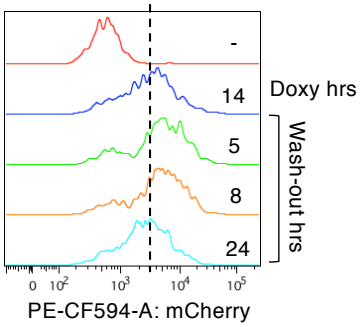
HeLa_EF1a-rtTA TRE3G-DDmCherry		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	520±8.4	1±0.1
1ng/mL	10μM	550±3.5	1±0.3
5ng/mL	10μM	1625±35.6	33.3±1
50ng/mL	10μM	13858±308.6	78±0.3
100ng/mL	10μM	16676±108.4	80.9±0.5
500ng/mL	10μM	17958±84.2	81.9±0.8
1000ng/mL	10μM	18145±127.6	82.8±0.5
1000ng/mL	-	3786±115.8	57.5±1
1000ng/mL	1nM	6764±299.3	72.8±1
1000ng/mL	10nM	15445±347.1	83.9±0.2
1000ng/mL	100nM	18805±164.4	83.6±0.3
1000ng/mL	1μM	19018±379	83.8±0.4
1000ng/mL	10μM	19760±789.1	85.1±0.6



Supplementary Figure 2

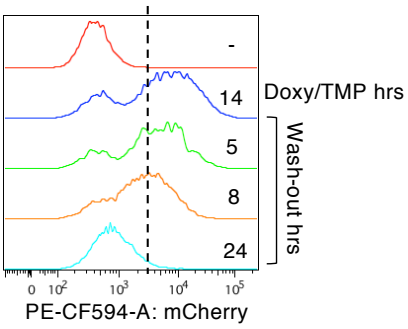
y

HeLa_EF1a-rtTA TRE3G-mCherry	Dynamic Time-Course	
Doxy	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	584.5±9.5	3.17±1.83
14hrs 100ng/mL	3399.5±196.5	54.3±0.2
5hrs wash-out	5107±127	72.25±2.15
8hrs wash-out	4724±112	69.55±1.25
24hrs wash-out	2565.5±96.5	44.25±2.55



z

HeLa_EF1a-rtTA TRE3G-DDmCherry		Dynamic Time-Course	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	**10nM**	405.7±13.1	0.77±0.16
14hrs 100ng/mL	**10nM**	5414±99	65.3±0.35
5hrs wash-out		4062±18.2	59.8±0.14
8hrs wash-out		2560±41.3	45.7±0.7
24hrs wash-out		769±3.9	5.1±0.07

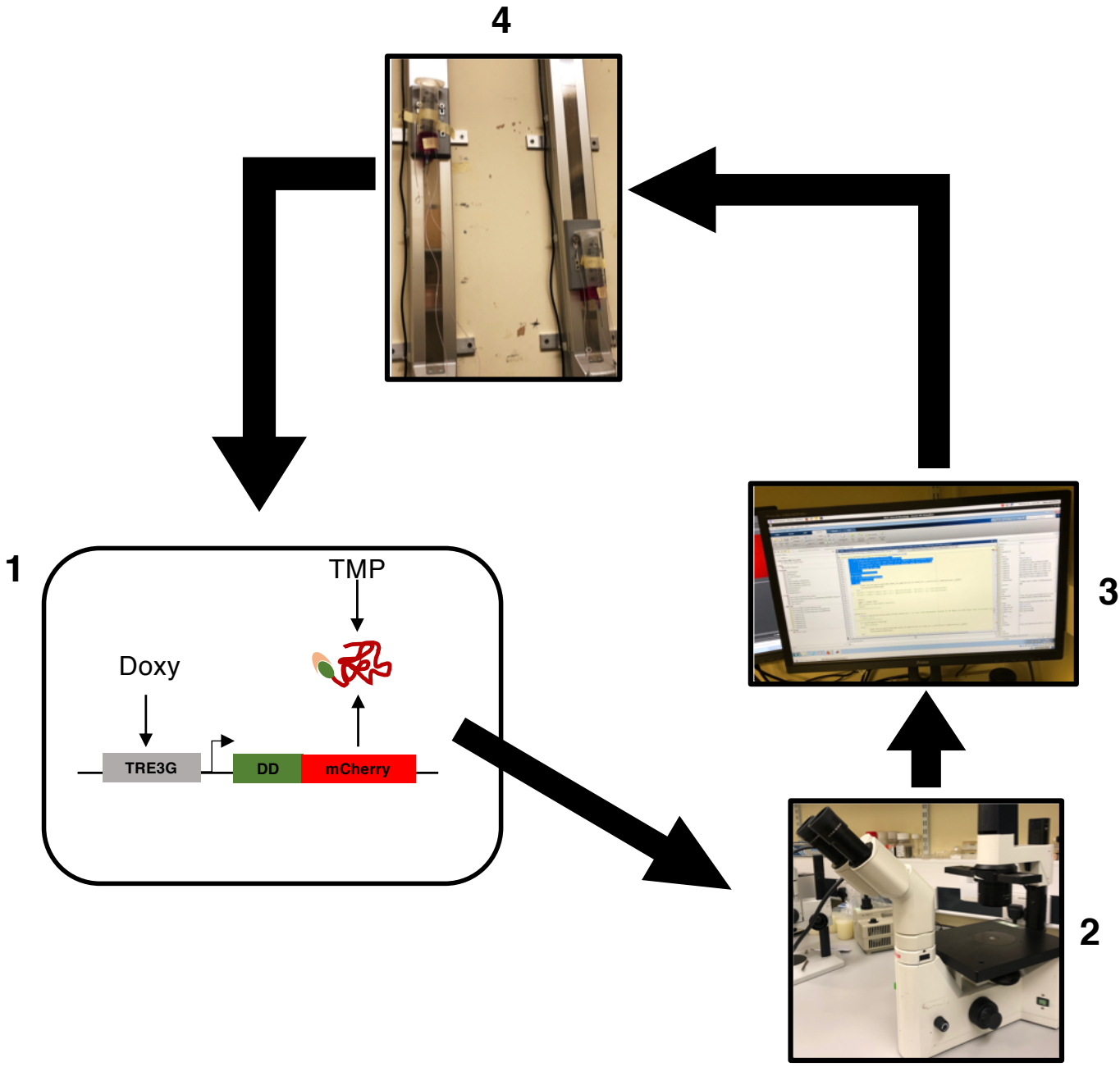


Supplementary Figure 2

Supplementary Figure 2. Flow cytometry profiling, dose response, switch-off dynamics, single cell analysis and dynamic range of inducible cells.

(a-d, p-s, x-z) Median Fluorescence Intensity (MFI) and % of mCherry+ cells measured by flow cytometry in EF1a-rtTA_TRE3G-mCherry (a, c), EF1a-rtTA_TRE3G-DDmCherry (b, d), EF1a-DDrtTA_TRE3G-mCherry (p, r), EF1a-DDrtTA_TRE3G-DDmCherry (q, s) mESCs, HeLa_EF1a-rtTA_TRE3G-mCherry (y) and HeLa_EF1a-rtTA_TRE3G-DDmCherry (x, z) cells. Inducer titration (a, b, p, q, x) and dynamic (c, d, r, s, y, z) response were performed using the concentrations and incubation times indicated in the tables. (e, f) Representative flow cytometry histograms of the dynamic switch-off analysed in 13 single clones sorted from EF1a-rtTA_TRE3G-mCherry (e) and EF1a-rtTA_TRE3G-DDmCherry (f) mESCs. (g) Box-plot representing mCherry+ cells in EF1a-rtTA_TRE3G-mCherry and EF1a-rtTA_TRE3G-DDmCherry single clones in a time-course experiment of 38hrs, in which inducers were washed out after incubation in the first 14hrs. The coefficient of variation, calculated on the mCherry+ cells, is also shown (g, inset). (h, i) Dual input regulation system consisting of the conditionally destabilised transactivator (DDrtTA) and a stable (h) or a conditionally destabilised (i) mCherry fluorescent protein. Protein expression following 24hrs Doxy/TMP treatment (1000ng/mL and 100nM, respectively) in EF1a-DDrtTA_TRE3G-mCherry (h, right panel) and EF1a-DDrtTA_TRE3G-DDmCherry (i, right panel) mESCs. (j-m) Fitted model simulations (dashed lines) and experimental data (dots) of EF1a-DDrtTA_TRE3G-mCherry (j, k, RMSE 0.0449 and 0.0669, respectively) and EF1a-DDrtTA_TRE3G-DDmCherry (l, m, RMSE 0.0907 and 0.084, respectively) mESC steady-state response. Dots represent experimental data of MFI in Supplementary Fig. 2p, q, normalised over the maximum activation point. (n, o) Model

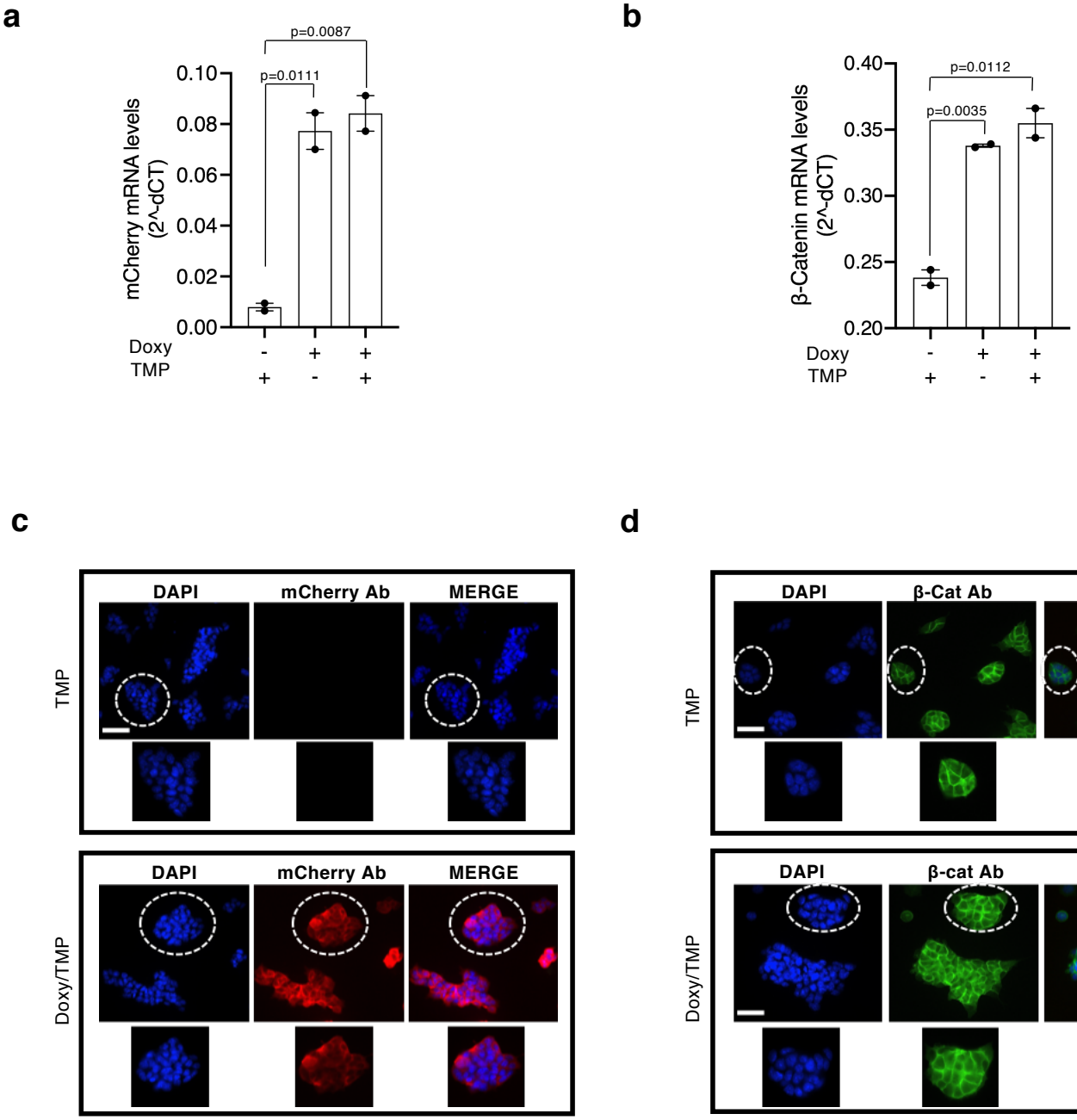
predicted dynamic response (dashed lines) and experimental data (dots) of EF1a-DDrtTA_TRE3G-mCherry (n, RMSE 0.1195) and EF1a-DDrtTA_TRE3G-DDmCherry (o, RMSE 0.1916) mESCs. Dots represent experimental data of MFI in Supplementary Fig. 2r, s, normalised over the maximum activation point. (t-w) Simulations of the model (t, v) and experimental validation (u, w) of EF1a-DDrtTA_TRE3G-mCherry (t, u) and EF1a-DDrtTA_TRE3G-DDmCherry (v, w) mESC steady-state response following 24hrs induction with constant concentration of Doxy (100ng/mL) and varying TMP (0, 10nM; 10μM) or constant TMP (10nM) and varying Doxy (0, 50, 1000ng/mL). Maximum concentrations of Doxy and TMP (1000mg/mL and 10μM, respectively) are used as control. mCherry values are shown as heatmaps of scaled values across the entire dynamical range of expression levels. Data are means ±SEM (n=3, a-d, n-s, u, x, z; n=13, g; n=2, w, y); ±SD (n=3, j-m). Source data are provided as a Source Data file.



Supplementary Figure 3

Supplementary Figure 3. *In-silico* feedback control microfluidics/microscopy platform

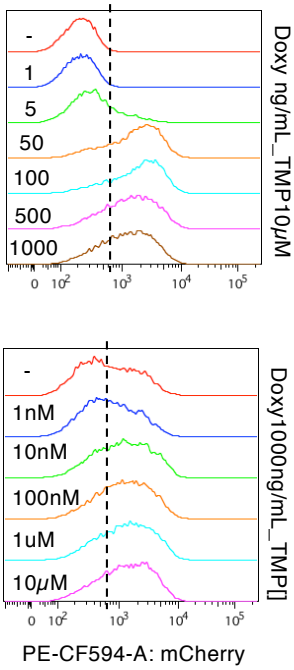
Schematic representation of the *in-silico* feedback control platform used in the present study. It consists of the controlled biological system (1), in our case mouse Embryonic Stem Cells (mESCs) stably carrying an inducible exogenous gene (for specific control inputs used in different experiments see Supplementary Movies' legends and Main text); an inverted fluorescence microscope (2) measuring fluorescence every 60mins during the time-lapse; a computer (3) to implement real-time cell segmentation, fluorescence quantification and a Relay control algorithm (the latter computing the error from the comparison of the desired over the measured fluorescence); an actuation system (4), consisting of motor-controlled syringes dispensing media +/- inducers (+ inducers if the measured fluorescence is below the set-point and vice versa)[1].



Supplementary Figure 4

g

EF1a-rtTA TRE3G-DDmCherryβ-cateninS33Y		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	210.5±2.5	0.38±0.01
1ng/mL	10μM	217.5±0.5	0.34±0.06
5ng/mL	10μM	336.5±3.5	13.05±0.95
50ng/mL	10μM	1837.5±17.5	69.05±0.15
100ng/mL	10μM	2053.5±4.5	71.65±0.05
500ng/mL	10μM	1446±25	61.5±0.3
1000ng/mL	10μM	1257.5±7.5	57.35±0.25
1000ng/mL	-	604.5±9.5	35.3±0.3
1000ng/mL	1nM	720.5±42.5	38.55±2.45
1000ng/mL	10nM	1146.5±52.5	53.75±1.65
1000ng/mL	100nM	1293.5±45.5	57.55±0.65
1000ng/mL	1μM	1374±14	59.8±0
1000ng/mL	10μM	1306±43	58.15±1.25



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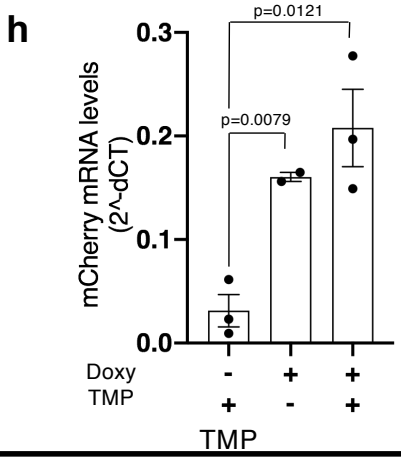

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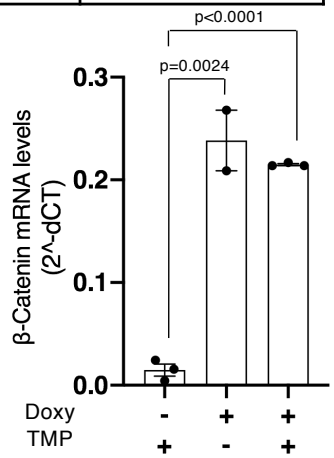

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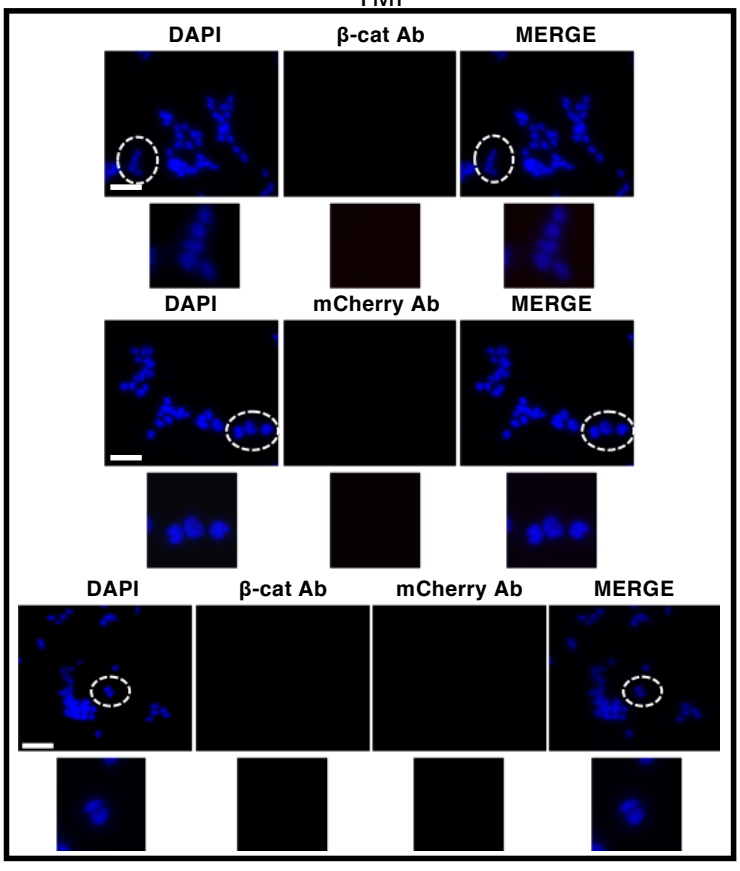

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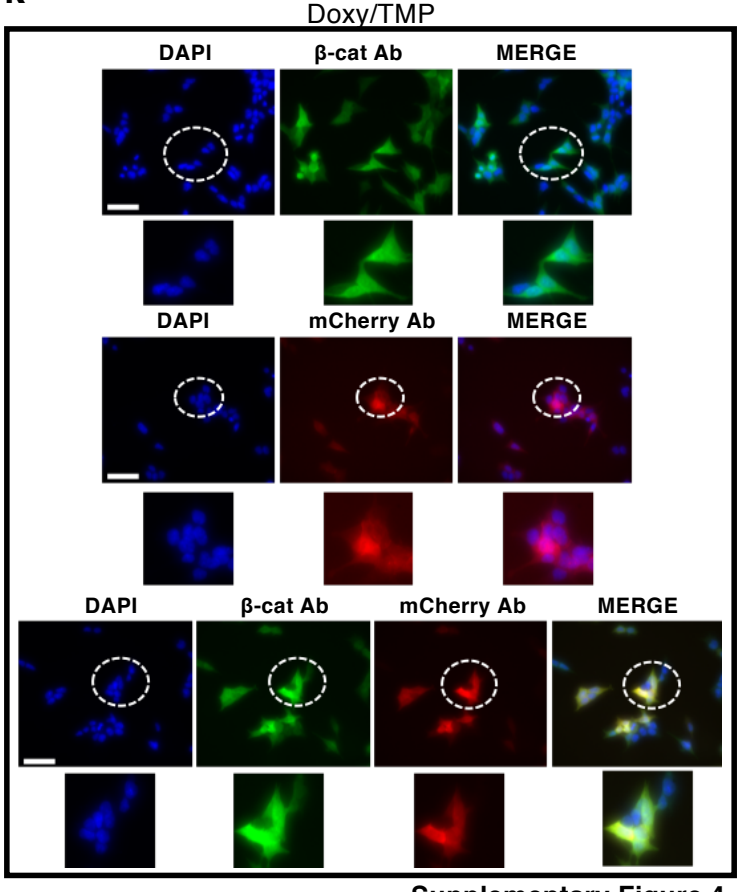
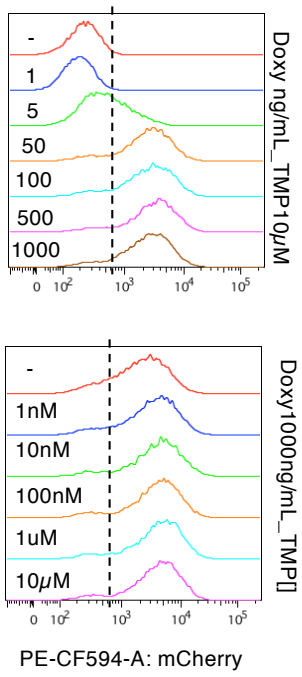


Supplementary Figure 4

l

C1-EF1a-rtTA TRE3G-DDmCherryβ-cateninS33Y		Doxy/TMP Titration	
Doxy	**TMP**	**Median Fluorescence Intensity (MFI)**	**% mCherry+ Cells**
-	10μM	246.5±6.5	0.6±0.05
1ng/mL	10μM	241±56	0.65±0.33
5ng/mL	10μM	476±4	21.3±0.1
50ng/mL	10μM	2683.5±82.5	81.7±1
100ng/mL	10μM	2754.5±55.5	80.1±1.2
500ng/mL	10μM	3105±221	84.3±1.6
1000ng/mL	10μM	2559.5±0.5	79.75±0.25
1000ng/mL	-	2261±25	74.4±0.2
1000ng/mL	1nM	3094.5±477.5	81.1±2.3
1000ng/mL	10nM	3750.5±583.5	85.4±2.3
1000ng/mL	100nM	4115±129	87.5±0.2
1000ng/mL	1μM	4216±310	87.2±0.9
1000ng/mL	10μM	3836±391	86.55±1.15



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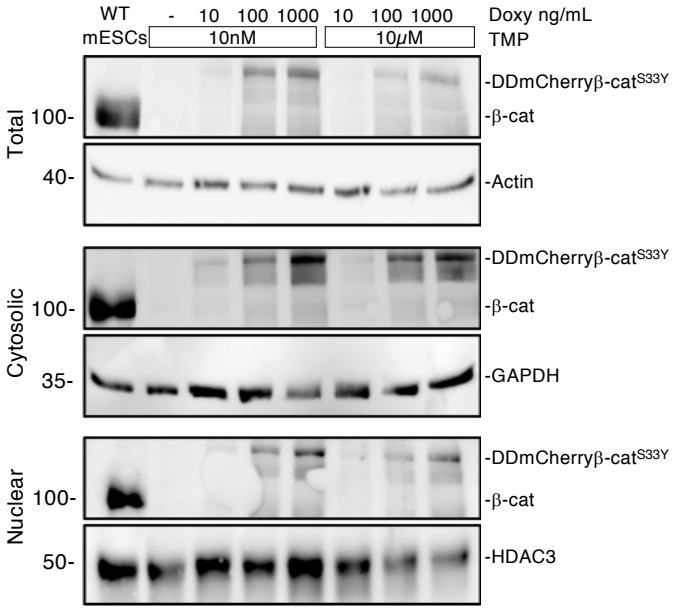

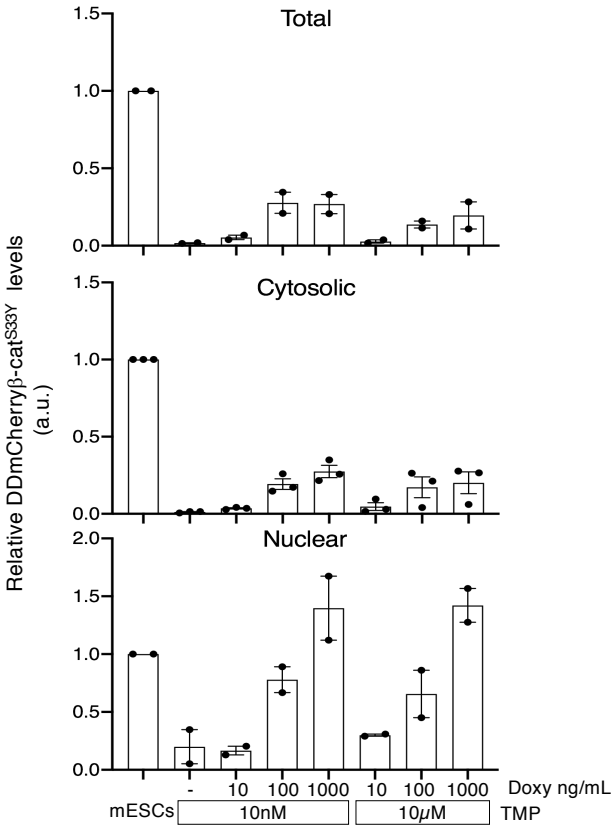

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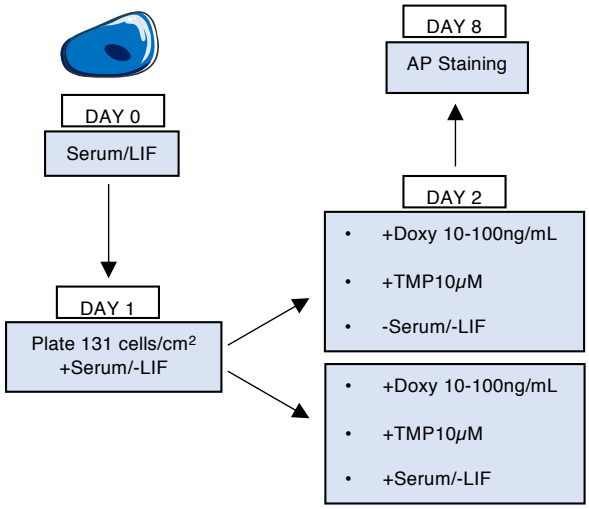

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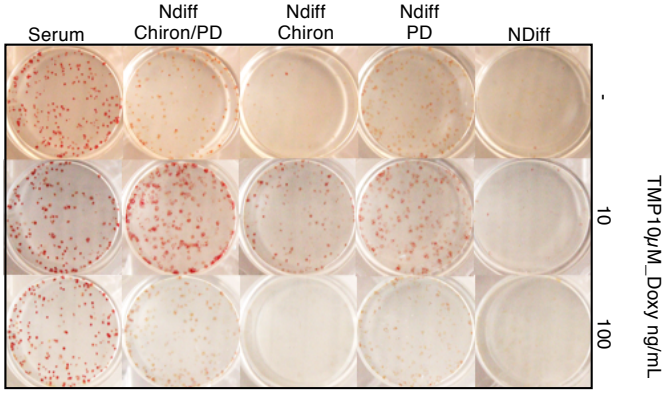
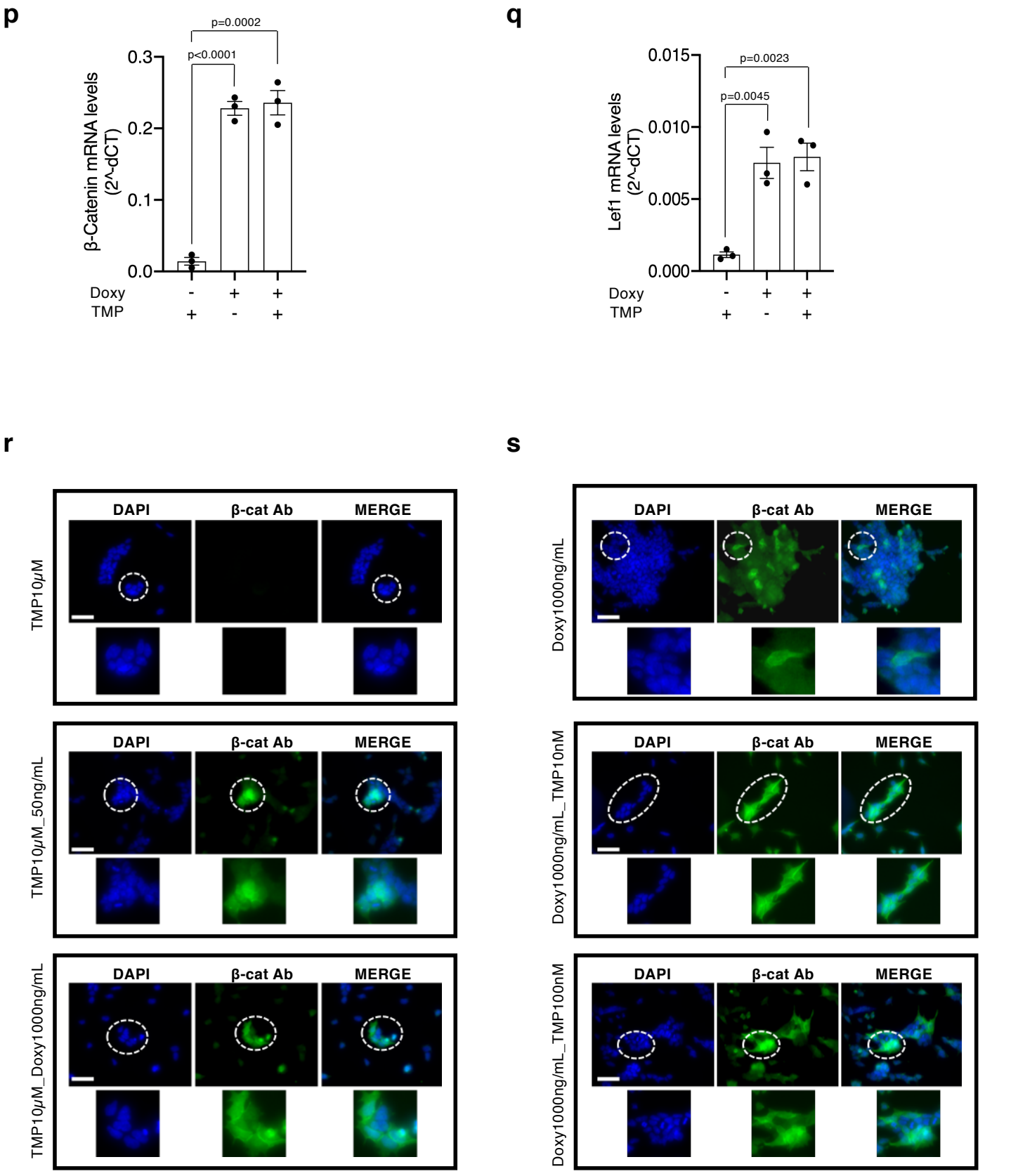
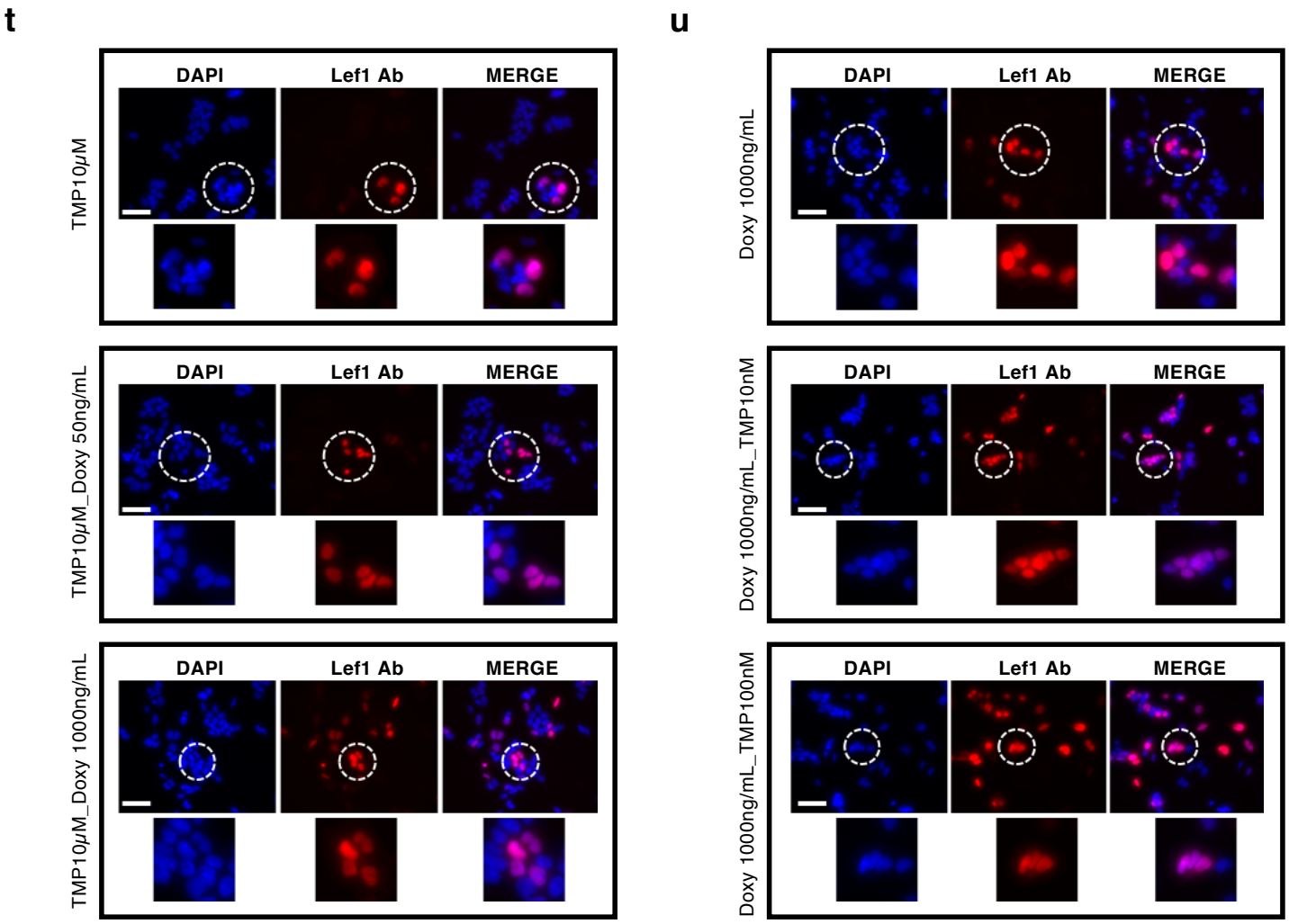


Supplementary Figure 4



Supplementary Figure 4



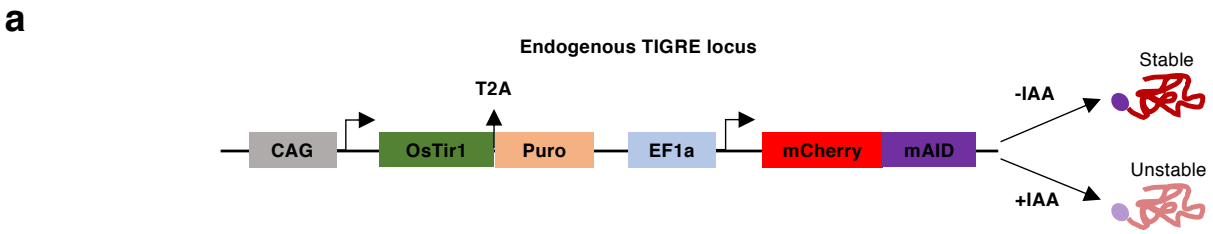
Supplementary Figure 4

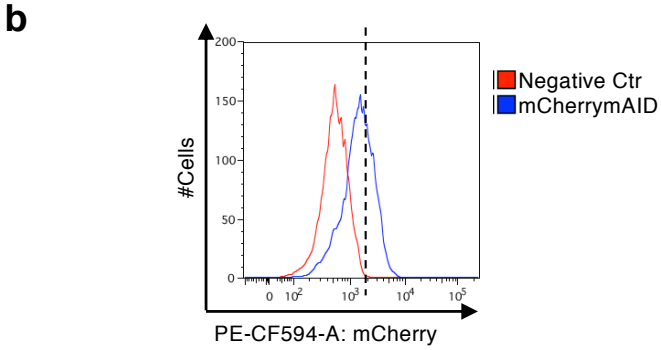
Supplementary Figure 4. Cell line characterisation and automatic control of Wnt/β-catenin pathway genes

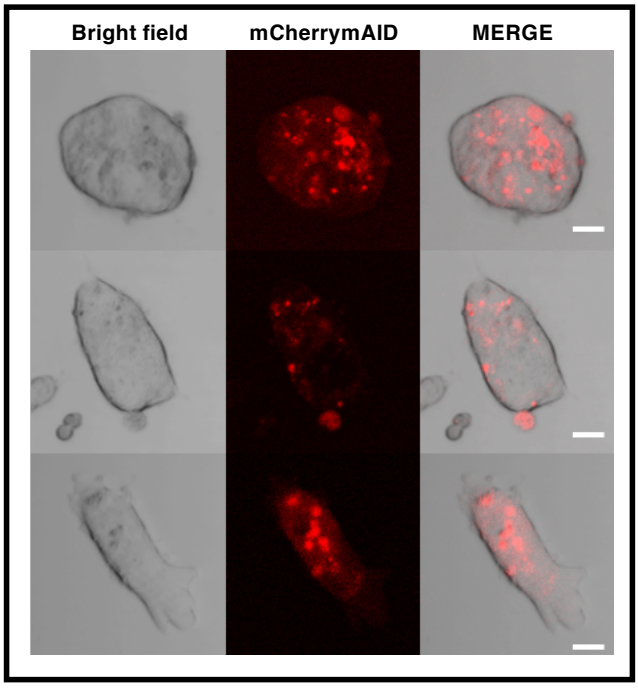
(a, b, h, i) DDmCherryβ-catenin^{S33Y} mRNA levels measured by qPCR using mCherry (a, h) and β-catenin (b, i) specific primers in EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} (a, b) and C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} (h, i) mESCs induced with Doxy (1000ng/mL) and/or TMP (100nM) for 24hrs. (c, d) β-catenin immunostaining in EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs treated for 24hrs with Doxy (1000ng/mL) and/or TMP (100nM); mCherry (c) and β-catenin (d) antibodies were used. DAPI was used to stain the nuclei. Zoomed pictures of selected cells are shown. Scale bars 25μm. (e) Western-blot of nuclear and cytosolic fractions from EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs treated for 24hrs with Doxy (1000ng/mL) and/or TMP (100nM), and blotted with an anti-mCherry antibody. (f) Set-point control experiment using inducers (Doxy 1000ng/mL and TMP 100nM) as control inputs in EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} cells. In red the measured output (normalised mCherry fluorescence), in blue the control reference fluorescence, set at 50% of the average value measured during the calibration phase (120mins with continuous Doxy/TMP administration). The control performance indexes are reported in Supplementary Table 3 and Note 2; details about feedback control implementation are in Supplementary Note 2. (g, l) Median Fluorescence Intensity (MFI) and % of mCherry+ cells measured by flow cytometry in EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} (g) and C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} (l) mESCs after 24hrs of treatment with indicated concentrations of Doxy and TMP. (j, k) β-catenin immunostaining in C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs treated for 24hrs with the indicated concentrations of TMP (j), or Doxy and TMP (k), using

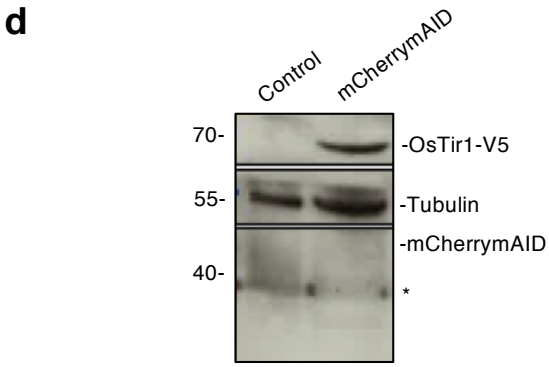
mCherry (red signal) and β-catenin (green signal) antibodies. DAPI was used to stain the nuclei. Zoomed pictures of selected clones are shown. Scale bars 25μm. (m) Western-blot of total, cytosolic and nuclear fractions from C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs treated for 24hrs with TMP 10nM or 10μM and increasing concentration of Doxy (0, 10, 100, 1000ng/mL) and blotted with an anti-β-catenin antibody. Wild-type mESCs were used as control. Western-blot densitometric quantifications are shown (m, inset). Total, Cytosolic and Nuclear DDmCherryβ-catenin^{S33Y} values are normalised against the corresponding housekeeping gene Actin, GAPD and HDAC3. (n) Clonogenicity assay experimental scheme. At Day 1, 131cells/cm^2 C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs were plated in complete mESCs growth media without LIF (+Serum/-LIF); the following day, inducers were added (TMP10μM and Doxy at 0, 10, 100ng/mL) and cells were either grown in Serum/-LIF or -Serum/-LIF supplemented with Chiron and/or PD media for 6 days; inducers were refreshed every 72hrs. At Day 8, cells were fixed and the Alkaline Phosphatase (AP) staining was performed. (o) Representative AP pictures from C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} mESCs treated with TMP10μM and Doxy (0, 10, 100ng/mL). The media used were either serum-based (Serum) or Serum-free (NDiff-Chiron/PD, NDiff-Chiron, NDiff-PD and NDiff). Red spots represent AP+ pluripotent colonies. (p, q) Exogenous DDβ-catenin^{S33Y} (p) and DDLef1 (q) mRNA levels measured by qPCR in EF1a-rtTA_TRE3G-DDβ-catenin^{S33Y} (p) and EF1a-rtTA_TRE3G-DDLef1 (q) mESCs. (r-u) β-catenin (r, s) and Lef1 (t, u) immunostaining in EF1a-rtTA_TRE3G-DDβ-catenin^{S33Y} (r, s) and EF1a-rtTA_TRE3G-DDLef1 (t, u) mESCs treated for 24hrs with the indicated inducer concentrations. DAPI was used to stain the nuclei. Zoomed pictures of selected cells are shown. Scale bars 25μm.

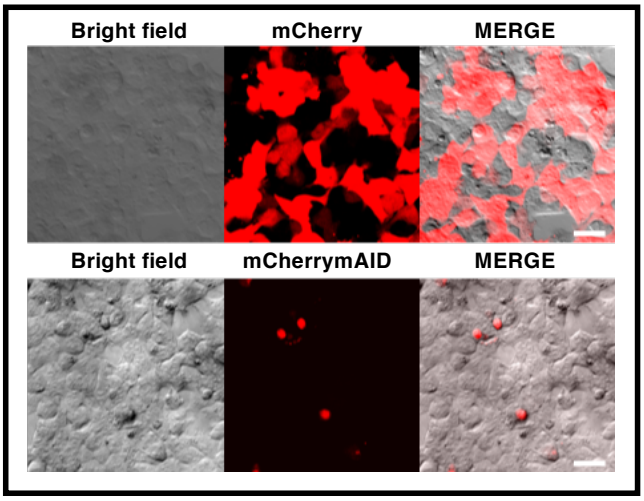
Data are means ± SEM (n=2, a, b, g, l, m, inset; n=3, h, i, p, q). p values from two-tailed unpaired t test are shown. Source data are provided as a Source Data file.

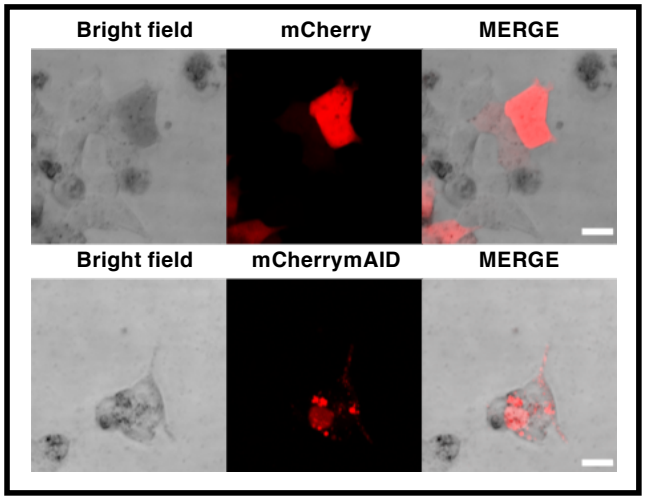


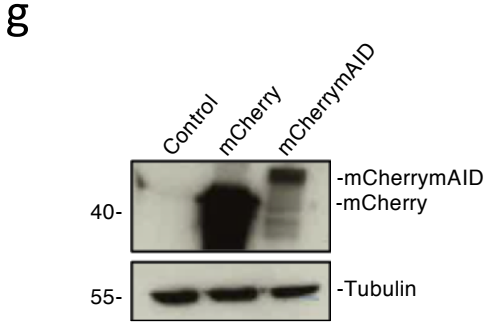












Supplementary Figure 5

Supplementary Figure 5. AID (auxin-inducible degradation) system characterisation

(a) The AID-system consisting of the auxin responsive F-box protein, Tir1 (CAG-OsTir1- V5-2A-PuroR) and the mini-AID fused mCherry (EF1a-mCherrymAID). (b) Representative flow cytometry profiles of negative control and mCherrymAID mESCs. (c) Confocal microscopy images of mCherrymAID mESCs. (d) mCherry and OsTIR1 protein levels measured in mCherrymAID mESCs by western-blot using anti-mCherry and anti-V5-tagged OsTIR1 antibodies. * indicates a non-specific band. (e, f) Confocal microscopy images of HEK293T cells transiently transfected with mCherry or mCherrymAID using equal (e) or cell-line adjusted (i.e. higher exposure in mCherrymAID cells, f) laser settings. (g) mCherry protein levels measured in mCherry and mCherrymAID HEK293T cells by western-blot using an anti-mCherry antibody. Scale bars 10μm. Source data are provided as a Source Data file.

Supplementary Note 1: Mathematical modelling

The mathematical models for the EF1a-rtTA_TRE3G-mCherry, EF1a-rtTA_TRE3G-DDmCherry, EF1a-DDrtTA_TRE3G-mCherry and EF1a-DDrtTA_TRE3G-DDmCherry systems are based on sets of 3 ODEs, describing transactivator protein, and fluorescent gene mRNA and protein concentrations, as the result of a production term and a degradation term. We formulated the parameters estimation problem as a constrained optimization problem:

$$\min_{\gamma} J(x, u; p)$$

$$s.t.:$$
$$\dot{x} = f(x, u; p)$$
$$p \geq 0$$

where x is the state vector, u is the vector of inputs acting on the systems (e.g. Doxy and TMP), f is the vector field of the system dynamics and p is the vector of all parameter to be identified. J is the cost function to be minimized, defined as

$$J = \sum_{i=1}^{n} \left(\frac{y_i - \hat{y}_i}{\hat{y}_i} \right)^2$$

where n is the number of experimental data points, y are the predicted values of the mathematical model and $\hat{y}$ are the experimental data points used for fitting.

For the parameters identification, we used the Interior Point algorithm implemented in *fmicon* function (Matlab Optimization toolbox™, MathworksMatlab R2018a).

The parameters were first identified for EF1a-rtTA_TRE3G-mCherry and EF1a-rtTA_TRE3G-DDmCherry mESCs (equations (1)-(3), and (4)-(6), respectively). The Doxy and TMP Michaelis-Menten constants (K_1 and K_3, respectively) were directly fixed from dose-response experimental data (Fig. 2a-c), and the degradation of mCherry protein (d_3) was fitted to have mCherry protein half-life in range to that

measured experimentally (Fig. 1f; Supplementary Fig.1a). The remaining parameters were fitted as aforementioned, with a constrain on the term describing the degradation of DDmCherry protein in equation (6) $\left(d_3 + d_4\left(\frac{K_3^{h_3}}{K_3^{h_3}+TMP^{h_3}}\right)\right)$ so that, in presence of saturating TMP concentration, the DDmCherry protein half-life would be equal to that of untagged mCherry protein.

For the EF1a-DDrtTA_TRE3G-mCherry and EF1a-DDrtTA_TRE3G-DDmCherry systems (equations (7)-(9), and (10)-(12), respectively), the Hill coefficients for Doxy (h_1) and for TRE3G promoter (h_2), the TRE3G Michaelis-Menten constant (K_2) and degradation rate of mCherry mRNA (d_3) were fixed equal to the corresponding parameter values identified for the EF1a-rtTA systems. The Doxy and TMP Michaelis-Menten constants (K_1 and K_3, respectively) were fixed from dose-response experimental data (Supplementary Fig. 2j-m). For EF1a-DDrtTA_TRE3G-DDmCherry mESCs, parameters related to TMP-dependent degradation were kept identical in the transactivator and DDmCherry protein equations. The remaining parameters were fitted as aforementioned, with a constrain on TMP-dependent degradation of the transactivator and DDmCherry proteins $\left(d_1 + d_2\left(\frac{K_3^{h_3}}{K_3^{h_3}+TMP^{h_3}}\right)\right)$ and $\left(d_4 + d_2\left(\frac{K_3^{h_3}}{K_3^{h_3}+TMP^{h_3}}\right)\right)$, respectively, so that, in presence of saturating TMP concentration, the degradation rates would be equal to those of the corresponding untagged proteins. Letting x_1 be the rtTA (DD-tagged or not) protein concentration, x_2 the mCherry mRNA concentration and x_3 the mCherry (DD-tagged or not) protein concentration, the models of the systems considered in this work are reported below. The description of the estimated parameters and their values can be found in Supplementary Tables 1

and 2. Of note, variables concentrations are in [a.u.], as the model was fitted on normalised FACS data; the latter was used for model fitting or validation as described in the main text. The Root Mean Squared error (RMSE) reported to quantify goodness of fitting and predictions was calculated using the formula $\sqrt{\frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n}}$ where n is the number of experimental data points, y are the predicted values of the mathematical model and $\hat{y}$ are the experimental data points used for fitting.

EF1a-rtTA_TRE3G-mCherry model

$$\frac{dx_1}{dt} = \gamma_1 - d_1 x_1 \ (1)$$

$$\frac{dx_2}{dt} = \alpha_1 + \alpha_2 \left(\frac{x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)}{K_2^{h_2} + x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)} \right) - d_2 x_2 \ (2)$$

$$\frac{dx_3}{dt} = \gamma_2 x_2 - \ d_3 x_3 \ (3)$$

EF1a-rtTA_TRE3G-DDmCherry model

$$\frac{dx_1}{dt} = \gamma_1 - d_1 x_1 \ (4)$$

$$\frac{dx_2}{dt} = \alpha_1 + \alpha_2 \left(\frac{x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)}{K_2^{h_2} + x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)} \right) - d_2 x_2 \ (5)$$

$$\frac{dx_3}{dt} = \gamma_2 x_2 - d_3 x_3 - d_4 \left(\frac{K_3^{h_3}}{K_3^{h_3} + TMP^{h_3}} \right) x_3 \ (6)$$

Supplementary Table 1: Parameters identified for the EF1a-rtTA_TRE3G-mCherry and EF1a-rtTA_TRE3G-DDmCherry systems.

Parameter	Description	Value

γ_1[a.u. hrs^{-1}]	Production rtTA protein	54.61
d_1[hrs^{-1}]	Degradation rate rtTA protein	55.12
α_1 [a.u.hrs^{-1}]	Basal activity TRE3G	0.58
α_2 [a.u.hrs^{-1}]	Maximal transcription rate TRE3G	22.80
K_1 [ng/mL] EF1a-tTA_TRE3G-mCherry	Doxy Michaelis-Menten constant	64.00
K_1 [ng/mL] EF1a-tTA_TRE3G-DDmCherry	Doxy Michaelis-Menten constant	87.07
K_2 [a.u.]	TRE3G Michaelis-Menten constant	0.91
h_1	Hill coefficient for Doxy	2.34
h_2	Hill coefficient of the TRE3G promoter	6.21
d_2[hrs^{-1}]	Degradation rate mCherry mRNA	72.09
γ_2[hrs^{-1}]	Production rate mCherry protein	0.57
d_3[hrs^{-1}]	Degradation rate mCherry protein	0.11
d_4[hrs^{-1}]	TMP-dependent degradation rate DDmCherry protein	0.40
K_3 [ng/mL]	TMP Michaelis-Menten constant	0.40
h_3	Hill coefficient for TMP	1.20

EF1a-DDrtTA_TRE3G-mCherry model

$$\frac{dx_1}{dt} = \gamma_1 - d_1 x_1 - d_2 \left(\frac{K_3^{h_3}}{K_3^{h_3} + TMP^{h_3}} \right) x_1 \quad (7)$$

$$\frac{dx_2}{dt} = \alpha_1 + \alpha_2 \left(\frac{x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)}{K_2^{h_2} + x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)} \right) - d_3 x_2 \quad (8)$$

$$\frac{dx_3}{dt} = \gamma_2 x_2 - d_4 x_3 \quad (9)$$

EF1a-DDrtTA_TRE3G-DDmCherry model

$$\frac{dx_1}{dt} = \gamma_1 - d_1 x_1 - d_2 \left(\frac{K_3^{h_3}}{K_3^{h_3} + TMP^{h_3}} \right) x_1 \quad (10)$$

$$\frac{dx_2}{dt} = \alpha_1 + \alpha_2 \left(\frac{x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)}{K_2^{h_2} + x_1^{h_2} \left(\frac{Dox^{h_1}}{K_1^{h_1} + Dox^{h_1}} \right)} \right) - d_3 x_2 \quad (11)$$

$$\frac{dx_3}{dt} = \gamma_2 x_2 - d_4 x_3 - d_2 \left(\frac{K_3^{h_3}}{K_3^{h_3} + TMP^{h_3}} \right) x_3 \quad (12)$$

Supplementary Table 2: Parameters identified for the EF1a-DD::rtTA_TRE3G-mCherry and EF1a-DD::rtTA_TRE3G-DD::mCherry systems.

Parameter	**Description**	**Value**
γ_1[a.u. hrs^{-1}]	Production rtTA protein	0.37
d_1[hrs^{-1}]	Degradation rate rtTA protein	0.51
α_1[a.u.hrs^{-1}]	Basal activity TRE3G	0.47
α_2 [a.u.hrs^{-1}]	Maximal transcription rate TRE3G promoter	73.70
K_1 [ng/mL] EF1a-DDrtTA tTA_TRE3G-mCherry/ TRE3G-DDmCherry	Doxy Michaelis-Menten constant	103
K_2 [a.u.]	TRE3G Michaelis-Menten constant	0.91
h_1	Hill coefficient for Doxy	2.34
h_2	Hill coefficient of the TRE3G promoter	6.21
d_3[hrs^{-1}]	Degradation rate mCherry mRNA	72.09
γ_2[hrs^{-1}]	Production rate mCherry protein	0.83
d_4[hrs^{-1}]	Degradation rate mCherry protein	0.161
d_2[hrs^{-1}]	TMP-dependent degradation rate	0.14

	DDmCherry protein and DDrtTA protein	
K_3 [ng/mL]	TMP Michaelis-Menten constant	0.70
h_3	Hill coefficient for TMP	1.05

Supplementary Note 2: Microfluidics/microscopy-based time lapses

Microfluidics/microscopy-based experiments were performed using the device designed and optimised for mammalian cells in the laboratory of Prof Jeff Hasty at the University California in San Diego[1,2]. The topology of the device ensures controlled flow perfusion, minimised cell stress and controlled CO_2 diffusion. As described in[1], cells from a sub-confluent 60cm petri dished were washed with sterile Phosphate-Buffered Saline (PBS, Gibco), trypsinised for 2-3' at room temperature and centrifuged at 8000 xg for 5'. Pelleted cells were resuspended in 200uL of complete mESC media supplemented with Doxy/TMP (Figs. 3a-c, e, 4e and Supplementary Fig. 4f; Supplementary Movies 1-3, 5, 7, 8) or Doxy (Fig. 3d, f; Supplementary Movies 4, 6) and chip-loaded. Before loading, the chip was fulfilled with media containing Doxy/TMP (Figs. 3a-c, e, 4e and Supplementary Fig. 4f; Supplementary Movies 1-3, 5, 7, 8) or Doxy (Fig. 3d, f; Supplementary Movies 4, 6) from port 5 first and port 1 after. Cell suspension was loaded from port 1 while the vacuum applied to ports 3 and 4. The vacuum allows air to be released from chambers, facilitating cell trapping. Cells were cultured for 24hrs in a tissue culture incubator (5%CO2, 37◦C) under constant perfusion with Doxy (Fig. 3d, f; Supplementary Movies 4, 6) or Doxy/TMP (Figs. 3a-c, e, 4e and Supplementary Fig. 4f; Supplementary Movies 1-3, 5, 7, 8) containing media. Media was perfused with a syringe directly connected to port 2 *via* 24-gauge PTFE tubing (Cole-Parmer Inc.). Port 5 was used for waste media whereas ports 1, 6 and 7

were plugged to avoid media spillage. The day after, the device was transferred on the widefield microscope for *in-silico* feedback control experiments. The actuation system consists of two motor-controlled syringes (http://biodynamics.ucsd.edu/dialawave/) connected to port 6 and 7. One syringe contains Doxy- (Fig. 3a, d, f; movies 1, 4, 6) or TMP- (Fig. 3b; Supplementary Movie 2) or Doxy/TMP- (Figs. 3c, e, 4e and Supplementary Fig. 4f; Supplementary Movies 3, 5, 7, 8) enriched media, whereas the other contains plain media (Figs. 3c-f, 4e and Supplementary Fig. 4f.; Supplementary Movies 3-8), or TMP- (Fig. 3a; Supplementary Movie 1) or Doxy- (Fig. 3b; Supplementary Movie 2) supplemented media, depending on experimental set-up. Ports 1, 2 and 5 are also connected to static syringes working as waste tanks. If the height of the waste tanks is fixed, the one of the perfusing media ports is automatically adjusted during the experiment to change the input provided to cells. Input administration was measured using a green dye (1μM Atto488 dye from ThermoFisher), added to Doxy/TMP or Doxy containing syringe.

Image segmentation

The segmentation algorithm follows the methodology illustrated in[1]. Briefly, a threshold is defined to generate a binary image selecting only pixels belonging to cell edges. Then, by using dilation and filling operators, it derives a binary image (mask) that selects the portion of the original image covered by cells. The mask obtained is applied to the red field image. In order to calculate the average intensity fluorescence of pixels belonging to cells, the background signal measured in a cell-free portion of the chamber is subtracted from the value of mask pixels.

Fluorescence microscopy

The control platform consists of a Leica DMi8 inverted microscope equipped with the digital camera AndoriXON 897 ultra back-illuminated EMCCD (512x512 16µm pixels, 16 bit, 56 fps at full frame), and an environmental control chamber (PeCon) for long-term temperature control and CO2 enrichment. The Adaptive Focus Control (AFC) ensures focus is maintained during the entire time-course experiment. The experimental set-up includes consecutive acquisition in three channels (phase contrast, green and red fluorescence) with a 20X objective every 60mins. Of note, for the control experiments in Fig. 3d, f; Supplementary Movies 4, 6, the power lamp was increased from 33 to 50% given the lower cell fluorescence of EF1a-rtTA_TRE3G-DDmCherry mESCs activated with Doxy only (Supplementary Fig. 2d, main text).

Relay control

The Relay control strategy can be expressed as follows:

$$u(t) = \begin{cases} u_{MAX} = 1 & if \quad e(t) > 0 \\ u_{MIN} = 0 & if \quad e(t) < 0 \end{cases}$$

where the control error $e(t) = r(t) - y(t)$ is the difference between the control reference signal r and the system output y; u is the control input. The Relay controller requires only the computation of the control error at each sampling time $e(kT)$, where $T = 60$ mins, whose sign dictates which input must be provided to the cells. Specifically, cells are treated with inducers-containing medium for the next 60mins if $e(kT) > 0$ or medium without inducer otherwise. A 5% hysteresis interval to the controller, corresponding to a tolerance interval around the set-point in which the Relay algorithm ignores the control error, was added to avoid chattering[1].

In control experiments, we controlled EF1a-rtTA_TRE3G-DDmCherry mESCs using differently control inputs: Doxy (1000ng/mL) in Fig. 3a, d, f; TMP (100nM) in Fig. 3b;

combination of Doxy (1000ng/mL) and TMP (100nM) in Fig. 3c, e and Supplementary Fig. 4f; combination of Doxy (100ng/mL) and TMP (10nM) in Fig. 4e.

The Relay control strategy, although being simple, succeeds in keeping the system output close to the desired static or varying reference. Typically, the controlled variable oscillates around the reference and it is acceptable if the oscillation amplitude is sufficiently small[3].

Control Performance Indexes

To measure the performance of the control strategies adopted, we considered the following metrics defined as integral expressions related to the control defined above.

1. The Integral of Squared Error (ISE)

$$ISE = \int_0^\infty [e(t)]^2 \, dt$$

ISE integrates the square of the error over time penalising large errors more than smaller ones.

2. The Integral Absolute Error (IAE)

$$IAE = \int_0^\infty |e(t)| dt$$

IAE integrates the absolute error over time without adding weight to any of the errors in a systems response.

3. The Integral Time-weighted Absolute Error (ITAE)

$$ITAE = \int_0^\infty t|e(t)| dt$$

ITAE integrates the absolute error multiplied by the time over time. It is a weighted version of IAE. It weights errors that exist after a long time much more heavily than those at the start of the response.

Supplementary Table 3: Parameter Indexes calculated for the control experiments in Figs. 3a-f (Supplementary Movies 1-6), 4e (Supplementary Movie 8) and Supplementary Fig. 4f (Supplementary Movie 7).

Figure/ Supplementary Movie	**ISE**	**IAE**	**ITAE**
Fig. 3a, movie 1	6.56	10.2939	83.4885
Fig. 3b, movie 2	3.2176	58.8588	7.5369
Fig. 3c, movie 3	0.7159	2.3828	12.7121
Fig. 3d, movie 4	0.3188	1.9998	14.3546
Fig. 3e, movie 5	0.3772	1.9926	18.4638
Fig. 3f, movie 6	1.0318	3.9879	42.6792
Fig. 4e, movie 8	0.839	2.73	12.5834
Supplementary Fig. 4f, movie 7	6.7844	11.5592	101.7628

Supplementary references

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