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Supplemental information

Multi-range ERK responses shape the proliferative trajectory of single cells following oncogene induction

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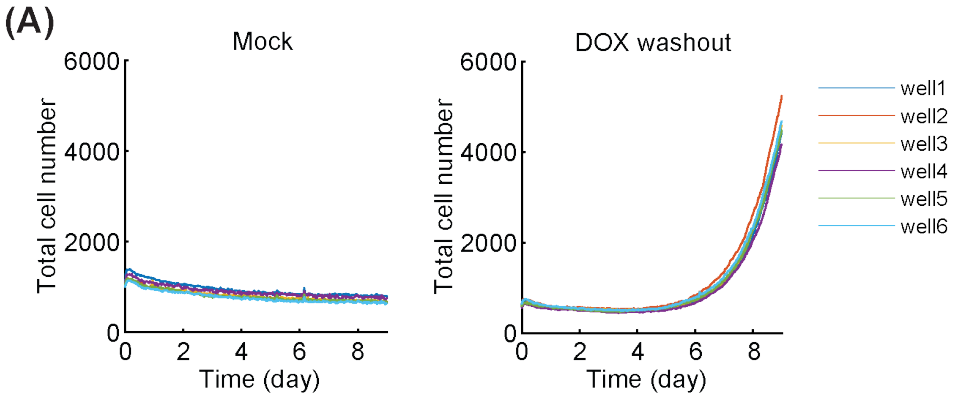
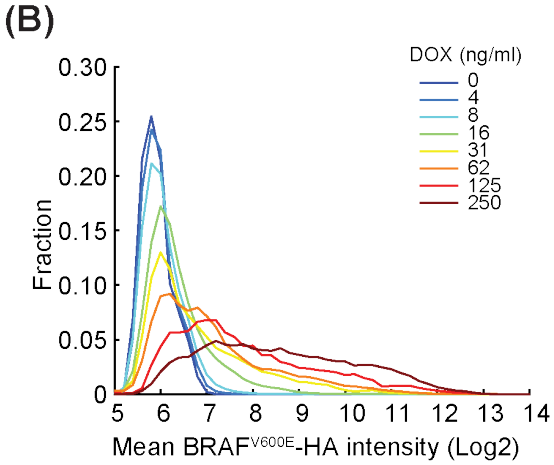
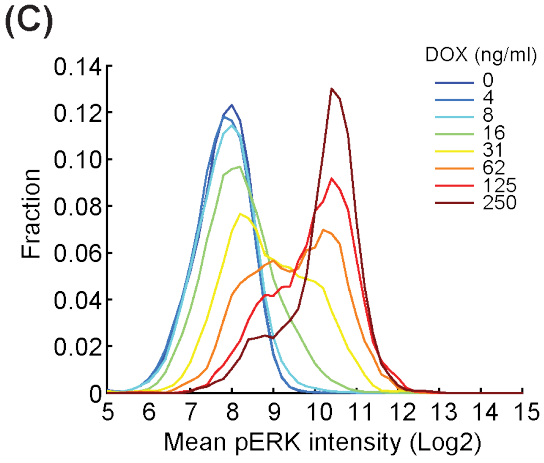
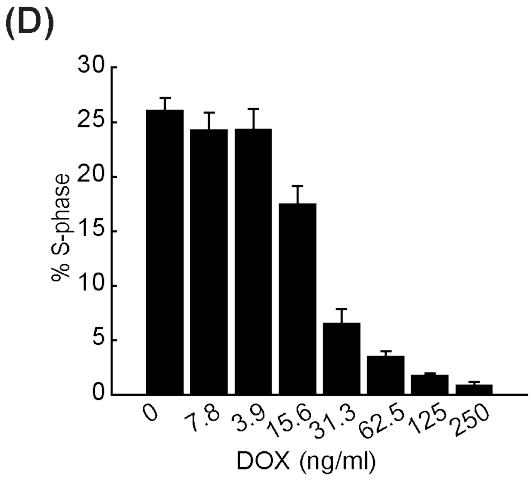
Supplemental Figures and Legends









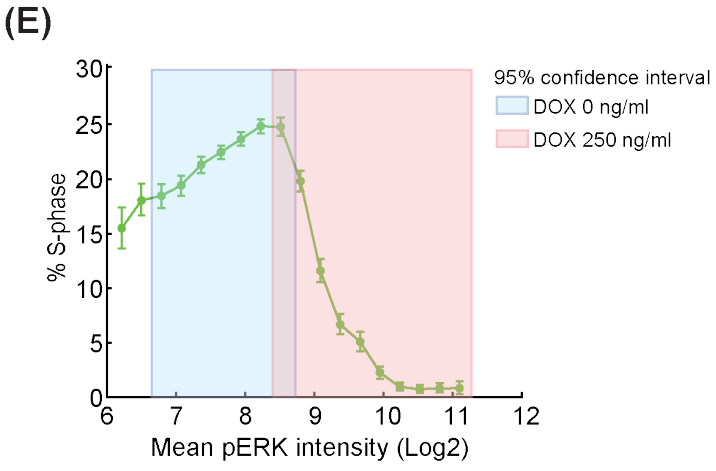


Figure S1. Tunable expression of $BRAF^{V600E}$ combined with heterogeneity in ERK activation and proliferation achieves a wide range of downstream responses. Related to Figure 1.

(A) Total cell numbers over time following mock (DOX present at all time) or DOX washout at time 0 in RPE/tet-$BRAF^{V600E}$ cells pre-treated with DOX (250 ng/ml) for 8 days. Cells were imaged every 45 min for 8 days after DOX washout. Each line represents a single well in a 96-well plate.

(B & C) Frequency plots showing $BRAF^{V600E}$ (A) and pERK (B) distribution following DOX treatment. RPE/tet-$BRAF^{V600E}$ cells were treated with serial doses of DOX (0-250 ng/ml, 2-fold dilution) for 72 h and immunostained for HA and pERK.

(D) RPE/tet-BRAFV600E cells were pulsed with EdU for 30 min after 72 h of DOX treatment and the percent of cells in S phase (% S) was quantified and plotted as a function of the DOX dose.

(E) Ranges of proliferation (percent of cells in S phase) observed at lower pERK levels in the absence of DOX or at higher pERK levels induced by DOX (250 ng/ml) 72 h post treatment. Shaded area shows a 95% confidence interval of pERK intensity in cells treated with 0 or 250 ng/ml DOX.

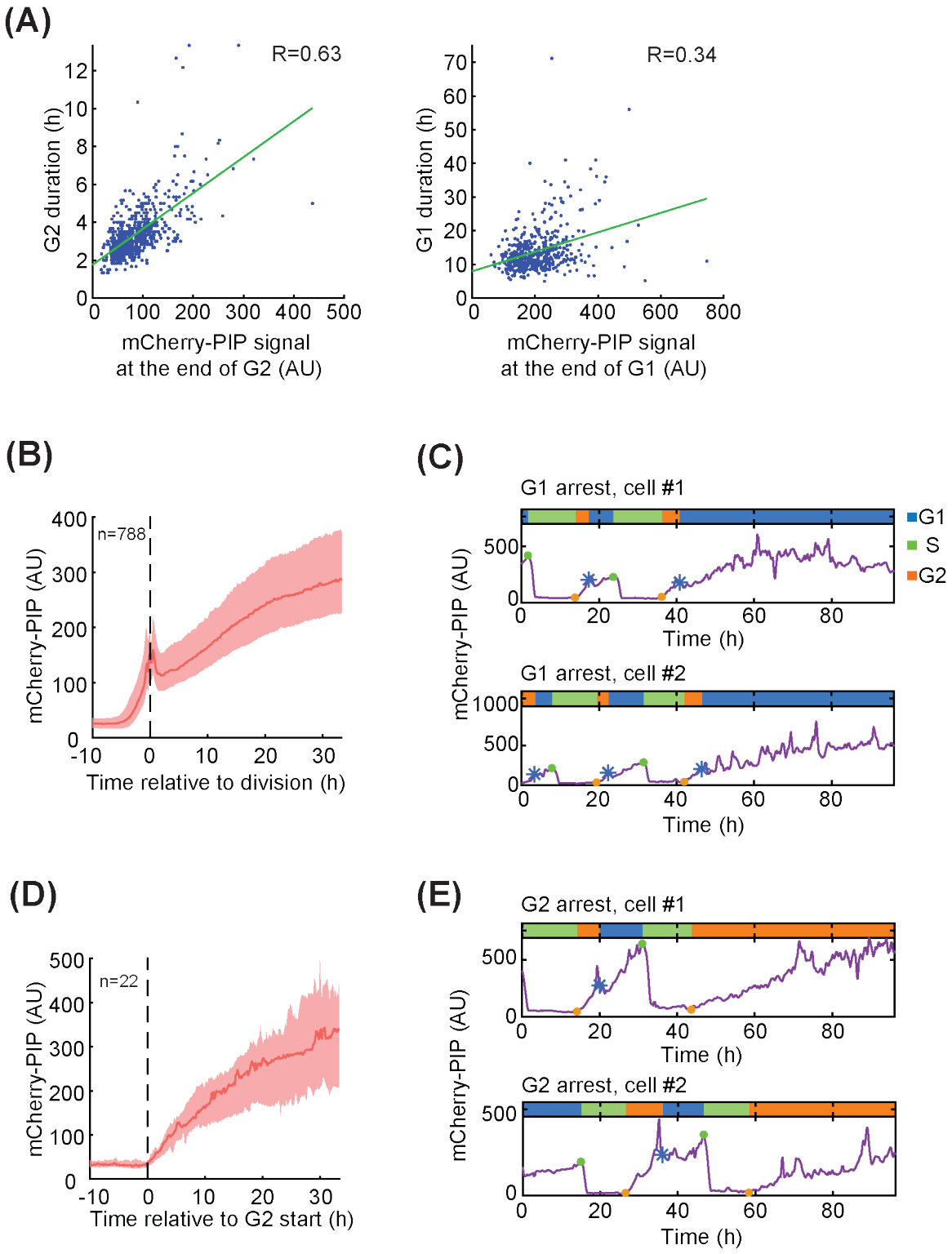

(A)
R=0.63
G2 duration (h)
mCherry-PIP signal
at the end of G2 (AU)
R=0.34
G1 duration (h)
mCherry-PIP signal
at the end of G1 (AU)
(B)
n=788
mCherry-PIP (AU)
Time relative to division (h)
(C)
G1 arrest, cell #1
G1 arrest, cell #2
mCherry-PIP (AU)
Time (h)
G1
S
G2
(D)
n=22
mCherry-PIP (AU)
Time relative to G2 start (h)
(E)
G2 arrest, cell #1
G2 arrest, cell #2
mCherry-PIP (AU)
Time (h)

Figure S2. S-phase reporter accumulates with increasing G1 and G2 lengths. Related to Figure 2.

(A) mCherry-PIP signal at the end of G2 (left, n=607 cells) or G1 (right, n=458 cells) in single cells plotted as a function of G2 or G1 duration, respectively. RPE cells stably expressing mCherry-PIP were imaged for 96 h. Single cell traces of mCherry-PIP were quantified and used in combined with nuclear divisions to derive G1 and G2 lengths. Each dot represents a single cell. The weak but significant correlation between mCherry-PIP signal and the G1 or G2 duration indicates that mCherry-PIP reporter accumulates with increasing G1 or G2 duration.
(B) S-phase reporter dynamics following G1 arrest. $BRAF^{V600E}$ Dual Reporter cells (see also **Fig 3B-D** & **Fig S3A-F**) were treated with DMSO, 62.5, or 500 nM ERKi in the absence or presence of DOX at 24 h after the start of imaging and imaged for an additional 3 days. Cells remaining longer than 30 h in G1 phase at the end of imaging were defined as G1-arrested. mCherry-PIP traces from G1 arrested cells were pooled together and *in silico* synchronized at the latest mitosis prior to the G1 arrest. Bold lines and shaded areas correspond to median and interquartile range, respectively (n=788).
(C) Two representative single-cell traces of mCherry-PIP (with DOX added at 24 h after the start of imaging) in G1-arrested RPE cells. Blue star indicates cell division and the start of G1 phase. Green and orange dots mark the start of S and G2 phases, respectively. Respective length of each cell cycle phase is shown above.
(D) S-phase reporter dynamics following G2 arrest. $BRAF^{V600E}$ Dual Reporter cells were treated as in **(B)** and cells that remained longer than 30 h in G2 phase at the end of imaging were defined as G2-arrested. mCherry-PIP traces from G2 arrested cells were pooled together and *in silico* synchronized at the start of G2 phase prior to the G2 arrest (n=22).
(E) Two representative G2-arrested cells depicted as in **(C)**.

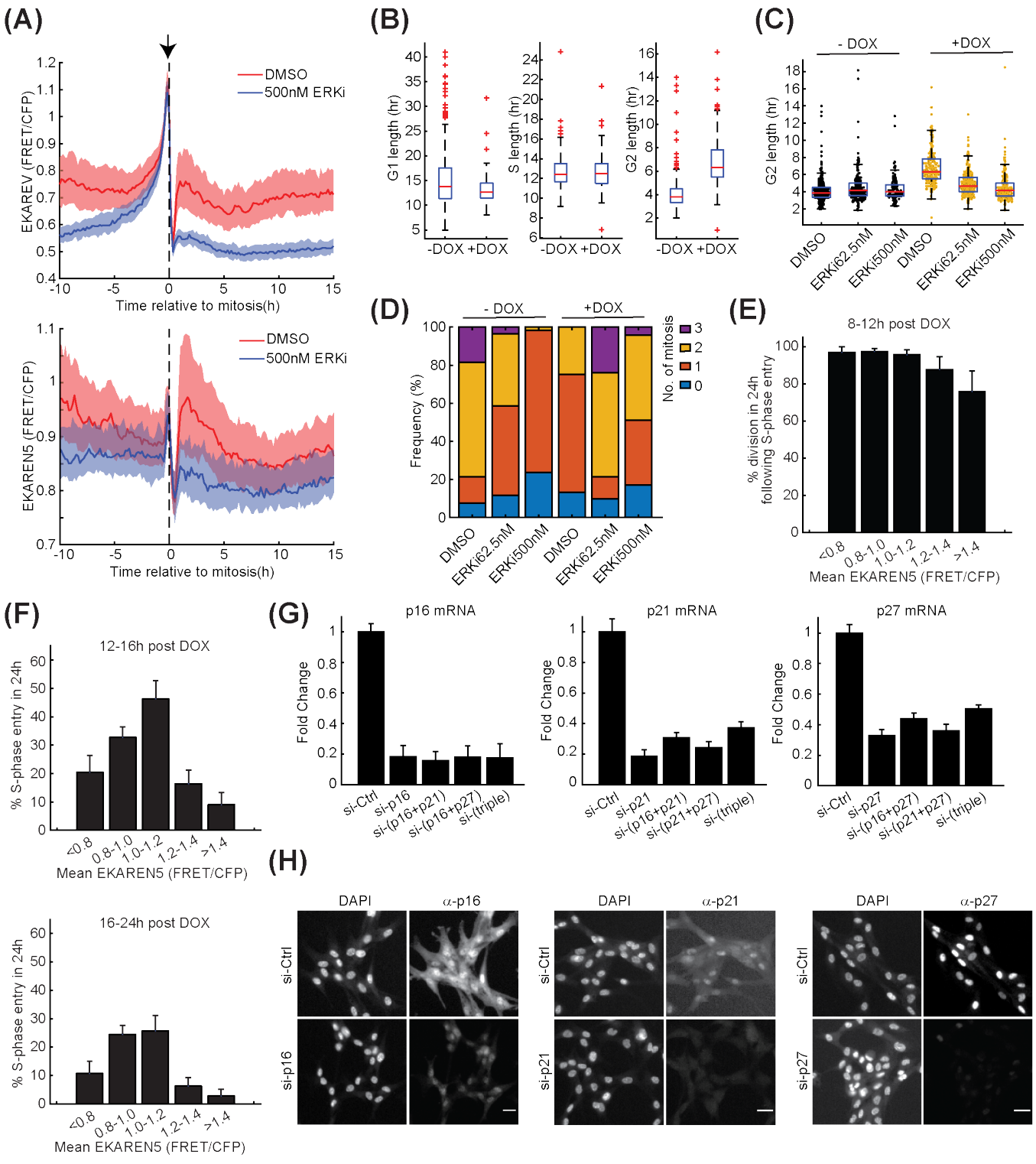


Figure S3. ERK activation lengthens G2 duration and alters proliferation in a non-monotonic fashion. Related to Figure 3.

(A) Dynamics of ERK activity measured by the traditional EKAREV (top) or the improved EKAREN5 probe (down) in the vicinity of mitosis. RPE cells stably expressing the EKAREV reporter or the EKAREN5 reporter were treated with DMSO or 500nM ERK inhibitor followed by 48 h of live imaging (EKAREV DMSO, n=294; EKAREV ERKi, n=100; EKAREN5 DMSO, n=139; EKAREN5 ERKi, n=48). Cells were *in silico* synchronized at mitosis. Bold lines and shaded areas correspond to median and interquartile range, respectively. Non-specific activity of EKAREV prior to mitosis is indicated with an arrow.

(B) Box plots comparing G1 (left), S (middle) and G2 (right) duration in untreated or DOX-treated $BRAF^{V600E}$ Dual Reporter cells. Duration of each cell cycle phase in individual cells was computationally derived based on the mCherry- PIP reporter. n=260 (-DOX, G1), 61 (+DOX, G1), 334 (-DOX, S), 164 (+DOX, S), 399 (-DOX, G2), and 236 (+DOX, G2).

(C) Box plots comparing G2 duration in $BRAF^{V600E}$ Dual Reporter cells treated with DMSO, 62.5 nM ERKi or 500 nM ERKi in the absence or presence of DOX. Each dot represents a single cell (n>130 cells per condition).

(D) Frequency of divisions in $BRAF^{V600E}$ Dual Reporter cells treated with DMSO, 62.5, or 500 nM ERKi in the absence or presence of DOX. Individual cells were tracked for 72 h; after division, one daughter cell was randomly selected for further tracking (also see **Fig 3B**). n>200 cells for each condition.

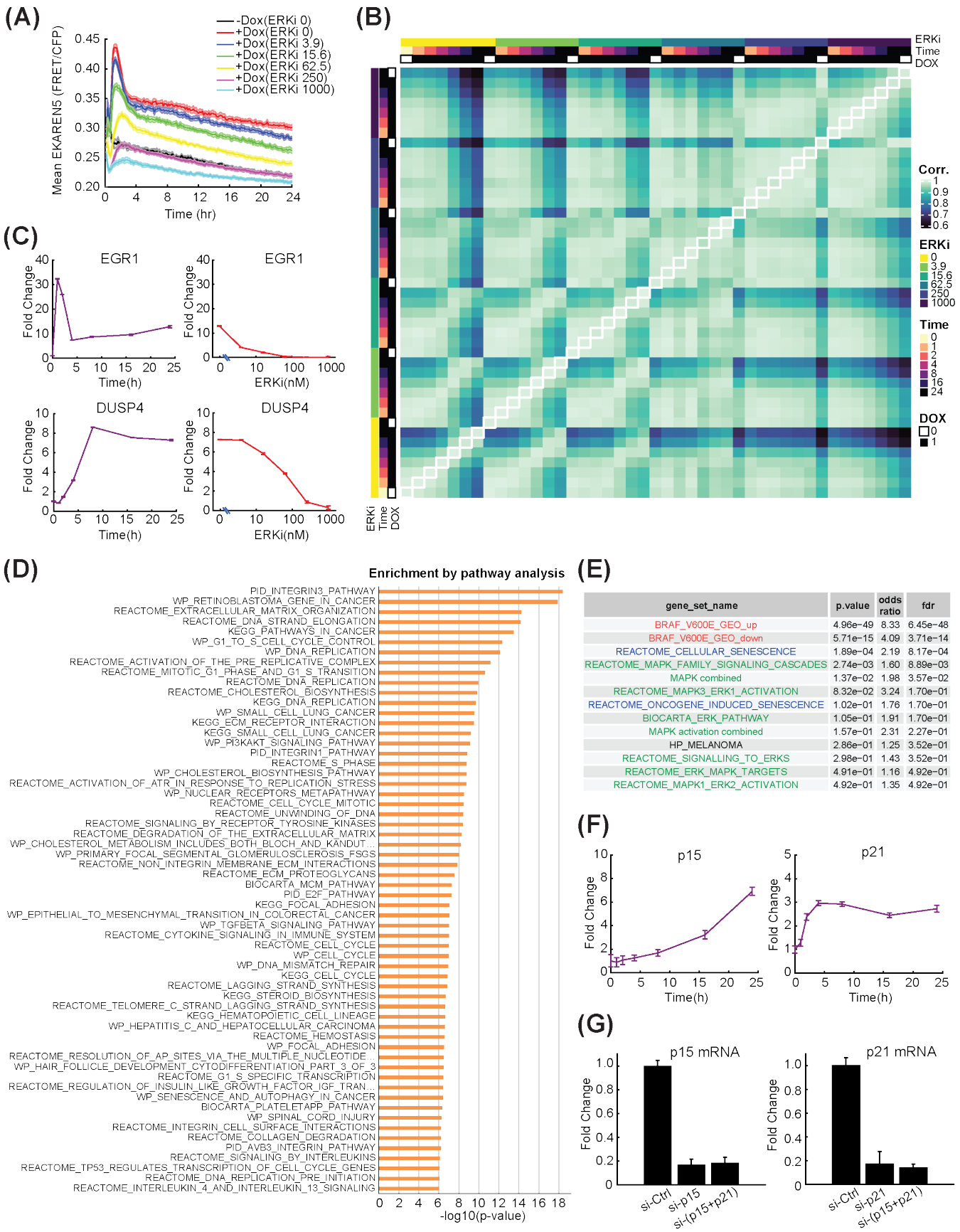
(E) Percent of cells entering S-phase (**Fig 3D**) that subsequently divide. The percent of dividing cells within 24 h after the S-phase entry in each mean ERK activity span (between 8-12h post treatment) shown in **Fig 3D** was quantified. Cells that reached the end of live imaging before 24 h following S-phase entry without dividing were not counted (mean ± 95% confidence interval, n=33, 309, 186, 57 and 54 cells per ERK activity bin).

(F) Percent of S-phase entry in response to increasing ERK activity. Similar to **Fig 3D**, data from all treatments were pooled together and the mean ERK activity between 12-16 h and 16-24 h post treatment was calculated. The percent of cells entering into S-phase was quantified within 24 h after the time-frame in which ERK activity was monitored (mean ± 95% confidence interval, n>150 for each binning ERK activity).

(G) p16, p21 and p27 mRNA levels after siRNA-mediated depletion of the indicated CDK inhibitors (individually or in combination) in RPE/tet-$BRAF^{V600E}$ cells (mean ± SD, n=4 replicates). The values were normalized to HPRT and reported relative to control knockdown

cells (si-Ctrl). Cells were transfected with siRNAs for 24 h and then treated with DOX for 2 days before subjecting to RNA isolation and qPCR.

(H) RPE/tet-BRAF^{V600E}-HA cells were transfected with the indicated siRNA for 24 h and treated with DOX for 2 days before fixation and immunostaining. Scale bars, 20μm.



gene_set_name	p.value	odds ratio	fdr
BRAF_V600E_GEO_up	4.96e−49	8.33	6.45e−48
BRAF_V600E_GEO_down	5.71e−15	4.09	3.71e−14
REACTOME_CELLULAR_SENESCENCE	1.89e−04	2.19	8.17e−04
REACTOME_MAPK_FAMILY_SIGNALING_CASCADES	2.74e−03	1.60	8.89e−03
MAPK combined	1.37e−02	1.98	3.57e−02
REACTOME_MAPK3_ERK1_ACTIVATION	8.32e−02	3.24	1.70e−01
REACTOME_ONCOGENE_INDUCED_SENESCENCE	1.02e−01	1.76	1.70e−01
BIOCARTA_ERK_PATHWAY	1.05e−01	1.91	1.70e−01
MAPK activation combined	1.57e−01	2.31	2.27e−01
HP_MELANOMA	2.86e−01	1.25	3.52e−01
REACTOME_SIGNALLING_TO_ERKS	2.98e−01	1.43	3.52e−01
REACTOME_ERK_MAPK_TARGETS	4.91e−01	1.16	4.92e−01
REACTOME_MAPK1_ERK2_ACTIVATION	4.92e−01	1.35	4.92e−01

Figure S4. ERK activity and pairwise correlations between RNA-seq replicates as a function of time and ERK inhibitor dose, gene set enrichment analysis of ERK-responsive genes, and p15 and p21 expression profiles upon $BRAF^{V600E}$ induction. Related to Figure 4.

(A) Dynamics of ERK activity following DOX and ERK inhibitor treatments. $BRAF^{V600E}$ Dual Reporter cells were treated with DMSO (ERKi 0) in the absence or presence of DOX or with different concentrations of ERK inhibitor in the presence of DOX followed by 24 h of live imaging (median ± SD of fourteen imaging positions).

(B) Heatmap of pairwise Pearson correlation coefficients between all RNA-seq replicates, with one replicate on the x-axis and the other on the y-axis. Replicates with the same treatments are marked with white stroke. In the margins of the heatmap, the treatment of samples with ERK inhibitor, doxycycline, and time of collection are shown. Correlations are computed using normalized gene counts from DESeq2, considering only the 1000 most differentially expressed genes.

(C) Relative *EGR1* and *DUSP4* transcripts levels as measured by qPCR in response to variable time of DOX treatment (left) or variable ERKi concentrations in the presence of 250 ng/ml DOX for 24 h (right). Data represent mean ± SD (n=4 replicates). The values were normalized to HPRT and reported relative to target gene levels at time 0 h in the absence of DOX and ERKi treatment.

(D) Gene set enrichment analysis (GSEA) of the 1958 genes that were significantly differentially expressed across all conditions (see **Fig 4E**). Shown are the most enriched MsigDB gene sets in the Hallmark (H), curated pathways (C2: CP), and ontology (C5) categories, excluding Human Phenotype Ontology (HPO). P-values are derived from Fisher's exact test on a contingency table showing the association between membership in the set of differentially expressed genes versus membership in the given external gene sets.

(E) GSEA of selected $BRAF^{V600E}$ and ERK-related gene sets amongst 1958 significantly expressed genes. ERK-related gene sets include all gene sets in MSigDB that mentioned "ERK", "MAPK", "senescence", or "melanoma". $BRAF^{V600E}$ related gene sets include the set of differentially expressed genes from a $BRAF^{V600E}$ overexpression experiment from GEO (GSE46801). Odds ratio, p-value, and FDR adjusted p-value (fdr) are derived from Fisher's exact test as in **(D)**.

(F) Relative p15 and p21 transcripts levels as measured by qPCR over time following 250 ng/ml DOX treatment. Data represent mean ± SD (n=4 replicates). The values were normalized to HPRT and reported relative to target gene data at time 0 h in the absence of DOX treatment. **(G)** Bar plots showing knockdown efficiency after siRNA-mediated depletion of the indicated CDK inhibitors (individually or in combination) in RPE/tet-BRAF^{V600E} (mean ± SD, n=4 replicates). Cells were transfected with siRNAs for 24 h and then treated with DOX for 2 days before subjecting to RNA isolation and qPCR.

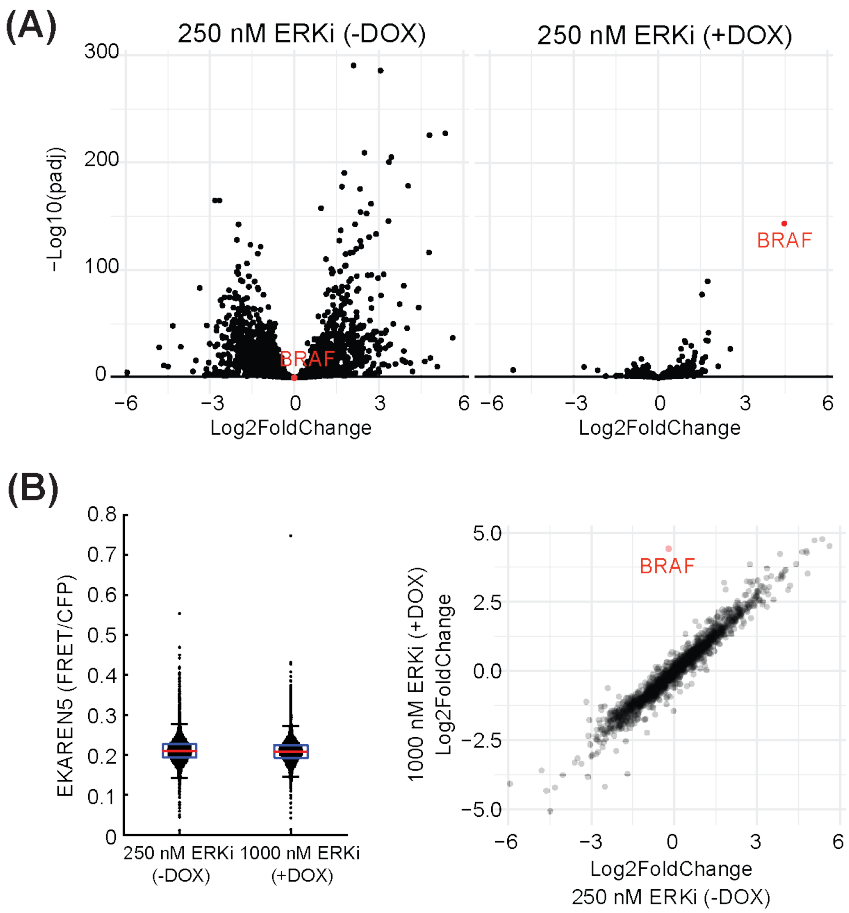


Figure S5. Rescue of ERKi effects on gene expression by BRAF^{V600E} overexpression. Related to Figure 5.

(A) Volcano plots of the differential gene expression between two treatment conditions and the untreated control condition. On the left, inhibition with 250 nM ERKi results in large changes in gene expression (438 genes; absolute log2FoldChange > 1; $p < 0.05$). On the right, in addition to 250 nM ERKi, DOX was used to induce BRAF^{V600E} overexpression (highlighted in red). At this concentration of ERKi, its effects were largely rescued by BRAF^{V600E} overexpression, with only 42 differentially expressed genes remaining.

(B) (Left) Box plots showing similar ERK activities in BRAF^{V600E} Dual Reporter cells treated with 250 nM ERKi in the absence of DOX, or 1000 nM ERKi in the presence of 250 ng/ml DOX. The ERK activity of each cell was measured at 24 h post treatment. Each dot represents a single cell (n>5000 cells per condition). (Right) Correlation between the differential gene expression in cells treated with 250 nM ERKi (x-axis) and 1000 nM ERKi plus DOX to induce overexpression of BRAF^{V600E} (y-axis). For each condition, differential gene expression was

calculated in comparison to untreated control cells. The two conditions display extremely similar differential gene expression (R-squared 0.97; $p < 2.2e-16$), indicating that the effect of $BRAF^{V600E}$ overexpression can be rescued by increasing the concentration of the ERK inhibitor.

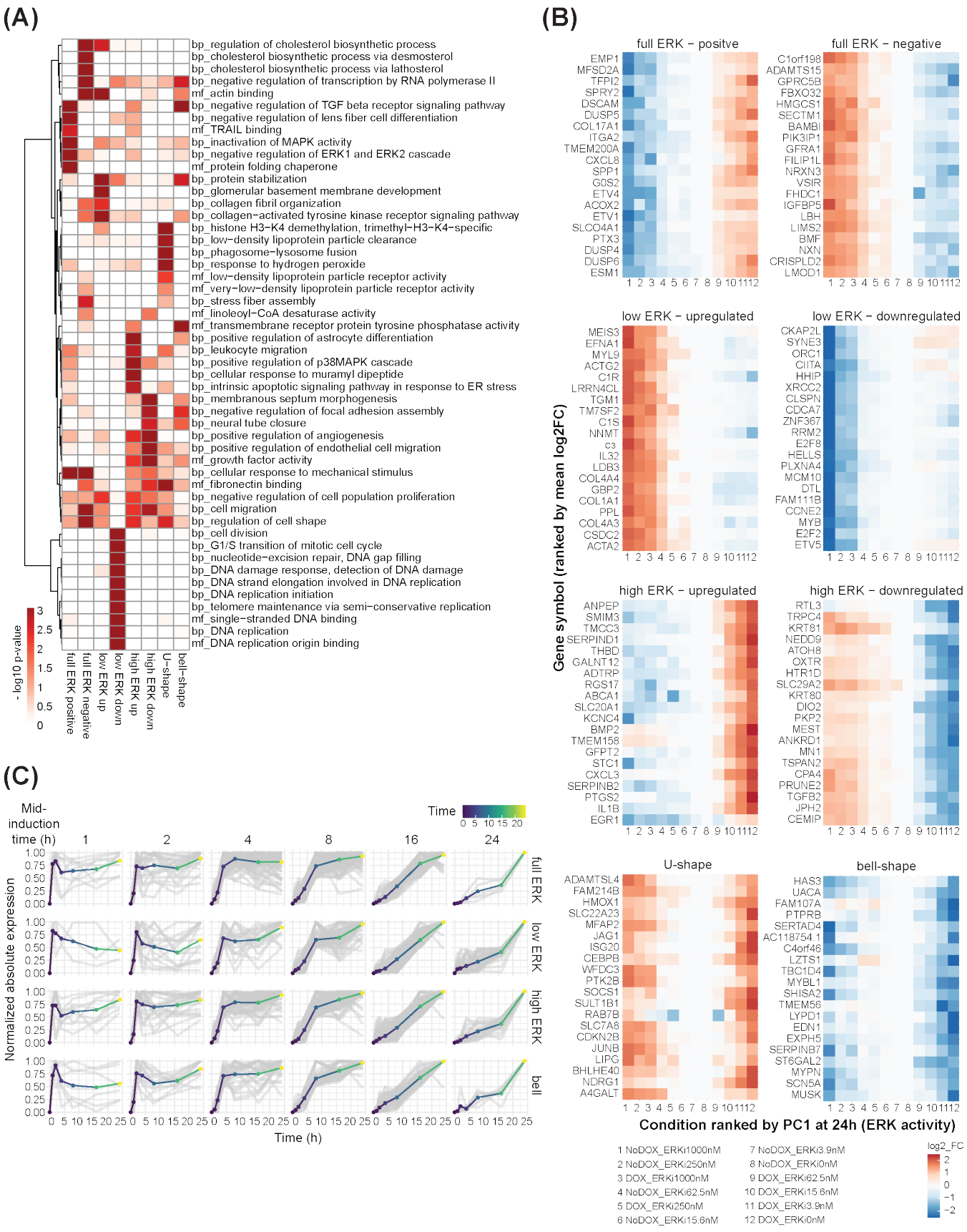

(A)
bp_regulation of cholesterol biosynthetic process
bp_cholesterol biosynthetic process via desmosterol
bp_cholesterol biosynthetic process via lathosterol
bp_negative regulation of transcription by RNA polymerase II
mf_actin binding
bp_negative regulation of TGF beta receptor signaling pathway
bp_negative regulation of lens fiber cell differentiation
mf_TRAIL binding
bp_inactivation of MAPK activity
bp_negative regulation of ERK1 and ERK2 cascade
mf_protein folding chaperone
bp_protein stabilization
bp_glomerular basement membrane development
bp_collagen fibril organization
bp_collagen−activated tyrosine kinase receptor signaling pathway
bp_histone H3−K4 demethylation, trimethyl−H3−K4−specific
bp_low−density lipoprotein particle clearance
bp_phagosome−lysosome fusion
bp_response to hydrogen peroxide
mf_low−density lipoprotein particle receptor activity
mf_very−low−density lipoprotein particle receptor activity
bp_stress fiber assembly
mf_linoleoyl−CoA desaturase activity
mf_transmembrane receptor protein tyrosine phosphatase activity
bp_positive regulation of astrocyte differentiation
bp_leukocyte migration
bp_positive regulation of p38MAPK cascade
bp_cellular response to muramyl dipeptide
bp_intrinsic apoptotic signaling pathway in response to ER stress
bp_membranous septum morphogenesis
bp_negative regulation of focal adhesion assembly
bp_neural tube closure
bp_positive regulation of angiogenesis
bp_positive regulation of endothelial cell migration
mf_growth factor activity
bp_cellular response to mechanical stimulus
mf_fibronectin binding
bp_negative regulation of cell population proliferation
bp_cell migration
bp_regulation of cell shape
bp_cell division
bp_G1/S transition of mitotic cell cycle
bp_nucleotide−excision repair, DNA gap filling
bp_DNA damage response, detection of DNA damage
bp_DNA strand elongation involved in DNA replication
bp_DNA replication initiation
bp_telomere maintenance via semi−conservative replication
mf_single−stranded DNA binding
bp_DNA replication
mf_DNA replication origin binding
full ERK positive
full ERK negative
low ERK up
low ERK down
high ERK up
high ERK down
U−shape
bell−shape
- log10 p-value
3
2.5
2
1.5
1
0.5
0
(B)
full ERK − positve
full ERK − negative
low ERK − upregulated
low ERK − downregulated
high ERK − upregulated
high ERK − downregulated
U-shape
bell-shape
Gene symbol (ranked by mean log2FC)
Condition ranked by PC1 at 24h (ERK activity)
1 NoDOX_ERKi1000nM
2 NoDOX_ERKi250nM
3 DOX_ERKi1000nM
4 NoDOX_ERKi62.5nM
5 DOX_ERKi250nM
6 NoDOX_ERKi15.6nM
7 NoDOX_ERKi3.9nM
8 NoDOX_ERKi0nM
9 DOX_ERKi62.5nM
10 DOX_ERKi15.6nM
11 DOX_ERKi3.9nM
12 DOX_ERKi0nM
log2_FC
2
1
0
−1
−2
(C)
Mid-induction time (h)
Time
Normalized absolute expression
Time (h)
full ERK
low ERK
high ERK
bell

Figure S6. Association of ERK responsive clusters with functional GO enrichment groups, and gene expression trajectories of each cluster over time. Related to Figure 6.

(A) GO term enrichment analysis for genes in all eight ERK response clusters. The 10 most significant GO terms per cluster are shown, with a maximum of 50 clusters in total. Rows are ordered using hierarchical clustering. Terms are prefixed with their GO domain. “mf” corresponds to Molecular Function and “bp” to Biological Function.

(B) Expression of 20 representative genes in each ERK response cluster. All data shown are at the 24 h time point. The x-axis corresponds to the different treatment conditions at 24 h ordered in terms of their ERK activity. Treatment conditions 1-12 are listed below. Genes were selected by picking the 20 genes with the highest “mean” absolute log2 fold change across all conditions at 24 h. Log2 fold changes compared to the control condition were scaled for unit variance but not zero centered.

(C) Gene expression trajectories over time. Gray lines represent normalized expression of individual genes, and colored lines show the average trajectory for the group. Each row represents an ERK-response cluster. Each column represents the time point at which genes in that group first reached half of their maximum induction. Vertically, the panels are divided into the four ERK response clusters that we identified. Gene expression was normalized to the maximum value observed in the dataset, and plotted as an absolute value ranging from 0 to 1.