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Supplemental Information

**Disentangling Pro-mitotic Signaling
during Cell Cycle Progression
using Time-Resolved Single-Cell Imaging**

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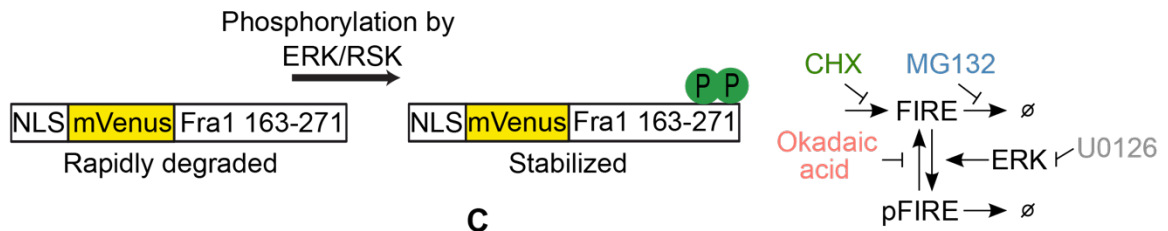
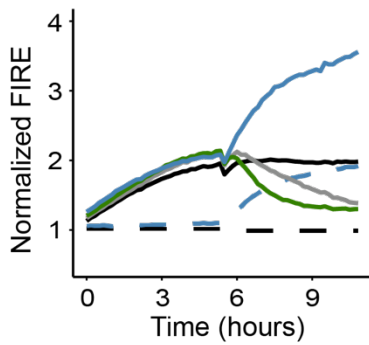
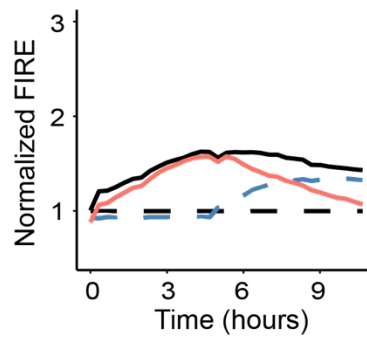
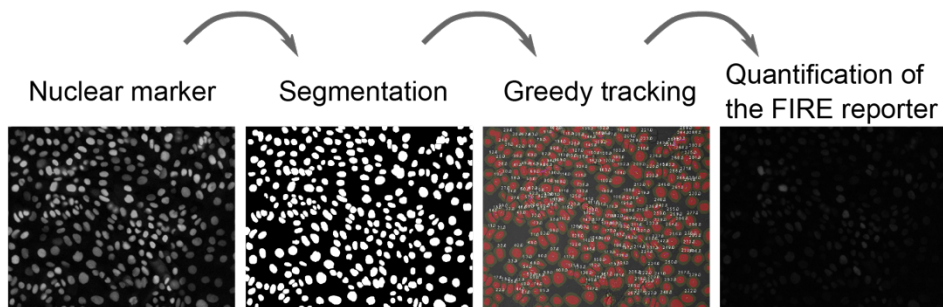
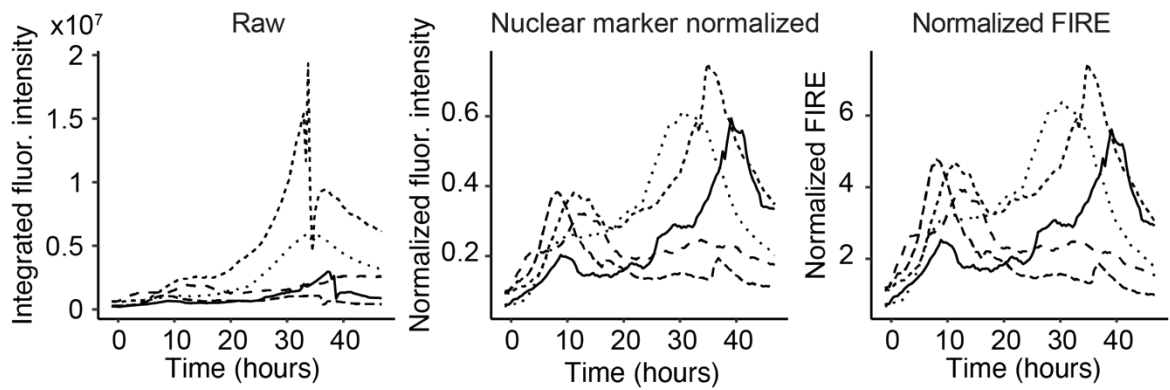
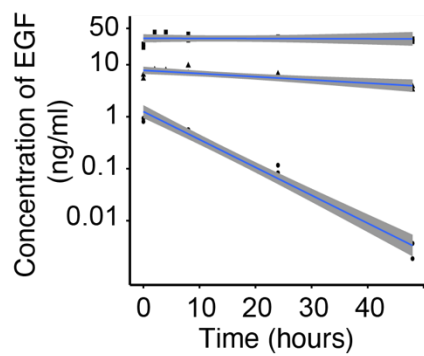
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Figure S1. Measuring ERK activity using the integrative FIRE reporter, related to Figure 1

A Scheme of the FRA1-integrative Reporter of ERK activity (FIRE). The reporter consists of the PEST domain of FRA1 fused to mVenus, a yellow fluorescent protein, and a nuclear localization sequence (NLS). The reporter is constitutively expressed by the MSCV LTR (murine stem cell virus long terminal repeat) and then rapidly degraded. ERK phosphorylates the FRA1 domain at multiple sites and thereby stabilizes the reporter. Validation experiments using pharmacological inhibitor are outlined.

B-C Mean time courses of the FIRE reporter for different perturbations. Control experiments are shown in black (0 ng/ml EGF = dashed line, 50 ng/ml EGF = solid line). Proteasome inhibitor (10 μ M MG132 = blue line), MEK inhibitor (10 μ M U0126 = gray line), or MEK inhibitor in combination with a protein synthesis inhibitor (10 μ M CHX = green line) or a phosphatase inhibitor (2.5 nM okadaic acid = red line) were added.

D Workflow for segmenting and tracking single cells. Cells stably expressed NLS-CFP as a nuclear marker (panel 1), which was used to segment the nucleus (panel 2). For tracking single cells, a customized greedy algorithm was used; in panel 3 tracked cells are labeled with their corresponding identifier. For each tracked cell, the total fluorescence intensity of the FIRE reporter was quantified (panel 4).

E Time courses of three cells are shown to exemplify the normalization procedure (light gray, dark gray and dashed lines). The raw integrated fluorescence intensities for the FIRE reporter in each cell are depicted over time (panel 1). The integrated fluorescence intensity of the FIRE reporter in each cell was normalized by the corresponding fluorescence intensity of the NLS-CFP nuclear marker to remove spurious spikes in single cell trajectories (panel 2). Subsequently, time courses were further normalized by the mean nuclear marker-normalized FIRE level of cells in the absence of EGF stimulation leading to a measure of FIRE induction upon EGF treatment.

F EGF degradation in medium over time. EGF concentration was measured in supernatants of cells initially treated with 1, 7.5 and 50 ng/ml EGF in duplicate at the indicated time points (dots). Blue lines indicate a linear fit on \log_{10} transformed EGF concentrations, gray ribbons standard error.

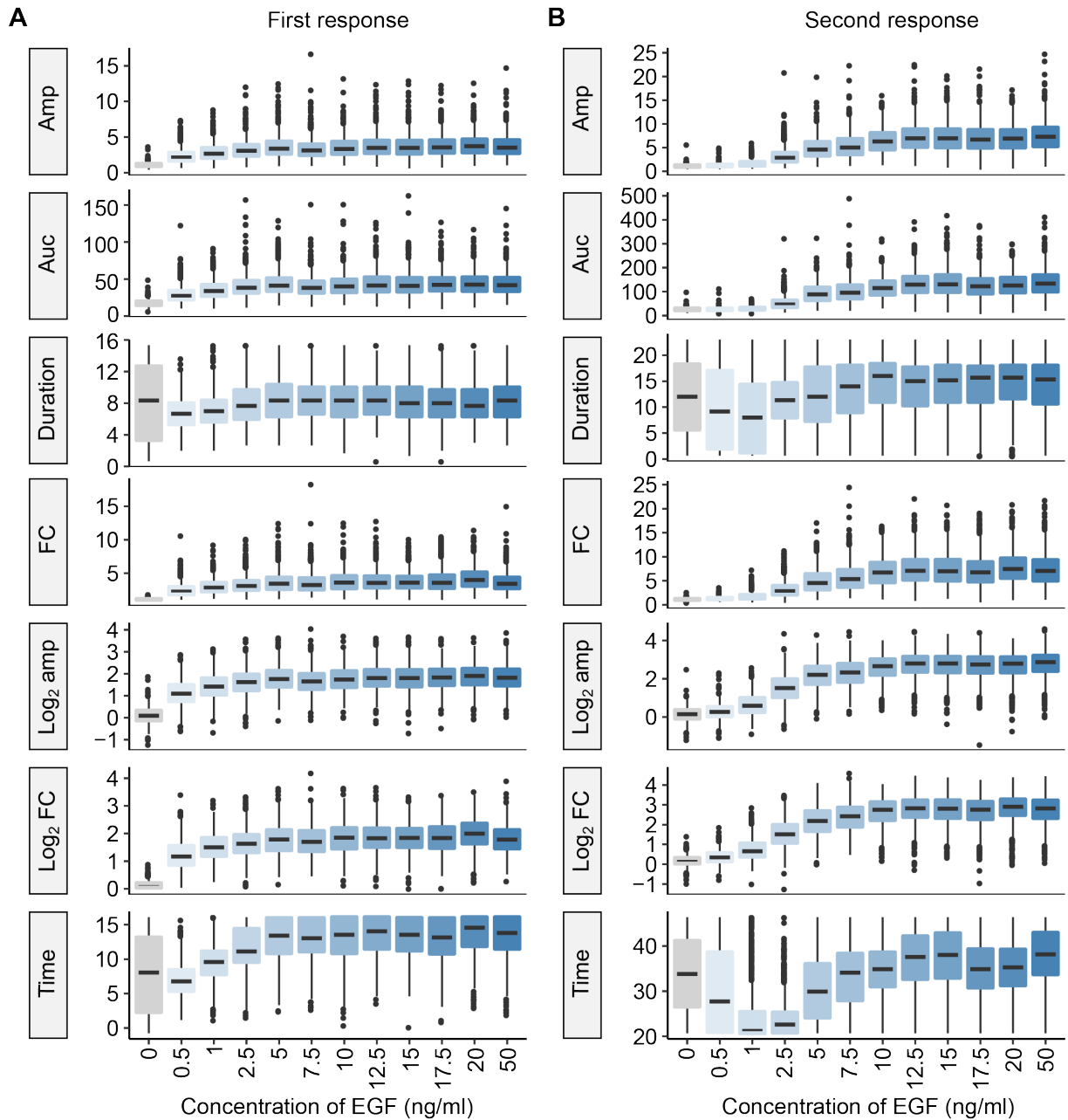


Figure S2. Dynamic features of the bi-phasic ERK activity profile, related to Figure 2

A Shown are box plots comparing the features of first response, namely amplitude (a.u.), time of the maximum (hours), fold-change of the amplitude (a.u.), duration of the response (hours), and area under the curve (a.u.), as well as \log_2 scaled amp and FC. We defined the amplitude as the mean of the three highest FIRE responses after normalization. The fold-change is taken with respect to the mean response of FIRE in the same cell before stimulation. The \log_2 amplitude and the \log_2 fold-change were also included as features. The area under the curve has been estimated using a spline interpolation; the duration is an estimation of full width at half maximum (as defined in the STAR methods section). Black lines indicate medians of distributions; boxes include data between the 25th and 75th percentiles; whiskers extend to the maximum values within 1.5 \times the interquartile range; dots represent outliers.

B Corresponding box plots comparing the features of the second response (as in A).

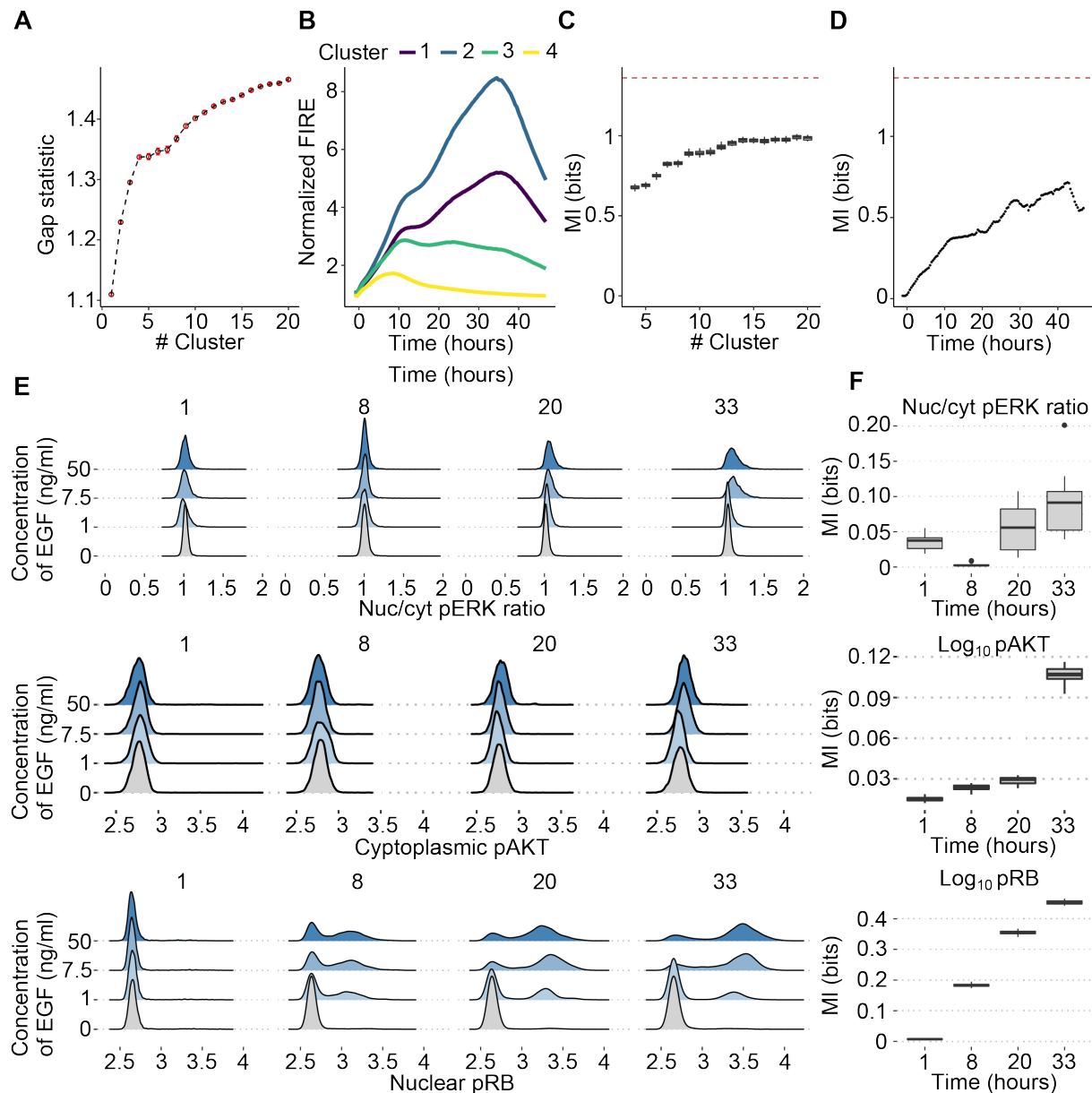


Figure S3. Information theoretical analysis of EGF signaling, related to Figure 2

A The gap statistic (dots) is calculated for each number of cluster (Euclidean distance) to identify the number of clusters, which best separates the time-courses. The error bars (red) are calculated via bootstrapping ($n = 60$).

B Mean time-courses for each cluster ($k = 4$) of all measured cells are indicated by bold lines, cells were stimulated with 12 different concentrations of EGF.

C Mutual information between concentrations of EGF and separation of FIRE reporter time series based on clustering with Euclidean distance. Mutual information was calculated for different number of clusters. The red line indicates the maximum mutual information based on feature analysis.

D Mutual information between concentrations and FIRE response at individual time-points. The red line indicates the maximum mutual information based on feature analysis.

E Distribution of immunofluorescence of pERK (nuc/cyt ratio), pAKT (cytosolic), and pRB (nuclear) at different time-points and for different concentrations of EGF.

F Mutual information between the different concentrations of EGF (see E) and immunofluorescence of pERK, pAKT, or pRB. Distributions are based on bootstrapping ($n = 20$).

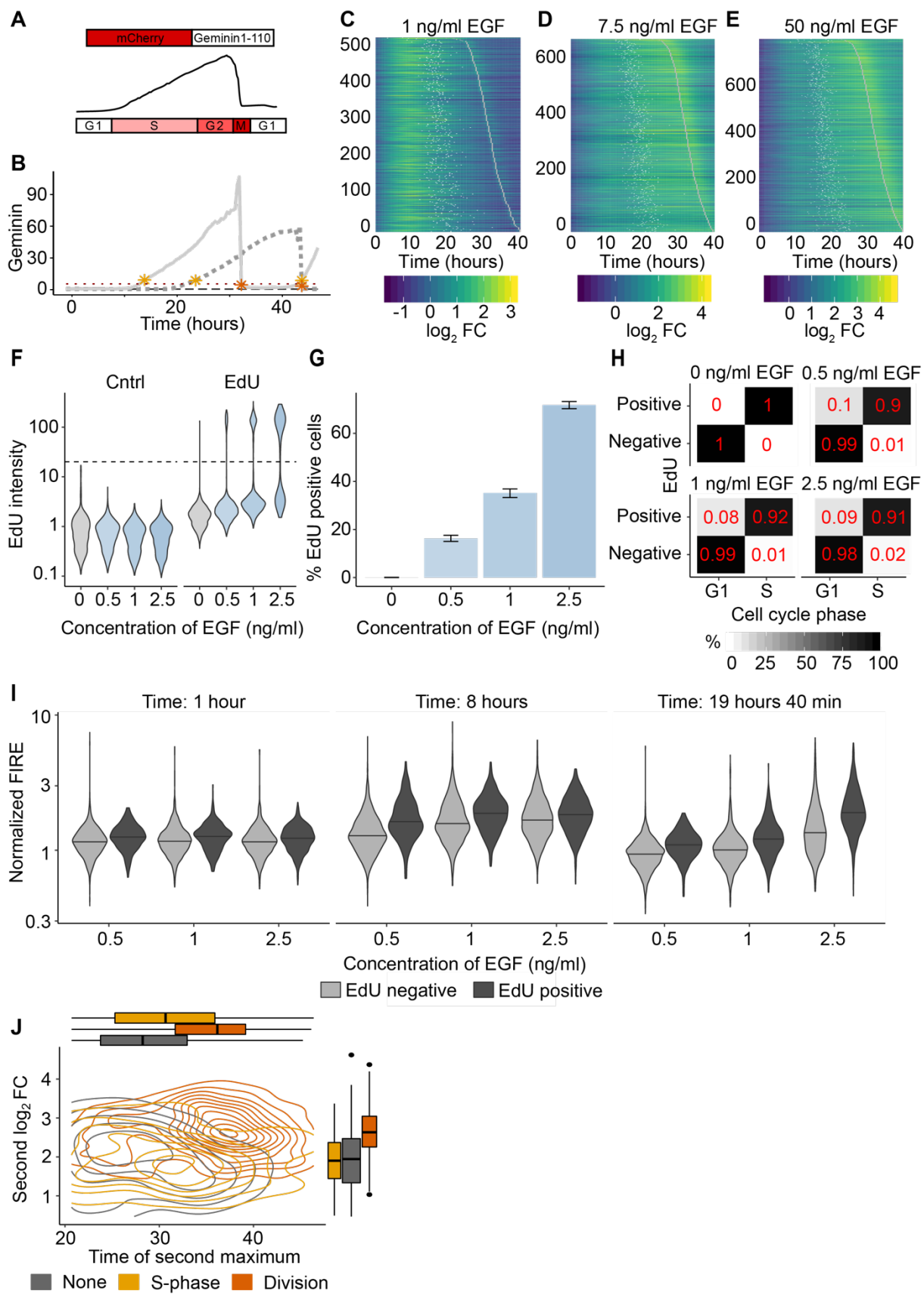


Figure S4. Following cell cycle progression in individual living cells, related to Figure 3

A Scheme of the geminin-based cell cycle reporter. The reporter consists of the first 110 amino acids of geminin fused to a red fluorescent protein. The reporter is kept at low levels during G1, accumulates over S/G2/M-phase and is rapidly degraded at the transition between meta- and anaphase of mitosis (Clijsters et al. 2013).

B Time courses of the normalized geminin reporter for 3 cells are shown to exemplify the detection of cell phases (light gray, dark gray and dashed lines). The red dotted line indicates the threshold for detecting cell cycle phases. Light orange asterisks show the time points for cells entering S-phase and dark orange asterisk show the time points immediately after division.

C-E Heatmap of single-cell reporter time series stimulated with the indicated EGF concentrations. Each line represents a single cell with logarithmic fold-change of the FIRE response (\log_2 FC) visualized according to the indicated color code. The onset of S/G2/M-phase (white) and the time of division (gray) are highlighted. Cells without division are not shown.

F Violin plots showing the EdU intensity (\log_{10} -normalized) distribution. Cells were stained with EdU 20 hours after stimulation with different concentration of EGF. Suggested threshold to separate EDU positive from EDU negative cells is indicated as black dashed line.

G Percent of EDU positive cells at indicated concentrations of EGF. Error bars indicate 95% confidence interval based on bootstrapping ($n = 1000$).

H Comparing EDU negative or positive cells with cells in G1 or S-phase, respectively, according to geminin levels.

G Normalized FIRE intensity at different time-points before EDU staining. Violin plots with EDU positive cells are dark gray and populations with EDU negative cells are light gray. Horizontal lines indicate the median FIRE intensity.

H Contour plot of \log_2 FC of the amplitude for the second response, and for the timing of the second response comparing the different stages in the cell cycle (EGF = 7.5 ng/ml). Marginal distributions are indicated as box plots.

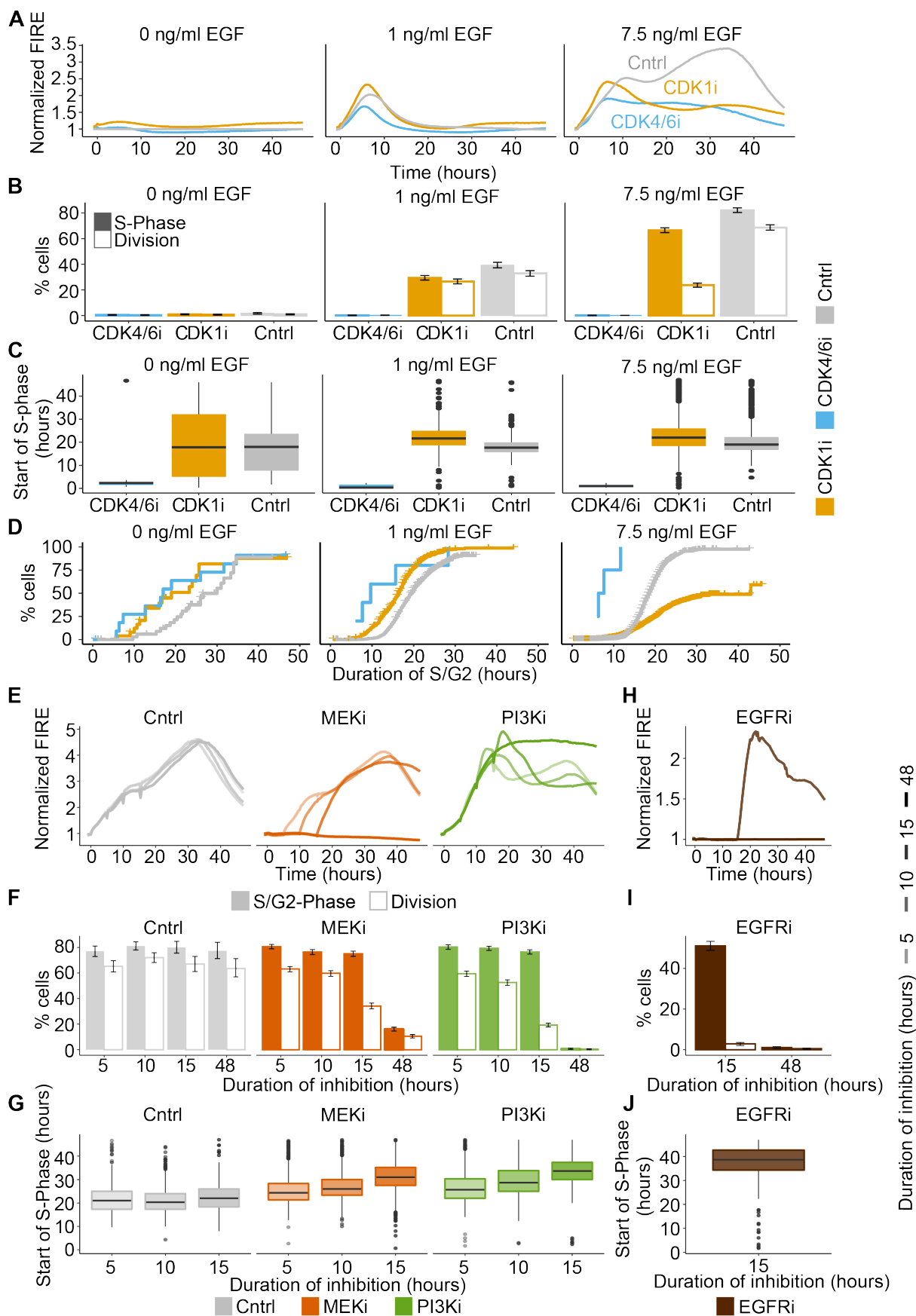


Figure S5. Pharmacological perturbation of EGF signaling, related to Figure 4 and 5

A Average time courses of the FIRE reporter after stimulation with different concentrations of EGF and pre-incubation with CDK inhibitors.

B Effect of CDK inhibitors on the percent of cells entering S-phase (solid bars) or undergoing division (open bars). Error bars indicate 95% confidence interval based on bootstrapping (n = 1000).

C Start of S-phase within 47 h after stimulation with different concentrations of EGF and CDK inhibition. Black lines indicate medians of distributions; boxes include data between the 25th and 75th percentiles; whiskers extend to the maximum values within 1.5× the interquartile range; dots represent outliers.

D Effect of CDK inhibition on the duration of S/G2-phases upon stimulation with different concentrations EGF using Kaplan-Meier analysis.

E Average time courses of the normalized FIRE reporter after stimulation with 7.5 ng/ml EGF alone or in combination with a MEK inhibitor or PI3K inhibitor. The inhibitors were washed from the medium 10 hours, 15hours or 20 hours later.

F Effect of pulsed inhibition on the percent of cells entering S-phase (solid bars) or dividing (open bars). Error bars indicate 95% confidence interval based on bootstrapping (n = 1000).

G Comparing the effect of pulsed inhibition using a MEK or a PI3K inhibitor on the duration of S/G2-phases using Kaplan-Meier analysis.

H Average time course of the normalized FIRE reporter stimulation with 7.5 ng/ml EGF in combination with an EGFR inhibitor. The inhibitor was either kept during the complete experiment (dark) or washed off after 15 hours (light).

I same as **F** for EGFR inhibition.

J same as **G** for EGFR inhibition.

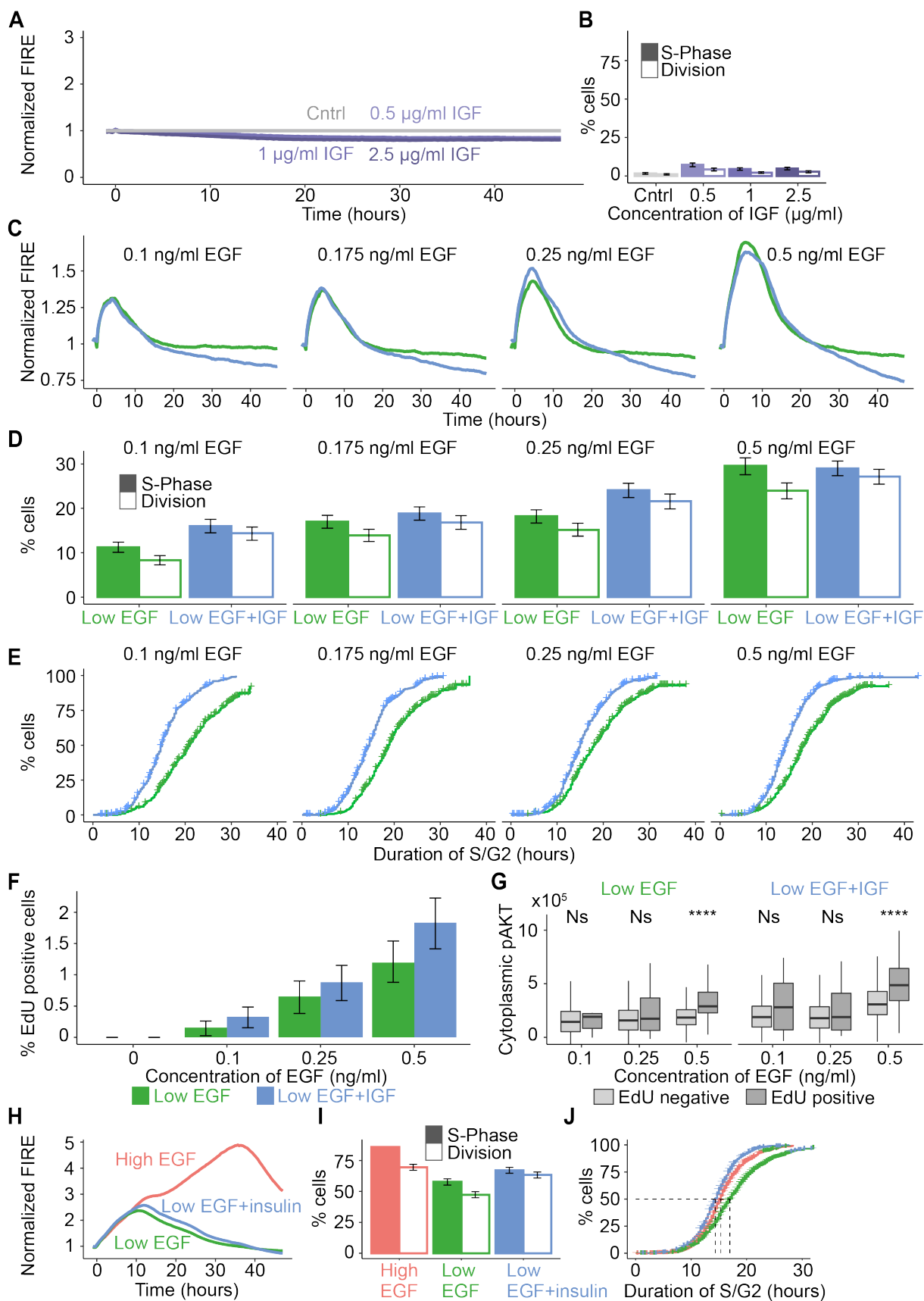


Figure S6. IGF or insulin-mediated activation of AKT rescues delayed cell cycle progression, related to Figure 6

A Average time courses of the FIRE reporter after stimulation with different concentrations of IGF alone.

B Effect of IGF stimulation alone on the percent of cells entering S-phase (solid bars) or undergoing division (open bars). Error bars indicate 95% confidence interval based on bootstrapping (n = 1000).

C Comparing the effect of insulin stimulation on the duration of S/G2-phases using Kaplan-Meier analysis.

D Average time-courses of the FIRE reporter after stimulation with different low concentrations of EGF (green lines) and in addition with 2.5 μ g IGF (blue lines).

E Effect of additional IGF stimulation on the percent of cells entering S-phase (solid bars) or dividing (open bars).

F Comparing the effect of additional IGF stimulation on the duration of S/G2-phases using Kaplan-Meier analysis.

G Percent of EdU positive cells. Cells were first stimulated with different concentrations of EGF (green bars) or in combination with 2.5 μ g IGF (blue bar) and after 20 hours cells were stained with EdU. Error bars indicate 95% confidence interval based on bootstrapping (n = 1000).

H Comparing the distribution of cytoplasmic pAKT levels between EdU negative (light gray) and EdU positive cells (dark gray). P-values based on a t-test are indicated with asterisk (ns: p > 0.05, *: p \leq 0.05, **: p \leq 0.01, ***: p \leq 0.001, ****: p \leq 0.0001).

I Average time courses of the FIRE reporter after stimulation with different concentrations of EGF in comparison to a rescue experiment with additional insulin.

J Effect of insulin stimulation on the percent of cells entering S-phase (solid bars) or dividing (open bars). Error bars indicate 95% confidence interval based on bootstrapping (n = 1000).

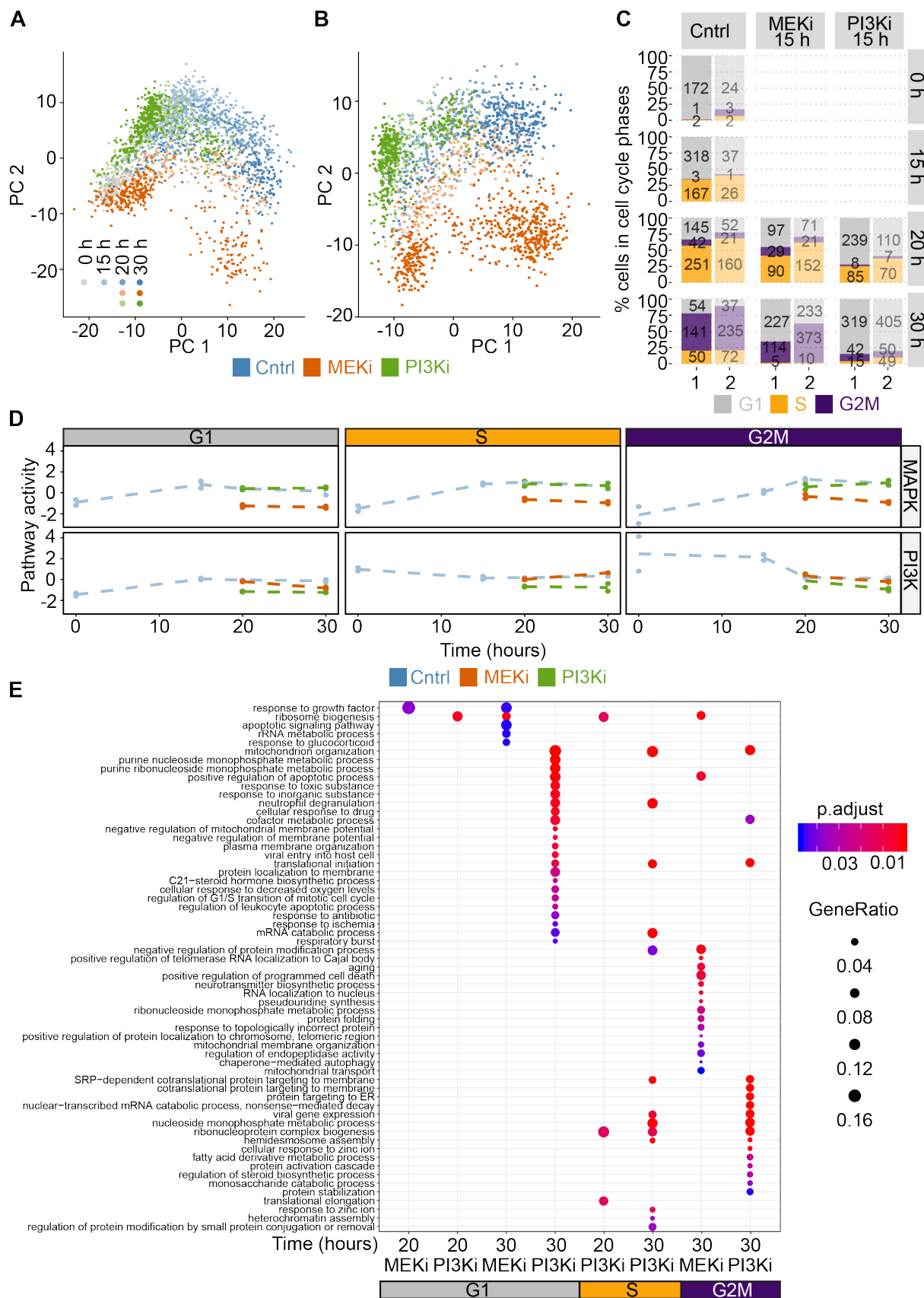


Figure S7. Cell cycle resolved expression analysis of single cells upon mitogenic stimulation, related to Figure 7

A Principle component analysis (PCA) of highly variable genes based on single-cell RNA sequencing from the first replicate. The first two principle components (PC) are plotted; dots indicate individual cells; colors correspond to stimulation with EGF (blue) or additional inhibition with MEK (orange) or PI3K (green) inhibitor. Cells were sampled at different time-points as indicated.

B PCA of highly variable genes based on single-cell RNA sequencing from a replicate experiment.

C Stacked bar plot indicating the number and fractions of cells in G1-phase (gray), S-phase (yellow) and G2M-phase (purple) for different conditions and for two replicates.

D Progeny pathway activity scores for MAPK pathway (upper panel) and PI3K pathway (lower panel) over time and for the different conditions. Each dot indicates one replicate and dashed lines connect the corresponding mean values as guidance for the eye.

E Pathway enrichment using differential gene expression for samples treated with MEK inhibitor or PI3K in comparison to EGF stimulation at the indicated time-points and cell cycle phases.