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Supplemental Information

Ki67 is a Graded Rather than a Binary Marker of Proliferation versus Quiescence

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Figure S1. Ki67 levels across the cell cycle as measured by immunofluorescence, Related to Figure 1

(A) Density scatter plots of EdU incorporation (left), α-phospho-Rb-S807/811 (middle), and α-phospho-Histone H3 (right) *vs.* DNA content as measured by Hoechst. These stains were used in combination to determine cell-cycle phases in asynchronously cycling cells, indicated by labeled boxed areas, as described previously (Gookin et al., 2017). G0 phase: ~2N DNA content/phospho-Rb^{low}/EdU^{low}; G1 phase: 2N DNA content/phospho-Rb^{high}/EdU^{low} ; Early S phase: ~2N DNA content /EdU^{mid}; S phase: 2-4N DNA content/EdU^{high}; Late S phase: ~4N DNA content/EdU^{mid}; G2 phase: 4N DNA content/EdU^{low}/phospho-$HH3^{low}$ and M phase: ~4N DNA content/phospho-$HH3^{high}$.

(B) Density scatter plot of α-Ki67 signal vs DNA content (left) was converted into a contour plot (middle) using cell-cycle phase information obtained from (A). Probability densities of α-Ki67 signal for cells in G0 and G1 phases were plotted from duplicate wells (right).

Figure S2. **Generation of an mCitrine-Ki67 knock-in line, Related to Figure 2-4**

(A) Schematic of mCitrine-Ki67 knock-in process. 3.5% of cells were classified as mCitrine-positive by flow cytometry and sorted into single-cell clones (right).

(B) Expected sizes (left) and PCR products (right) of the wild-type *MKI67* gene and the Citrine-MKI67 fusion gene. Both alleles are tagged in the mCitrine-Ki67 knock-in cell line resulting in the absence of a wild-type band.

(C) mCitrine-Ki67 protein response to 32 hr of 100 nM MEK inhibitor is equivalent to wild-type Ki67, as assessed by western blot. Note that addition of the small mCitrine tag (26 kDa) to the large Ki67 protein (359 kDa) is not discernable by western blot.

(D) The α-Ki67 signal linearly correlates with mCitrine fluorescence in the mCitrine-Ki67 knock-in line, but not in wild-type MCF10A cells that lack mCitrine. Treatment with 100nM MEK inhibitor for 32 hours resulted in a decrease in α-Ki67 and mCitrine-Ki67 signals in the knock-in cell line. Note that x-axis units are arbitrary fluorescence units in natural log.

(E) Top: Live-cell, time-lapse images of a single mCitrine-Ki67 cell stably expressing Histone 2B-mTurquoise and mCherry-tagged CDK2 activity sensor, in various phases of the cell cycle. Bottom: Single-cell images of wild-type MCF10A cells fixed and stained with Hoechst and Ki67 antibody. Fixed cell-cycle phases were determined as in Figure S1A. The subcellular localization of mCitrine-Ki67 fusion protein is consistent with wild-type Ki67 protein localization in all cell-cycle phases.

(F) Corresponding single-cell CDK2 activity and Ki67 traces for the live-cell images shown in (E, Top); magenta circles on the Ki67 trace mark the time points shown in (E, Top). Dashed lines mark anaphase.

Figure S3. Ki67 continuously decreases in G0/G1 and rises upon S phase entry, Related to Figure 2-3

(A) CDK2 activity and mCitrine-Ki67 traces for two individual MCF7 $CDK2^{inc}$ cells (top), $CDK2^{emerge}$ cells re-entering the cell cycle 10 hr after mitosis (middle), and $CDK2^{low}$ cells (bottom).

(B) Averaged single-cell MCF7 traces of CDK2 activity (top) or mCitrine-Ki67 (middle) computationally aligned to time of anaphase, for each group of cells indicated. Box plot quantifies Ki67 levels at the time CDK2 activity begins to rise (bottom). n = 13-500.

(C) MCF7 cells were perturbed with control medium change, serum withdrawal, CDK4/6 inhibition, or Nutlin addition. Averaged single-cell traces for CDK2 activity (left column) or mCitrine-Ki67 (right column) were computationally aligned to time of anaphase. Gray boxes indicate times of perturbation. Error bars represent standard error of the mean. n = 15 for Control G0; n = 280-700 for other conditions.

Figure S4. Retrospective estimation of quiescence duration with IF images of Ki67, Related to Figure 2

(A-B) Population fit of Ki67 degradation in $CDK2^{low}$ cells. Left: the logarithm (A) and the inverse (B) of the Ki67 signals are plotted against time since anaphase: average traces (red), 95% confidence intervals (red shades). The correlation coefficients of the fitted data are displayed in the upper right corner of the images. Right: Average Ki67 traces for $CDK2^{low}$ cells are plotted in red. The average trace is fit by 1^{st} (A) or 2^{nd} order (B) degradation laws and the fitted curves are plotted in black. Fitting was conducted by searching for the optimal Ki67 signal at 2 hr after anaphase, [Ki67](0), and the kinetic rate constant, k_1 or k_2, to minimize the Euclidean distance between the average Ki67 signals and fitted signals, normalized by the standard error of the mean (SEM) of the Ki67 signals at each time point.

(C) Population average estimation of quiescence durations using parameters inferred from MCF10A cells. Inference of Ki67 kinetic rate constants in MCF10A cells and prediction of quiescence durations with Ki67 IF images were performed as described in Methods. In each panel, each blue dot represents a $CDK2^{low}$ cell in the IF images, and the cell's measured (x-axis) and predicted (y-axis) quiescence durations are plotted. The red line represented the correct prediction where measured durations equal the predicted ones. The correlation coefficient (R) and the average Euclidean distance (Error, in hours) between the measured and predicted durations are displayed in the lower right corners. The model with MCF10A parameters shows predictive power for all four cell lines, suggesting that different cell lines follow similar Ki67 degradation kinetics.

(D) Normalized H2B signals at mitosis. The normalized H2B signal at mitosis of a cell is defined as its H2B signal at anaphase divided by its average H2B signal across all time points. The probability distributions of normalized H2B signals of all cells in a cell line were then estimated by nonparametric density estimation.

(E) Bayesian estimation of quiescence durations. The probability distribution of quiescence durations at a given Ki67 signal of $CDK2^{low}$ cells was computed as described in Methods. The 0.10, 0.25, 0.5, 0.75, 0.9 quantiles of the distribution are displayed in the figure. Each red dot represents a $CDK2^{low}$ cell in the IF images, and its Ki67 signal and measured quiescence duration are plotted on the x- and y-axes, respectively.

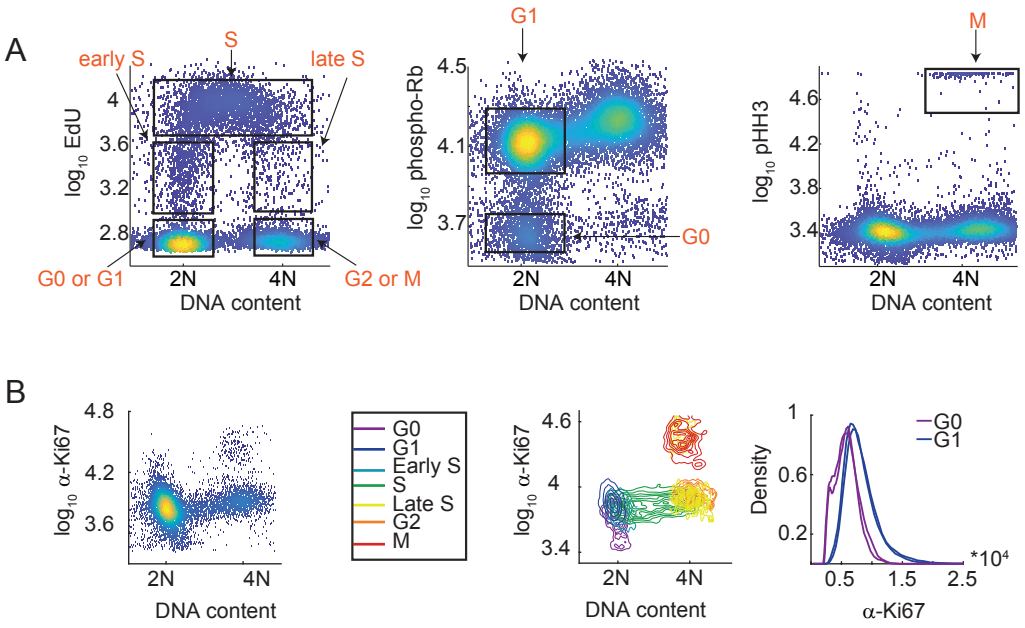
A
early S
S
late S
log10 EdU
4
3.6
3.2
2.8
G0 or G1
2N
4N
G2 or M
DNA content
G1
log10 phospho-Rb
4.5
4.1
3.7
G0
2N
4N
DNA content
M
log10 pHH3
4.6
4.2
3.8
3.4
2N
4N
DNA content
B
log10 α-Ki67
4.8
4.2
3.6
2N
4N
DNA content
G0
G1
Early S
S
Late S
G2
M
log10 α-Ki67
4.6
4
3.4
2N
4N
DNA content
Density
1
0.6
0.2
G0
G1
*10^4
0.5
1.5
2.5
α-Ki67

Figure S1

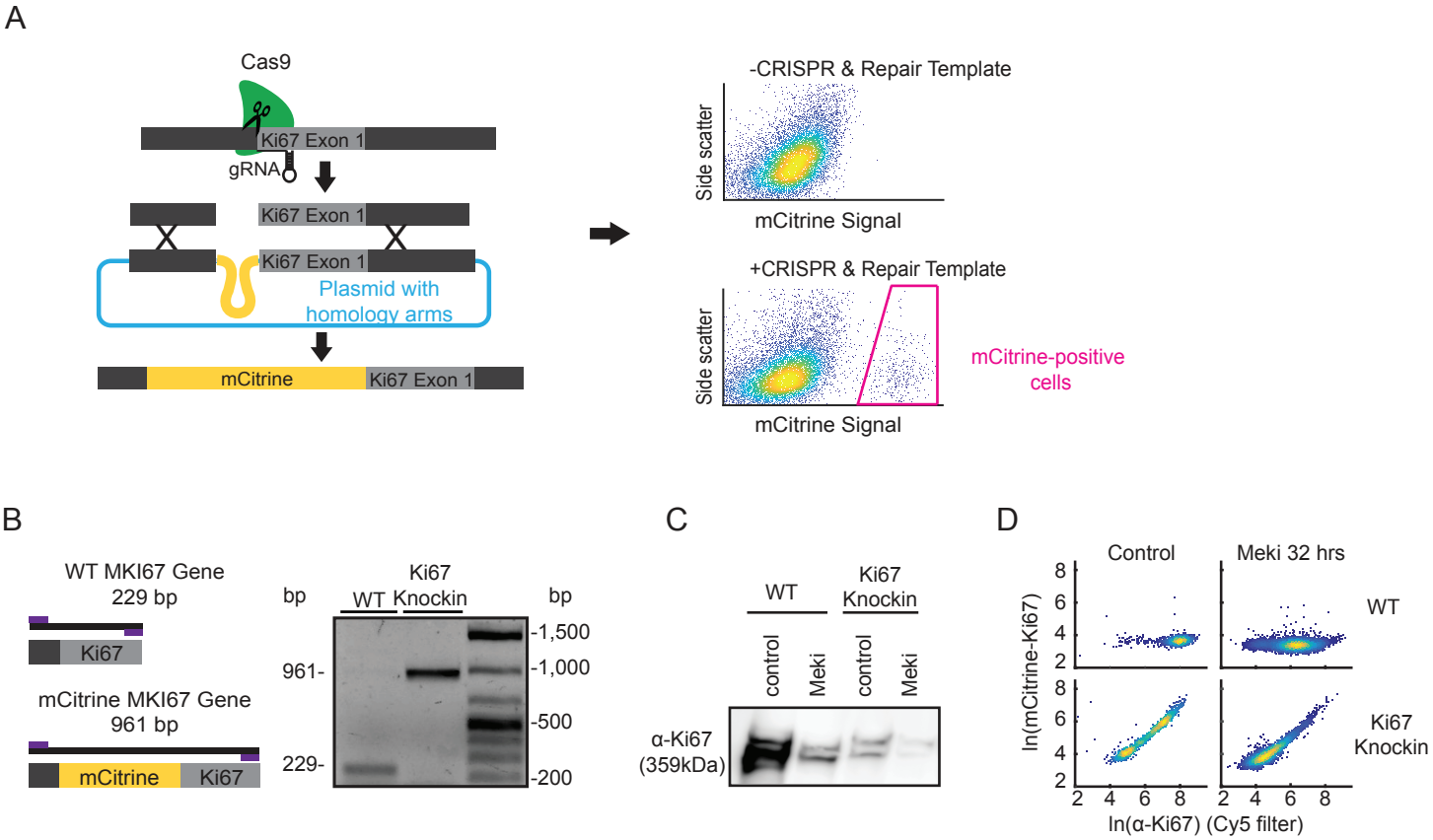
A
Cas9
gRNA
Ki67 Exon 1
Ki67 Exon 1
Ki67 Exon 1
Plasmid with homology arms
mCitrine
Ki67 Exon 1
-CRISPR & Repair Template
Side scatter
mCitrine Signal
+CRISPR & Repair Template
Side scatter
mCitrine Signal
mCitrine-positive cells
B
WT MKI67 Gene
229 bp
Ki67
mCitrine MKI67 Gene
961 bp
mCitrine
Ki67
Ki67
WT Knockin
bp
961-
229-
bp
-1,500
-1,000
-500
-200
C
WT
Ki67 Knockin
control
Meki
control
Meki
α-Ki67
(359kDa)
D
Control
Meki 32 hrs
WT
Ki67 Knockin
ln(mCitrine-Ki67)
ln(α-Ki67) (Cy5 filter)
E
G1
S
G2
Anaphase
G0
H2B-mTurquoise
Ki67-mCitrine
10 μm
Timepoint (hr):
7
10.4
16
22.4
45
Hoechst
α-Ki67
10 μm
F
Single-cell trace
G1
S/G2
G0
CDK2
Ki67
CDK2 Activity
mCit-Ki67 Signal
Time Relative to Anaphase (hr)

Figure S2

MCF7 cells

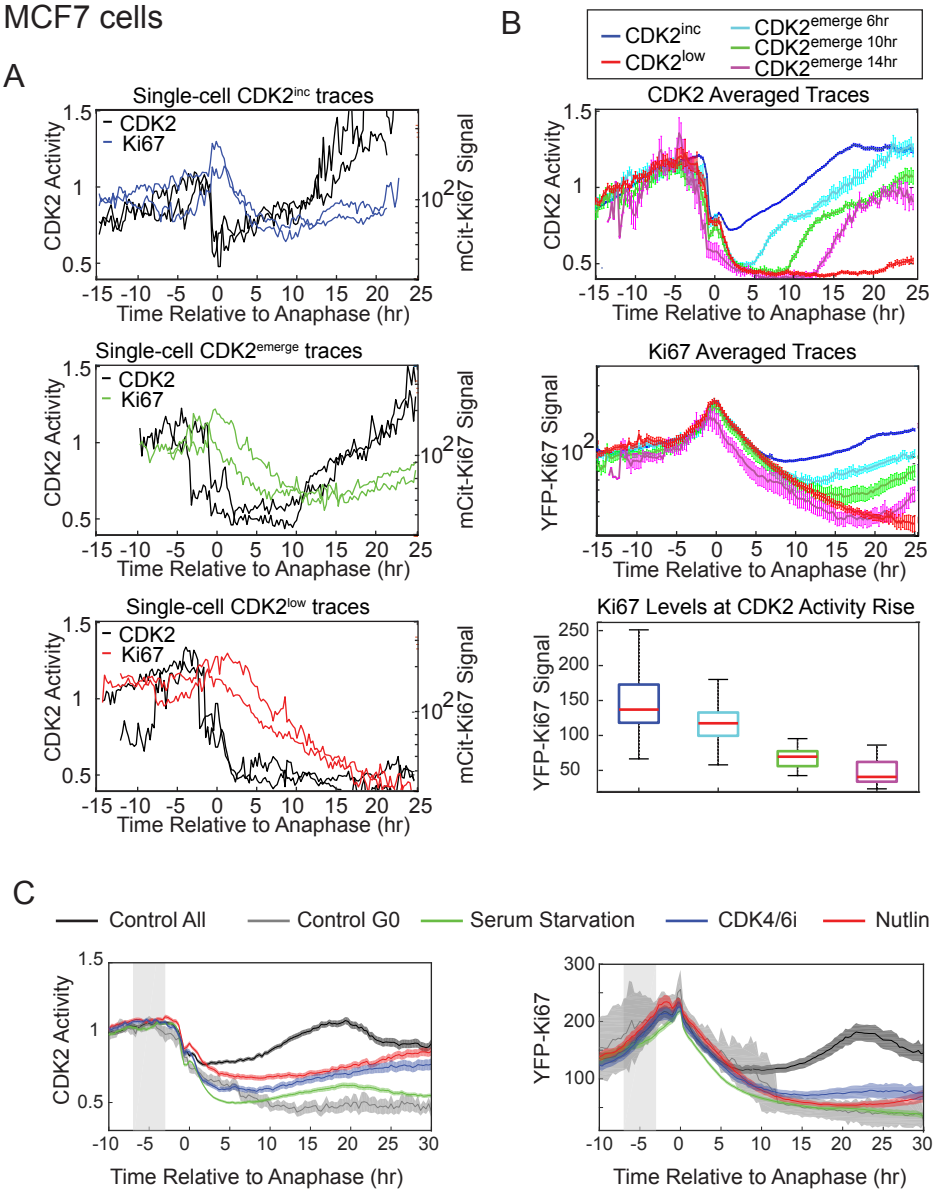

Figure S3

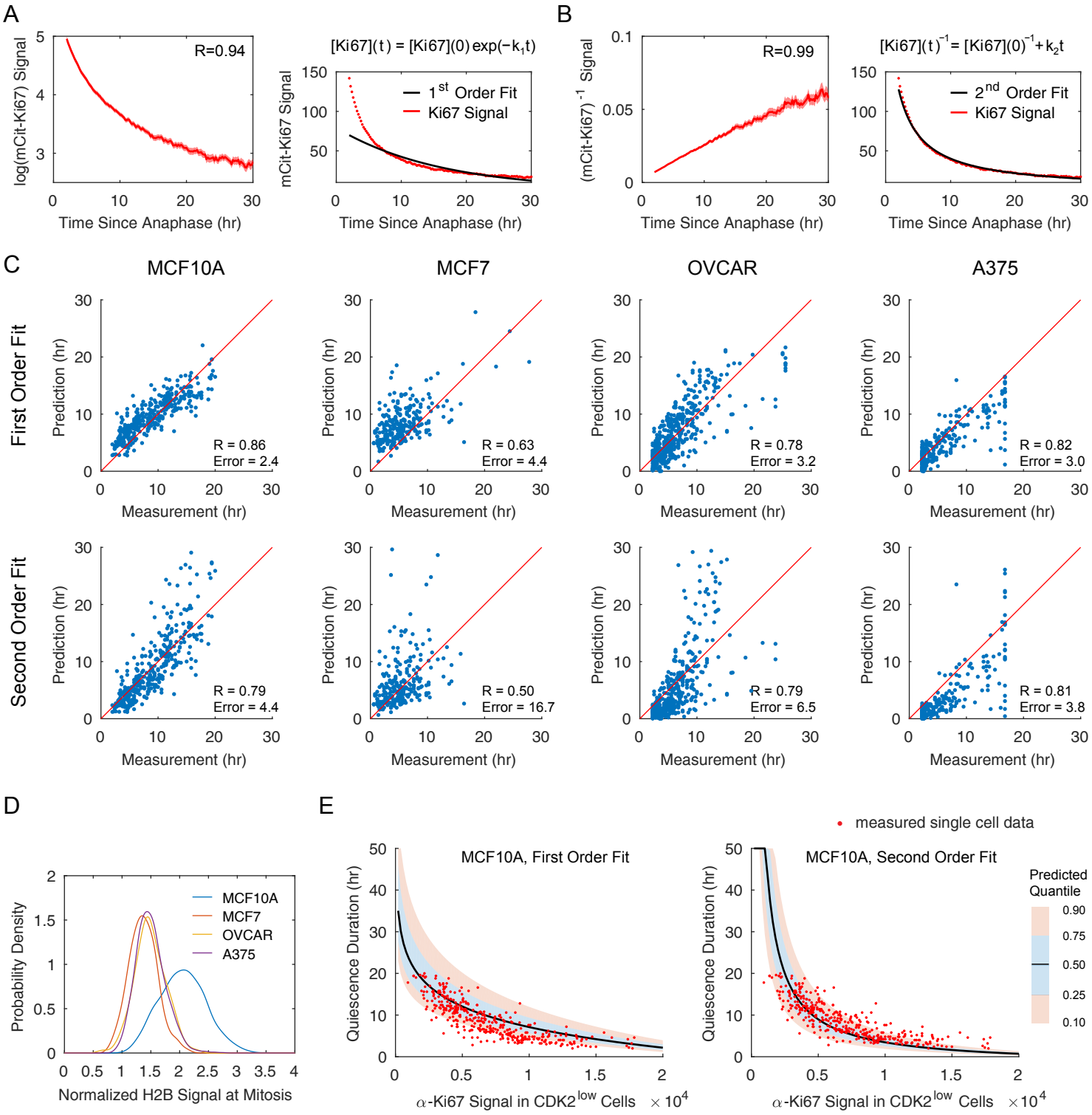
A
log(mCit-Ki67) Signal
R=0.94
Time Since Anaphase (hr)
[Ki67](t) = [Ki67](0) exp(−k1t)
mCit-Ki67 Signal
1st Order Fit
Ki67 Signal
Time Since Anaphase (hr)
B
(mCit-Ki67)-1 Signal
R=0.99
Time Since Anaphase (hr)
[Ki67](t)-1 = [Ki67](0)-1 + k2t
2nd Order Fit
Ki67 Signal
Time Since Anaphase (hr)
C
MCF10A
MCF7
OVCAR
A375
First Order Fit
Second Order Fit
Prediction (hr)
Measurement (hr)
R = 0.86
Error = 2.4
R = 0.63
Error = 4.4
R = 0.78
Error = 3.2
R = 0.82
Error = 3.0
R = 0.79
Error = 4.4
R = 0.50
Error = 16.7
R = 0.79
Error = 6.5
R = 0.81
Error = 3.8
D
Probability Density
MCF10A
MCF7
OVCAR
A375
Normalized H2B Signal at Mitosis
E
measured single cell data
MCF10A, First Order Fit
MCF10A, Second Order Fit
Quiescence Duration (hr)
α-Ki67 Signal in CDK2low Cells
×10^4
Predicted Quantile
0.90
0.75
0.50
0.25
0.10

Figure S4

Table S1. Definitions of cell populations in Figure 2 and Figure S3A-B

	MCF10A: CDK2 activity stayed between 1 and 1.5 between **a** to **b** hours after anaphase for at least 36 min, and remained below 0.8 from 3 to **c** hr after anaphase			MCF7: CDK2 activity stayed between 0.6 and 1.1 between **a** to **b** hours after anaphase for at least 2 hr, and remained below 0.6 from 3 to **c** hr after anaphase		
	a	**b**	**c**	**a**	**b**	**c**
$CDK2^{inc}$	3	5	NA	3.5	7.5	NA
$CDK2^{emeging\ 6\ hr}$	7	9	7	5.5	8.5	5
$CDK2^{emeging\ 10\ hr}$	11	13	11	9.5	14.5	9
$CDK2^{emeging\ 14\ hr}$	15	17	15	13.5	18.5	13
$CDK2^{low}$	CDK2 activity values below 0.5 at 2 hr after mitosis and remain below the level seen at anaphase for the remainder of the imaging period					

NA - Not applicable

Table S2. Definitions of cell populations in Figure 3 and Figure S3C

	MCF10A			MCF7	
	time point tested (hr after anaphase)		CDK2 activity	time period tested	CDK2 activity
	untreated	treated			
$CDK2^{inc}$	2	5	>0.5	NA	NA
$CDK2^{emerge}$	2	5	<0.5, but increase later to above the level seen at anaphase	NA	NA
$CDK2^{low}$	2	5	<0.5, and remain below the level seen at anaphase for the remainder of the imaging period	6 hr after anaphase to end of movie	<0.9

NA - Not applicable