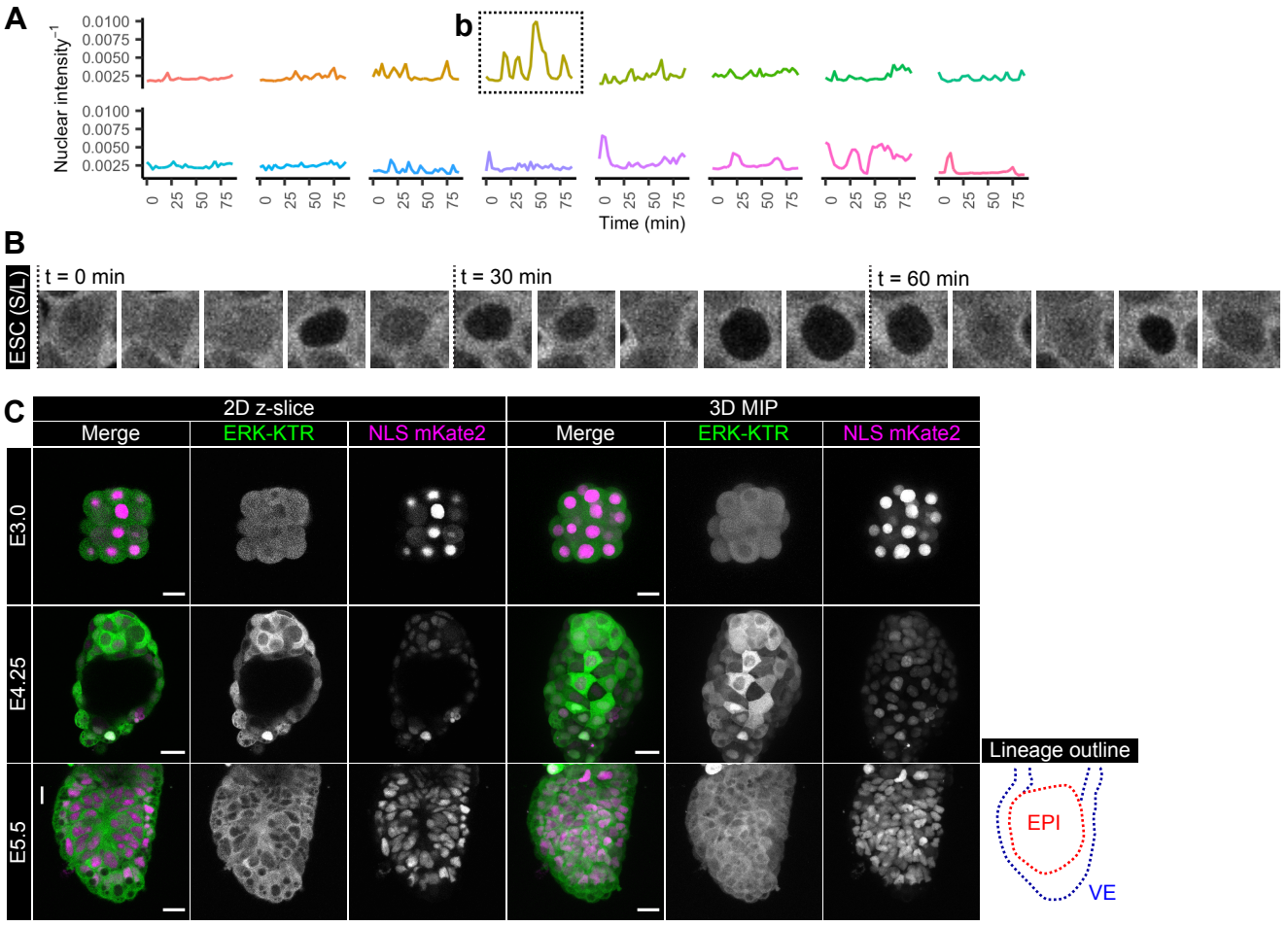
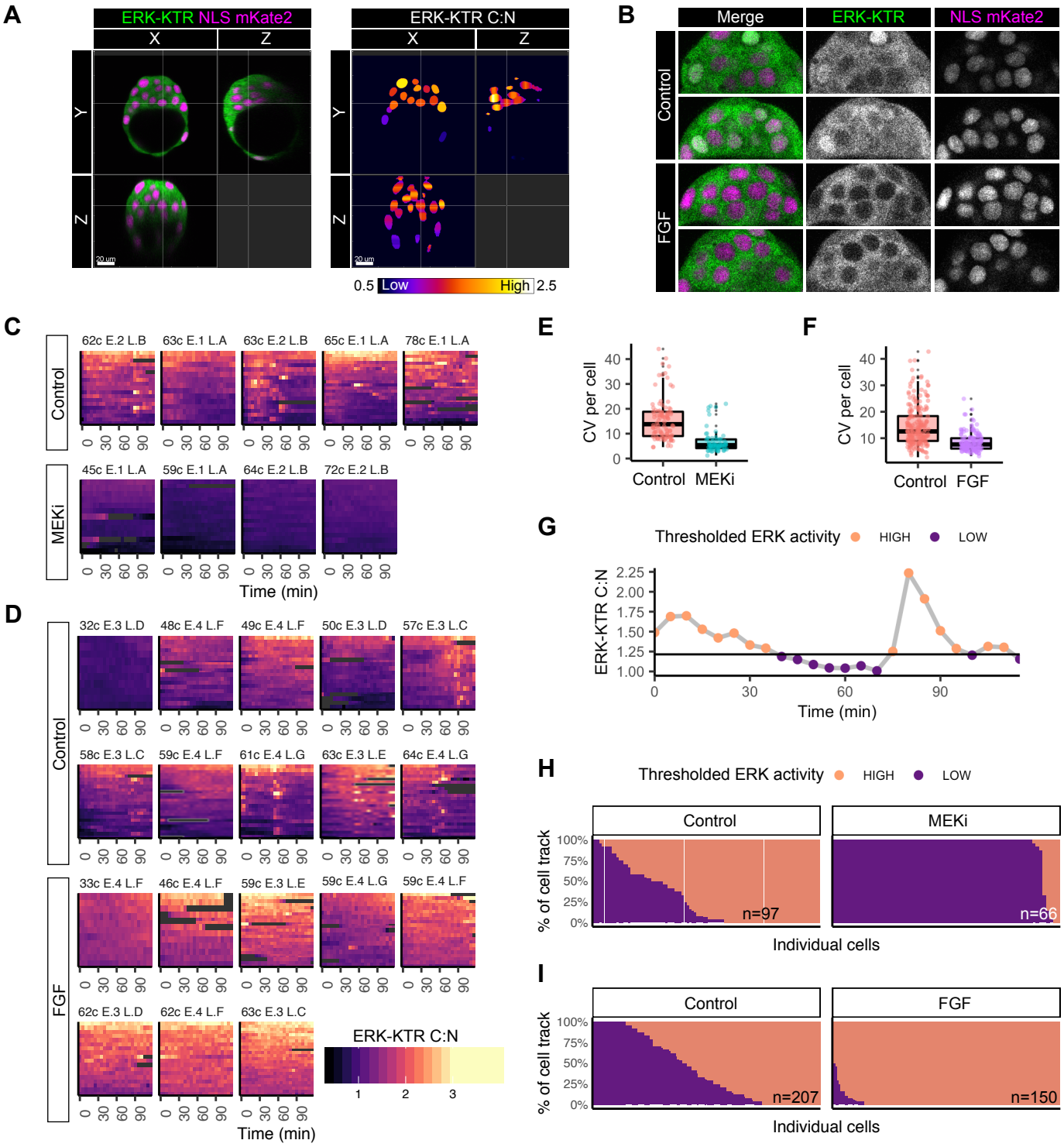


Supplemental Figure 1: An ERK-KTR targeted mouse line for live visualization of ERK activity in vivo. (Related to Figure 1)

(A) Quantification of ERK-KTR in individual mouse embryonic stem cells (ESC) cultured in serum/LIF (S/L). Line plots show individual cells tracked over 90min time-lapse with 3 min intervals within an ESC colony (Figure 1B). Due to the absence of a nuclear marker, inverse mean nuclear ERK-KTR intensity is shown as a proxy for ERK activity. Individual lines are randomly colored for each distinct cell (n=16). Dotted line box indicates cell shown in B.

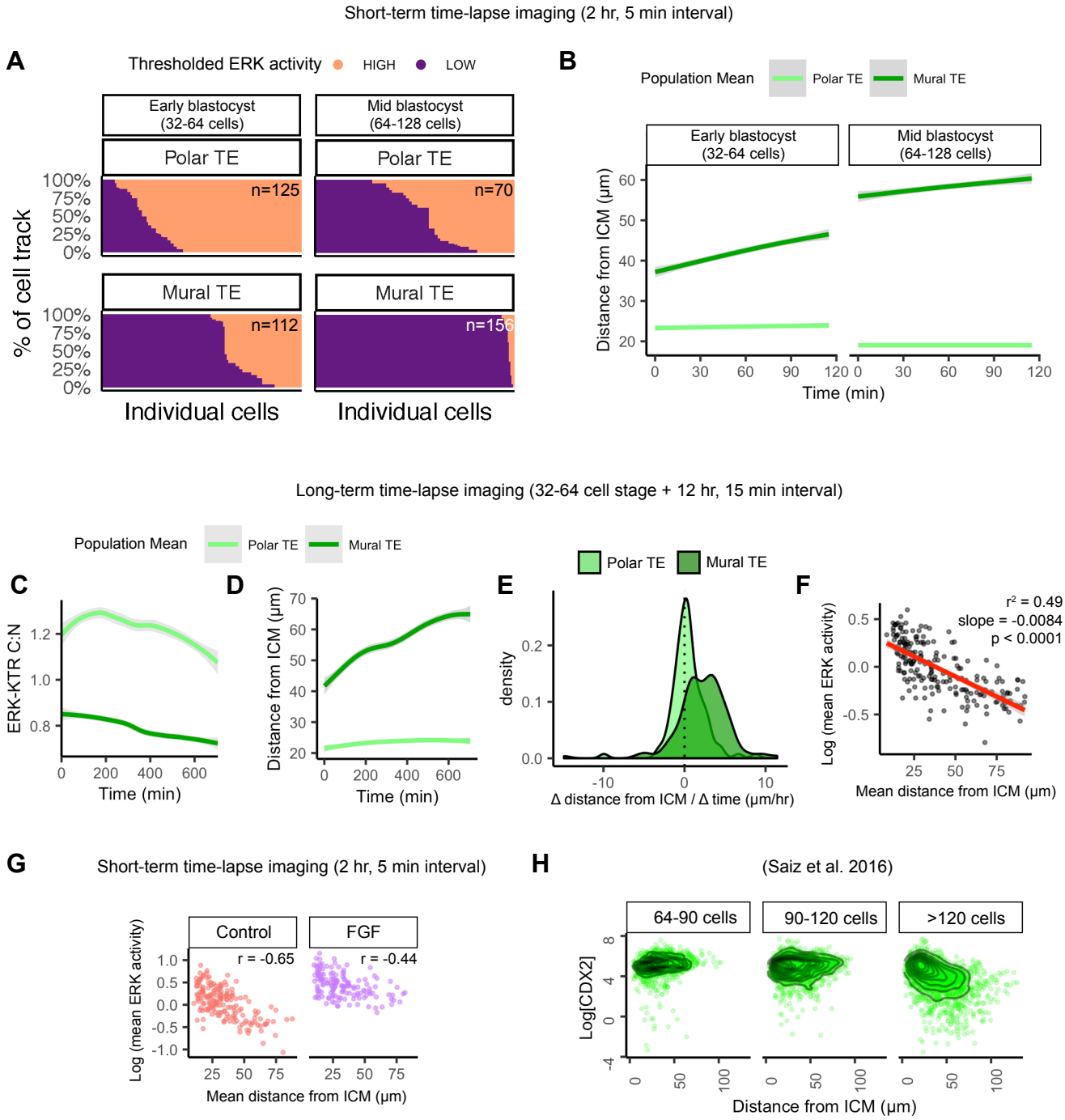
(B) Example of pulsing ESCs grown in S/L. Confocal time-lapse images of ERK-KTR (grayscale). Individual cell shown every 6 min, over the 90 min time-lapse.

(C) Confocal images of hemizygous *Hprt*$^{ERK\text{-}KTR}$ and homozygous *R26*$^{NLS\text{-}mKate2}$ embryos during pre-implantation development and early post-implantation development. Embryonic day (E) and cell number for pre-implantation embryos are indicated for staging. Max intensity projection (MIP). Epiblast (EPI). Visceral endoderm (VE). Proximal (P). Distal (D). Scale bars: 20μm.



Supplemental Figure 2: ERK-KTR is a read out of FGF/ERK signaling in the mouse blastocyst. (Related to Figure 2).

(A) Quantification pipeline of ERK-KTR for in toto embryo imaging. 3D reconstruction of ERK-KTR C:N z-slices. XY, XZ, and YZ optical planes of confocal images of ERK-KTR (green) and NLS mKate2 (magenta) (top), and computed ERK-KTR C:N values (bottom).
(B) First frame of time-lapse confocal images of hemizygous *Hprt*$^{ERK\text{-}KTR}$ and homozygous *R26*$^{NLS\text{-}mKate2}$ embryos in control and FGF culture conditions. Magnification of a single z-slice at mid-point through the ICM are shown.
(C-D) Heatmap of ERK-KTR C:N values in Control versus MEKi (B) and Control versus FGF -treated embryos (C). Each box represents an individual embryo. Embryonic stage by cell number (c), experiment (E: 1-4) and litter (L: A-G) are indicated for each embryo. Each line represents a tracked ICM cell over the 2hour, 5min interval time-lapse imaging. Grey values represent missing data, due to mitosis and nuclear envelope breakdown, or an incomplete track due to ICM cell moving out of the field of view or cell death.
(E-F) Boxplot showing coefficient of variation (CV) in ERK-KTR C:N within individual ICM cells over time. CV values per cell are shown as individual points overlaid on boxplot. Color coding for treatments Control: orange, MEKi: turquoise, FGF: light purple.
(G) Illustration of ERK-KTR C:N thresholding to delineate high versus low ERK activity based on MEKi and FGF experiments. Line plot of an individual ICM cell from control (untreated) experiment, showing temporal variation in ERK-KTR C:N. Time points with ERK-KTR C:N values > 1.21 (black horizontal line) assigned “HIGH” ERK activity (orange points), and values < 1.21 assigned “LOW” ERK activity (purple points).
(H-I) Stacked bar plots showing the relative amount of time individual cells spend in a high versus low signaling state throughout the time-lapse movie based on the threshold shown in (F). Control: n=5, MEKi: n = 4 embryos and Control: n=10, FGF: n=8 embryos.



Supplemental Figure 3: The trophectoderm is patterned by a spatial and temporal ERK activity gradient. (Related to Figure 3).

(A) Stacked bar plots showing the relative amount of time individual cells spend in a high versus low signaling state throughout the time-lapse movie based on the threshold shown in (Supplemental Figure 2E). Cells grouped by final position, polar or mural trophectoderm (TE) and developmental stage early blastocyst (32-64 cell) stage (n=14 embryos), and mid-blastocyst (64-128 cell) stage (n=10).

(B) Line plot showing the population mean of relative distance of TE cells from the nearest ICM cell over short term 2 hour time-lapse imaging. Lines color coded according to final position polar (light green), or mural (dark green) TE. Grey shading represents 95% confidence interval.

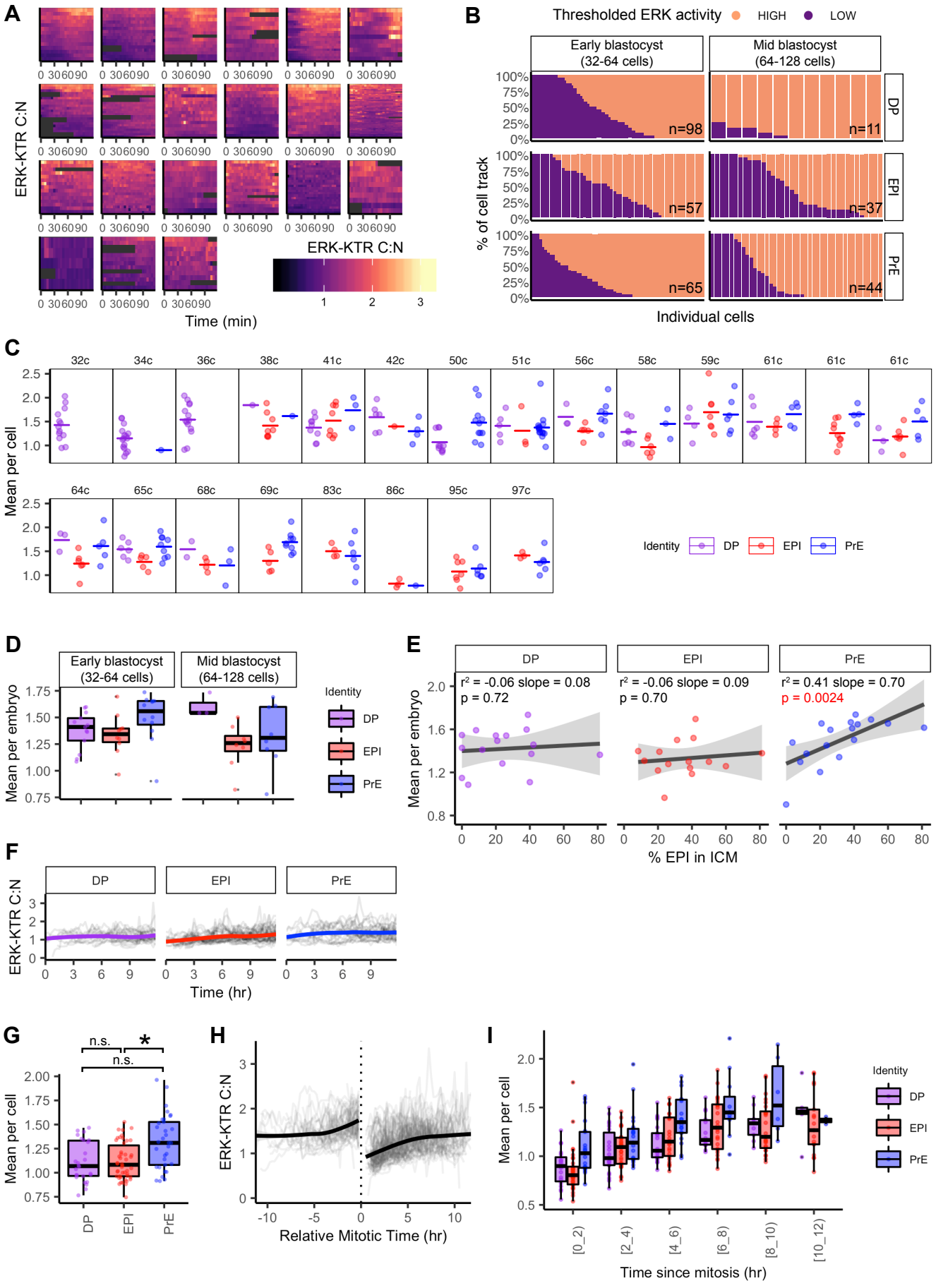
(C-D) Line plot showing the population mean of single-cell ERK-KTR C:N values (C) and relative dstance from the nearest ICM (D) over the long-term 12 hour time-lapse imaging. Lines color coded according to final position polar (light green), or mural (dark green) TE. Grey shading represents 95% confidence interval.

(E) Density plots showing the change ( ) in distance from the nearest ICM cell over the duration of the time-lapse for individual polar and mural TE cells.

(F) Scatterplot comparing the mean distance over the lifetime of a TE cell to its nearest ICM neighbor in each frame, with the mean integrated ERK activity of the TE cell in long-term 12 hour movies. Points (black) represent individual TE cells. Linear regression line of best fit shown in red. Slope, adjusted r2 goodness of fit, and p value are indicated. n=13 embryos.

(G) Scatterplot comparing the mean distance over the lifetime of a TE cell to its nearest ICM neighbor in each frame, with the mean integrated ERK activity of that TE cell. Points represent individual TE cells and are color coded based on embryo culture conditions. Control: orange, FGF: light purple. Pearson correlation (r) is shown. Control: n=10, FGF: n=8 embryos (as in Figure 2).

(H) Scatter plot comparing distance of a TE cell to its nearest ICM neighbor and nuclear intensity of CDX2 protein. Data analyzed from publicly available dataset of immunostained mouse blastocysts that were imaged by confocal microscopy (Saiz, et al., 2016b). Individual points represent a TE cell (green). Contour lines (black) are provided to illustrate density of data points. Embryos are grouped by cell number, 64-90 cell, 90-120 cells, or >120 cells. n = 94 embryos



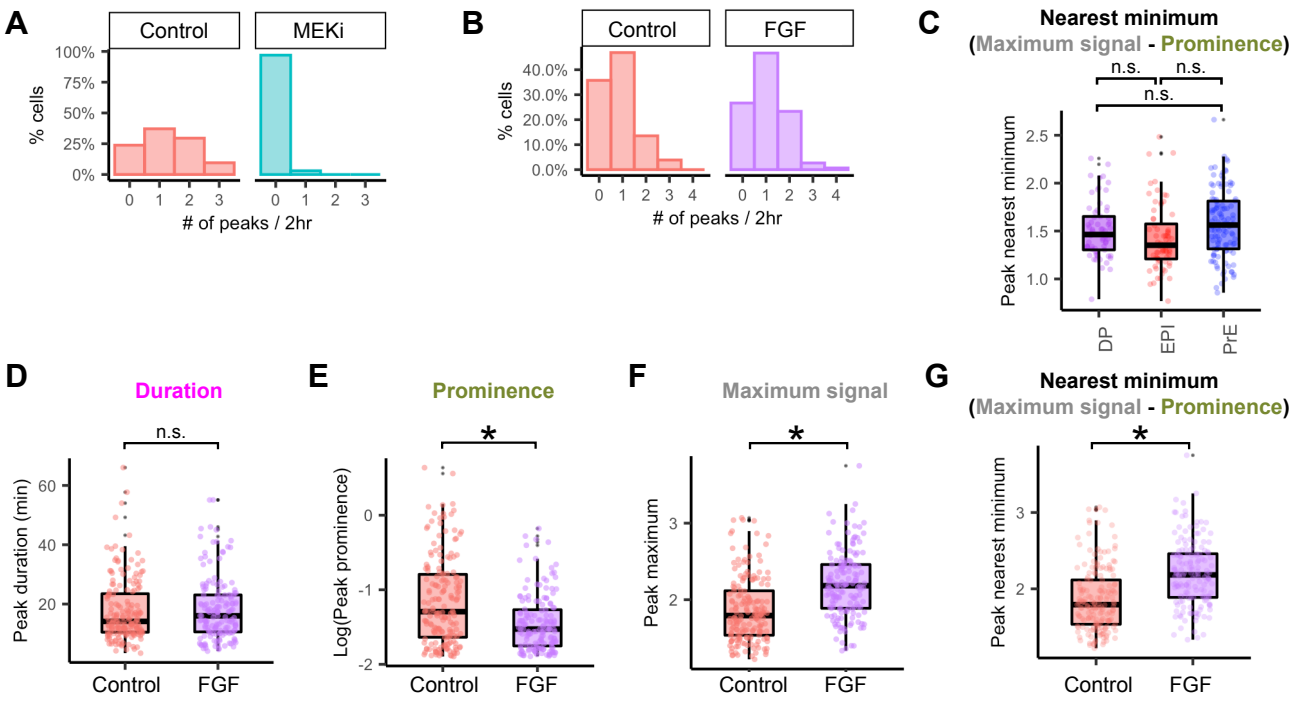
Supplemental Figure 4: Signaling heterogeneity between ICM cells corresponds to lineage identity. (Related to Figure 4).

(A) Heatmap of ERK-KTR C:N values. Each box represents an individual embryo. Each line represents a tracked ICM cell over the 2hour, 5min interval time-lapse imaging. Grey values represent missing data, due to mitosis and nuclear envelope breakdown, or an incomplete track due to ICM cell moving out of the field of view or cell death.

(B) Stacked bar plots showing the relative amount of time individual cells spend in a high versus low signaling state throughout the 2hr time-lapse movie based on the threshold shown in (Supplemental Figure 2E).

(C) Dot plots showing mean ERK-KTR C:N values in single cells from 2 hour movies. Individual boxes represent data from individual

embryos, and embryo cell number (c) is indicated above. Horizontal bars indicate mean ERK-KTR C:N values for each lineage and embryo. Colour indicates lineage DP (purple), EPI (red), PrE (blue).
(D) Boxplot showing mean ERK-KTR C:N values for each lineage and embryo from 2 hour movies.
(E) Scatter plots showing mean ERK-KTR C:N values for each lineage and embryo plotted against the % EPI cells within the ICM of that embryo from 2 hour movies. Linear regression line of best fit shown in black. Grey shading represents 95% confidence interval. Slope, adjusted r2 goodness of fit, and p value are indicated, n = 22 embryos.
(F) Traces of single-cell ERK-KTR C:N values over the course of long-term 12 hour time-lapse imaging. Single-cell traces are shown in black. Population mean are overlaid in color corresponding to lineage identity at the end of the time-lapse movie. DP: purple, PrE: blue, EPI: red.
(G) Boxplot showing integrated ERK activities in individual ICM cells over the long-term 12 hour time-lapse imaging. Mean ERK-KTR C:N values per cell are shown for every cell as individual points. Color coding for lineages as indicated before. Asterisk indicates statistical significance: p=0.0191, (linear mixed effects model, post-hoc Tukey pairwise test, see STAR Methods), n = 11 embryos.
(H) Traces of single-cell ERK-KTR C:N values over the course of long-term 12 hour time-lapse imaging. All ICM cells that were observed entering mitosis over the 12 hour time course are shown and aligned by the time of mitosis. Individual single-cell traces are shown in grey, black line represent the population mean. Timing relative to nuclear envelope break down of parental cell, dotted line = 0.
(I) Boxplot showing integrated ERK activities in individual ICM cells in 2 hour binned-windows from the start of mitosis in long-term 12-hour time-lapse movies. Mean ERK-KTR C:N values per cell during this time-period are shown for every cell as individual points. Color coding for final lineage identity as indicated before.



Supplemental Figure 5: Lineage-specific dynamics of ERK activity in ICM cells.
(Related to Figure 5).
(A-B) Histogram showing the number of peaks detected within each cell as a percentage of cell in that lineage, in Control versus MEKi (A) and Control versus FGF (B) treated embryos. Control: Orange, MEKi: Turquoise. FGF: light purple. (extracted from data in experiment shown in Figure 2).
(C) Boxplot showing dynamic information extracted from peak-calling (Figure 5D) in different ICM lineages. Nearest minimum as an approximation of baseline activity is calculated by subtracting peak prominence from peak maximum. Each peak is shown as individual points. Color coding for lineages DP: purple, EPI: red, PrE: blue. Not significant (n.s.) pairwise comparisons (Wilcoxon test) indicated where p > 0.05.
(D-G) Boxplot showing dynamic information extracted from peak-calling (Figure 5D). Each peak is shown as individual points. Color coding for treatment as indicated before. Asterisk indicates statistical significance: Peak prominence: p= 0.027. Peak maximum: p= 0.036. Peak nearest minimum: p=0.00099 (Wilcoxon test). Control: n=10, FGF: n=8 embryos. Not significant (n.s.) pairwise comparisons indicated where p > 0.05.