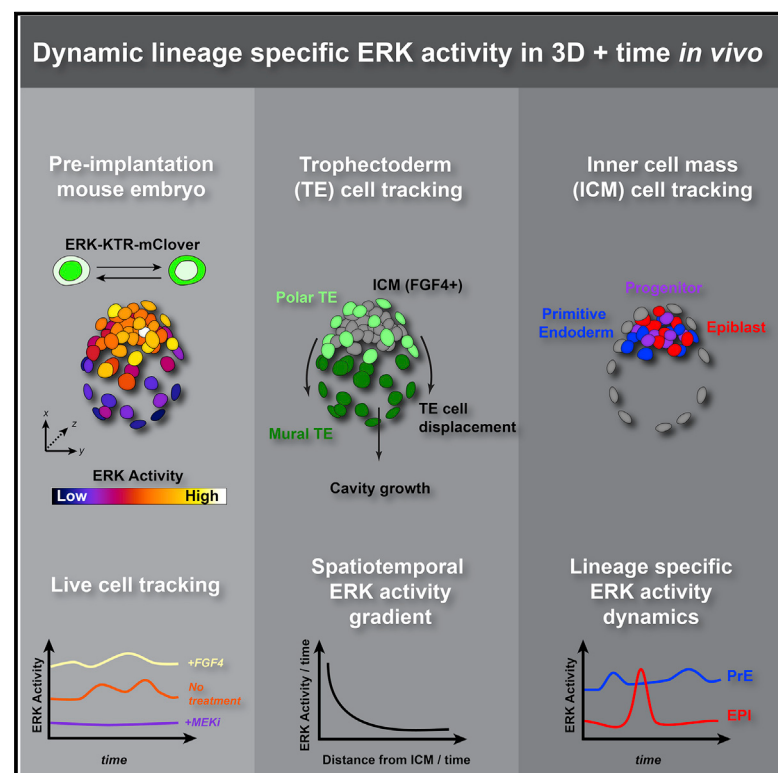


Developmental Cell

Live Visualization of ERK Activity in the Mouse Blastocyst Reveals Lineage-Specific Signaling Dynamics

Graphical Abstract



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In Brief

FGF/ERK signaling is crucial for the patterning and proliferation of cell lineages that comprise the mouse blastocyst. Using an ERK-kinase translocation biosensor, Simon et al. show that the trophectoderm is patterned by spatially graded ERK activity and that primitive endoderm and epiblast cells have distinct ERK dynamics.

Highlights

- Generation of a targeted ERK-KTR mouse line that reports ERK signaling in blastocysts
- FGF4 signals from the ICM spatially pattern the trophectoderm
- Signaling heterogeneity in ICM cells corresponds to distinct lineage identities
- PrE exhibit elevated ERK activity, while EPI show sporadic pulses of activity

Article

Live Visualization of ERK Activity in the Mouse Blastocyst Reveals Lineage-Specific Signaling Dynamics

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SUMMARY

FGF/ERK signaling is crucial for the patterning and proliferation of cell lineages that comprise the mouse blastocyst. However, ERK signaling dynamics have never been directly visualized in live embryos. To address whether differential signaling is associated with particular cell fates and states, we generated a targeted mouse line expressing an ERK-kinase translocation reporter (KTR) that enables live quantification of ERK activity at single-cell resolution. 3D time-lapse imaging of this biosensor in embryos revealed spatially graded ERK activity in the trophectoderm prior to overt polar versus mural differentiation. Within the inner cell mass (ICM), all cells relayed FGF/ERK signals with varying durations and magnitude. Primitive endoderm cells displayed higher overall levels of ERK activity, while pluripotent epiblast cells exhibited lower basal activity with sporadic pulses. These results constitute a direct visualization of signaling events during mammalian pre-implantation development and reveal the existence of spatial and temporal lineage-specific dynamics.

INTRODUCTION

Cell signaling, modulated by extracellular stimuli and cell intrinsic regulators, is critical for the patterning of tissues during development. In the mouse embryo, the fibroblast growth factor (FGF)/ERK signaling pathway plays a central role for the coordinated cell fate specification and tissue growth across the embryo before implantation in the maternal uterus (Simon et al., 2018a; Brewer et al., 2016). The first cell specification event during pre-implantation development allocates outer and inner cells to extra-embryonic trophectoderm (TE) or inner cell mass (ICM), respectively, at around embryonic day (E)3.0 (Mihajlović and Bruce, 2017). TE cells express FGF receptors *Fgfr1*, *Fgfr2*, and *Fgfr4* (Kurowski et al., 2019; Nowotschin et al., 2019; Molotkov et al., 2017; Guo et al., 2010). Although dispensable for initial TE fate decisions, FGF/ERK signaling is required for maintaining trophoblast stem cell identity and TE proliferation (Christodoulou et al., 2019; Kurowski et al., 2019; Nichols et al., 2009; Yang et al., 2006; Tanaka et al., 1998).

Progenitor cells within the ICM are progressively and asynchronously specified to either the pluripotent epiblast (EPI), or extra-embryonic primitive endoderm (PrE) lineages from E3.25 to E3.75 (~32–100 cells) (Saiz et al., 2016b; Schrode et al., 2014; Grabarek et al., 2012; Plusa et al., 2008; Chazaud et al., 2006). Abrogation of the FGF/FGFR/GRB2/MEK/ERK signaling cascade by genetic or pharmacological perturbation prevents

the formation of PrE and impairs pluripotency progression in the EPI (Kang et al., 2017; Molotkov et al., 2017; Saiz et al., 2016b; Ohnishi et al., 2014; Kang et al., 2013; Krawchuk et al., 2013; Yamanaka et al., 2010; Nichols et al., 2009; Chazaud et al., 2006). Conversely, administration of exogenous FGF to embryos induces a PrE fate in uncommitted ICM progenitors (Saiz et al., 2016b; Kang et al., 2013; Krawchuk et al., 2013; Yamanaka et al., 2010). Therefore, cell fate specification in the ICM is driven by FGF signaling acting predominantly via the ERK pathway. *Fgf4* is the only FGF ligand expressed in the blastocyst at the time of ICM cell fate specification, expressed from E3.0–E3.25 (~16–32 cell stage) in uncommitted ICM progenitors, and then later by EPI cells (Nowotschin et al., 2019; Ohnishi et al., 2014; Guo et al., 2010). The FGF receptor *Fgfr1* is expressed from E3.25 (~32 cells) by all cells of the ICM, and a second receptor *Fgfr2* is expressed in specified PrE cells (Nowotschin et al., 2019; Kang et al., 2017; Molotkov et al., 2017; Brewer et al., 2015; Ohnishi et al., 2014).

Given the early pan-ICM expression of *Fgfr1*, the predominant receptor for PrE specification, heterogeneity in FGF/ERK signaling is considered the main driver of ICM lineage segregation (Saiz et al., 2020; Simon et al., 2018a; Kang et al., 2017; Molotkov et al., 2017; Tosenberger et al., 2017; De Mot et al., 2016; Bessonard et al., 2014). How FGF signal transduction differs between cells of the blastocyst is a key open question in the field. However, previous approaches used to measure ERK activity in

the blastocyst have been unable to detect dynamic ERK activities described in other systems (Deathridge et al., 2019; Zhang et al., 2018; de la Cova et al., 2017; Gillies et al., 2017; Conlon et al., 2016; Ryu et al., 2016; Regot et al., 2014). For example, using a transcriptional reporter for *Spry4*, a target of FGF/ERK signaling, we could not detect differences between ICM lineages (Morgani et al., 2018). However, the design of this reporter was not permissive for visualizing short-term changes in signaling activity. By contrast, p-ERK immunostaining directly measures the amount of activated ERK in fixed embryos and provided direct evidence that prE cells have a higher proportion of p-ERK positive cells compared with epiblast cells (Azami et al., 2019). However, a direct comparison of p-ERK levels between lineages remains outstanding, and immunostaining only provides a snapshot of this dynamic process, in which heterogeneous labeling may be due to transient ERK phosphorylation. Quantification of FGF/ERK signaling transduction using rapidly responsive live reporters is, therefore, essential for furthering our understanding of signaling dynamics and how they impact cellular behaviors such as tissue patterning *in vivo*.

To achieve this, we generated a targeted mouse line expressing an ERK-kinase translocation reporter (KTR) in all cells. To perform *in toto* embryo imaging during pre-implantation development, we co-visualized this KTR reporter with a ubiquitously expressed nuclear reporter, which facilitated segmentation, tracking, and quantitation in 3D time-lapse data. In total, we manually tracked and analyzed ERK activity over time in approximately 2,000 cells from 60 blastocyst-stage embryos across different experiments. Live imaging of this ratiometric sensor in embryos revealed dynamic, lineage-specific ERK activities at single-cell resolution. We observed a spatiotemporal ERK activity gradient along the embryonic-abembryonic axis across the TE that presaged its differentiation to polar and mural subtypes. Within the ICM we found heterogeneous activities, where all cells relayed FGF/ERK signals to differing extents. PrE cells displayed higher overall levels of ERK activity, while pluripotent EPI cells exhibited lower basal activity with infrequent pulses over time. Together, these observations reveal how distinct modes of transduction of an individual signal within a population can mediate symmetry breaking and generate cellular diversity during embryonic development.

RESULTS

An ERK-KTR-Targeted Mouse Line for Live Visualization of ERK Activity *In Vivo*

To follow ERK activities *in vivo*, we used an ERK-kinase translocation reporter (ERK-KTR) (Kudo et al., 2018; Regot et al., 2014). ERK-KTR consists of a docking site (fragment of human Elk1, an ERK substrate), nuclear localization (NLS) and export (NES) signals, and a mClover fluorescent protein (Lam et al., 2012) (Figure 1A). When the ERK signaling pathway is active, activated p-ERK phosphorylates the sensor and nuclear export predominates, resulting in cytoplasmic enrichment. Conversely, when the ERK signaling pathway is inactive, the sensor is unphosphorylated and nuclear import predominates, resulting in increased nuclear enrichment of mClover fluorescence. Therefore, the cytoplasmic to nuclear ratio of ERK-KTR serves as proxy for ERK activity.

To generate a bright reporter suitable for 3D time-lapse imaging at single-cell resolution, we coupled the ERK-KTR-mClover construct to a strong CAG promoter (Niwa et al., 1991). We targeted the construct to the *Hprt* locus on the X chromosome to generate an *Hprt*^{CAG-ERK-KTR-mClover} allele (abbreviated to *Hprt*^{ERK-KTR}), thereby avoiding unpredictable site-specific integration and copy-number effects on expression levels associated with random-integrant transgenes. Mouse embryonic stem cells (ESCs) containing the targeted allele robustly expressed the ERK-KTR-mClover (Figure 1B). In 2i conditions (MEK and GSK-3 inhibitors) the reporter was homogeneously localized throughout the cell (data not shown) (Ying et al., 2008). By contrast, in serum/leukemia inhibitory factor (LIF) conditions, which are permissive for FGF/ERK signaling, the reporter was predominantly localized in the cytoplasm, indicating high ERK activity, consistent with p-ERK levels in these conditions (Figure 1B) (Deathridge et al., 2019; Chen et al., 2015; Ying et al., 2008). However, the localization of the reporter was variable between different cells and within a cell over time, typical of the heterogeneity seen in serum/LIF cultured ESCs (Lanner and Rossant, 2010) (Figures 1B, inset, S1A, and S1B; Video S1).

The *Hprt*^{ERK-KTR} ESCs were used to generate *Hprt*^{ERK-KTR} mice, which were crossed with mice ubiquitously expressing a nuclear-localized red fluorescent protein, mKate2 (*Rosa26*^{CAG-3xNLS-mKate2}, abbreviated to *R26*^{NLS-mKate2}) (Susaki et al., 2014) for segmentation of nuclei, cell counting, and tracking (Figure 1C). We collected hemizygous *Hprt*^{ERK-KTR}, homozygous *R26*^{NLS-mKate2} embryos for our analyses. To account for within-litter variation and unknown fertilization time, we also precisely staged embryos using total cell number (32 cells $\approx$ E3.25, 64 cells $\approx$ E3.5, and 100 cells $\approx$ E3.75). We visualized expression of the ERK-KTR (mClover) and NLS-mKate2 reporters in live embryos in 3D using laser-scanning confocal microscopy. At E3.0–E3.25 ERK-KTR showed a relatively even nuclear-to-cytoplasmic distribution in cells of the ICM and some cells with nuclear enriched reporter localization, indicative of low signaling activity. The reporter then progressively became more cytoplasmic from E3.5 to E4.25, and in the early post-implantation EPI at E5.5 (Figures 1D and S1C). We were unable to correlate the dynamic range of the ERK-KTR reporter and p-ERK staining within the same experiment as the staining procedure for p-ERK in embryos is incompatible with the localization based ratiometric readout of the reporter. The high percentage (8%) PFA fixation and Triton-X100 permeabilization protocols used for p-ERK immunodetection (Azami et al., 2019; Corson et al., 2003) do not preserve the nuclear and cytoplasmic localization of the KTR reporter, a common artifact encountered when fixing fluorescent proteins (Melan and Sluder, 1992; Schnell et al., 2012). However, we note these changes in reporter localization indicate an increase in ICM and EPI ERK activity with developmental stage, consistent with recent p-ERK immuno-localization (Azami et al., 2019).

ERK-KTR Is a Readout of FGF/ERK Signaling in the Mouse Blastocyst

The ERK-KTR ratiometric reporter system can be used to quantify dynamic ERK activity in individual cells. Although the blastocyst is relatively small, (E3.5 = $\sim$ 90 μ m, $\sim$ 64 cells), segmentation and tracking of nuclei during cellular rearrangements within the

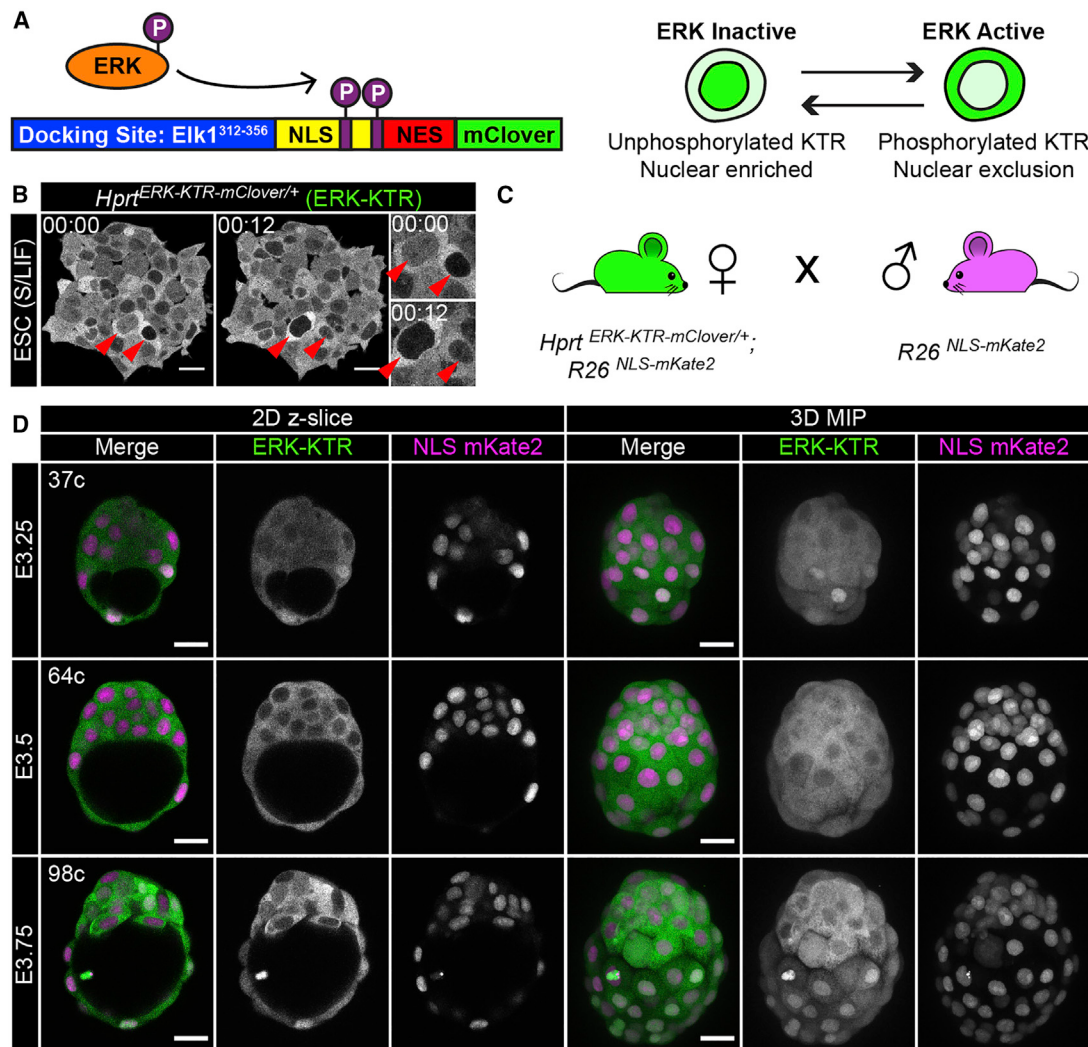


Figure 1. An ERK-KTR-Targeted Mouse Line for Live Visualization of ERK Activity In Vivo

(A) Schematic of ERK kinase translocation reporter (ERK-KTR). ERK-KTR is a biosensor, which shuttles between cytoplasm and nucleus as a readout of ERK-kinase activity. NLS, nuclear localization signal; NES, nuclear export signal; P, Phosphorylation.
(B) Time-lapse confocal images of mouse ESCs expressing *Hprt*^{ERK-KTR-mClover}, abbreviated to *Hprt*^{ERK-KTR}. Red arrows indicate two cells at different times. Inset shows higher magnification. Timestamp shows hour:min. S/LIF: serum LIF culture conditions.
(C) Summary diagram of mouse cross used to generate hemizygous *Hprt*^{ERK-KTR-mClover/+} and homozygous *R26*^{NLS-mKate2} embryos in this study.
(D) Confocal images of hemizygous *Hprt*^{ERK-KTR-mClover/+} and homozygous *R26*^{NLS-mKate2} embryos during pre-implantation development. Embryonic day (E) and cell number are indicated for staging. Scale bars, 20 μ m.

densely packed ICM presents a challenge for automated image analysis in 4D. Therefore, we developed a pipeline to segment and calculate the ratio between mean cytoplasmic and nuclear ERK-KTR fluorescence intensity (C:N ratio) per Z slice (optical section) as a proxy for ERK activity. Determination of a C:N ratio normalizes for KTR expression between cells within any given sample, and loss of fluorescence signal along the z axis due to imaging depth (Kudo et al., 2018). We then reconstructed these images in 3D and over time (3D + t) to manually track and measure the ERK activity of individual cells in time-lapse videos (Figures 2A and S2A; Video S2, and see STAR Methods).

To test the responsiveness of ERK-KTR as a readout of FGF/ERK signaling during pre-implantation development, we performed 3D time-lapse imaging of mid-stage blastocysts (E3.5,

~64 cells) in different treatment regimes. The sensitivity of embryos to pharmacological perturbation of FGF signaling is maximal in a narrow time window between E3.5 and E3.75 (Besonnard et al., 2017). Therefore, we collected E3.5 embryos, and cultured them in control (untreated) conditions, in the presence of a MEK inhibitor (1 μ M PD0325901, MEKi) or FGF ligand (1 μ g/mL FGF4 + 1 μ g/mL heparin). We imaged the embryos for a total of 2 h, with a 5-min time-lapse interval (Figures 2B, 2C, and S2B; Video S3).

We tracked individual ICM cells in control, MEKi and FGF cultures and measured the ERK C:N ratio (Figures S2D and S2E; Video S3). In control embryos, ERK-KTR localization was heterogeneous between individual cells within the ICM (Figures S2E and S2F), but it was predominantly cytoplasmic, corresponding

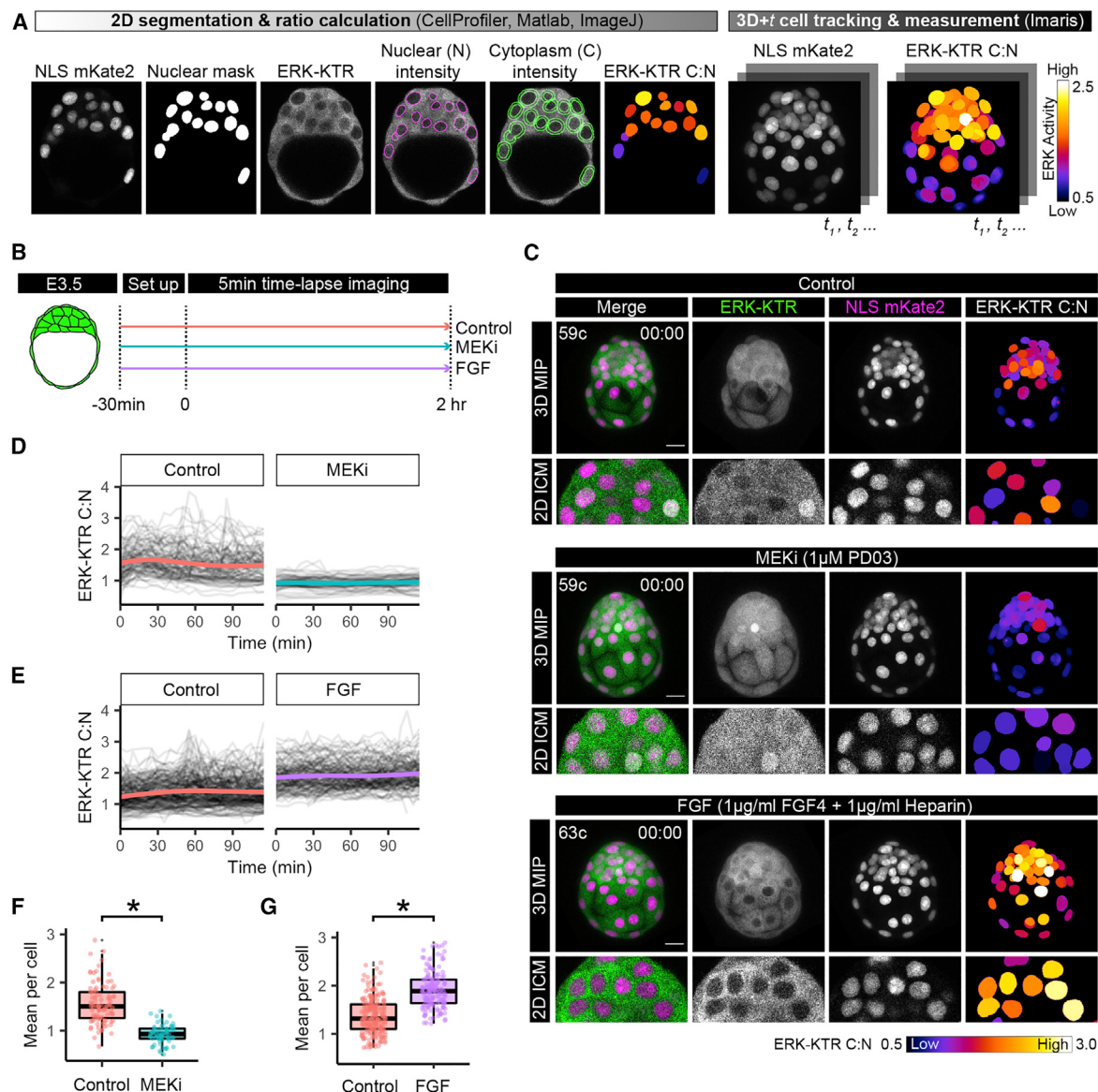


Figure 2. ERK-KTR Is a Readout of FGF/ERK Signaling in the Mouse Blastocyst

(A) Pipeline for quantification of ERK-KTR for 3D + time-lapse embryo time-lapse imaging. ERK-KTR cytoplasmic to nuclear (C:N) ratio is a proxy for ERK activity and can be visualized with a heatmap (see STAR Methods).

(B) Schematic of embryo culture treatments and time-lapse imaging.

(C) First frame of time-lapse confocal images of hemizygous *Hprt^{ERK-KTR}* and homozygous *R26^{NLS-mKate2}* embryos in control, MEKi, and FGF culture conditions. Embryonic day (E) and cell number are indicated for staging. Max intensity projections (MIPs) and magnification of a single z slice at midpoint through the ICM are shown. Scale bar, 20 μ m. ERK-KTR C:N values corresponding to ERK activity is shown as a heatmap as indicated.

(D and E) Traces of ICM cell ERK-KTR C:N values over the course of the 2-h time-lapse imaging in littermate control versus MEKi conditions (D) and control versus FGF conditions (E). Single-cell traces are shown in black, and population mean in color; control, orange; MEKi, turquoise; FGF, purple.

(F and G) Boxplot showing integrated ERK activities in individual ICM cells over the 2-h time-lapse imaging. Mean ERK-KTR C:N values per cell are shown for every cell as individual points. Color coding for treatments as indicated before. Asterisk indicates statistical significance. (F) Control versus MEKi: $p = 0.016$ (Wilcoxon test). Control: $n = 5$, MEKi $n = 4$ embryos. (G) Control versus FGF: $p = 0.003$ (Wilcoxon test). Control: $n = 10$, FGF: $n = 8$ embryos.

to higher C:N values and high ERK activity (Figure 2C, top). When we treated embryos with MEKi to block ERK-kinase activity, ERK-KTR fluorescence intensity became equivalent between the cytoplasm and the nuclei of ICM cells or nuclear enriched, corresponding to lower C:N values and low ERK activity (Figure 2C, middle). By contrast, when we treated embryos with

FGF, ERK-KTR localized almost exclusively to the cytoplasm, hence, high ERK activity (Figures 2C, bottom, S2B). When analyzing ERK-KTR C:N traces of single cells followed throughout the time-lapse video, we found that MEKi abolished fluctuations that were seen in control cells and cells treated with FGF (Figures 2D, 2E, and S2E). Due to this dynamic behavior, we

used the mean ERK-KTR C:N for each cell over the entire time course as an integrated measure of ERK activity. In MEKi-treated embryos, the mean ERK-KTR C:N per cell was significantly reduced when compared with control (untreated) embryos (Figures 2D, colored lines, and 2F), whereas FGF-treated embryos had significantly higher values of mean ERK-KTR C:N per cell than control embryos (Figures 2E and 2G).

We used the ERK-KTR C:N values obtained from the FGF and MEKi treatments, to set a threshold C:N value of 1.21 to delineate “high” and “low” signaling activity (Figure S2G, and see STAR Methods). Control embryos had a mix of cells with high and low signaling activity following thresholding for C:N values (Figures S2H and S2I). Upon embryo culture with FGF or MEKi, most treated ICM cells instead have low or high ERK activity, respectively (Figures S2H and S2I). In conclusion, these data demonstrate that ERK-KTR provides a quantifiable dynamic readout of FGF/ERK activity in live blastocysts in 3D at single-cell resolution.

The Trophectoderm Is Patterned by a Spatial and Temporal ERK Activity Gradient

FGF/ERK signaling is required for trophoblast stem cell identity, acting to inhibit differentiation, promote proliferation, and maintain *Cdx2* expression (a trophoblast stem cell marker) (Christodoulou et al., 2019; Nichols et al., 2009; Yang et al., 2006; Tanaka et al., 1998). In the embryo, FGF4 produced by EPI cells acts as a positional cue to drive the lineage divergence at E4.5 (~150–200 cell stage) of polar TE cells (*CDX2*^{high}) overlying the ICM, and mural TE cells (*CDX2*^{low}) overlying the blastocyst cavity (Christodoulou et al., 2019; Goldin and Papaioannou, 2003; Gardner, 2000; Chai et al., 1998). We applied our reporter system to understand how FGF4 signaling patterns the TE. In static images, we noticed differential ERK activity at E3.75 between both TE regions (Figures 1D and 3A) with high activity in polar TE cells and low activity in mural TE cells, consistent with p-ERK staining of the late-stage blastocyst (Azami et al., 2019).

To establish when these signaling patterns emerge, we performed time-lapse imaging of E3.25–E3.75 embryos for 2 h with a 5-min interval (Figure 3B; Video S4). To precisely stage each embryo, we counted the total cell number using the NLS-mKate2 nuclear marker and analyzed embryos between the early blastocyst (32–64 cells) and the mid-blastocyst (64–128 cells). Polar versus mural TE identities were manually assigned based on the final position of cells relative to the ICM. Individual ERK-KTR C:N traces showed differences within the TE (Figure 3C). By the mid-blastocyst stage, mural TE cells had a distinct signaling profile from polar TE cells, showing much lower activity (Figure 3C). At the early blastocyst-stage differences were already apparent between polar and mural TE cells (Figures 3D and S3A). This observed ERK activity in the polar TE from early blastocyst (32–64 cells) stage was earlier than reported p-ERK staining from E3.75 or E4.5 (Azami et al., 2019; Christodoulou et al., 2019), demonstrating the high sensitivity of the ERK-KTR reporter (Regot et al., 2014).

During embryo growth and expansion of the blastocyst cavity mural TE cells progressively move farther away from the ICM, the source of the FGF4 signal, concomitant with a flow of polar TE cells to a mural TE position (Figures 3E, 3F, and S3B) (Christodoulou et al., 2019; Gardner, 2000). Therefore, we hypothe-

sized that mean ERK activities within the TE may depend on changes in their signaling environment over time. To test this, we measured distance of each TE cell to its nearest ICM cell at every time point, then calculated the mean distance over the time course for that cell. We noticed an exponential decline in a cell's mean ERK activity with increasing mean distance from the ICM across early and mid-blastocyst stages (Figure 3G). A similar relationship was observed in longer time-lapse imaging experiments, where early blastocyst-stage embryos were imaged for a total of 12 h with a 15-min time interval (Figures S3C–S3F). Together, this suggests that ERK activity emerges in a spatiotemporal gradient along the embryonic-abembryonic axis of the TE, such that mean ERK activity is related to the spatial position of a cell within the TE of the blastocyst over time. This spatial-temporal gradient in ERK activity in the TE is in contrast to, and precedes, the binary difference, high (polar TE) versus low (mural TE) p-ERK immunostaining reported previously in pre- and peri-implantation embryos (Christodoulou et al., 2019).

Differential ERK signaling between TE subtypes could be caused by cell autonomous transcriptional changes (Frias-Aldeguer et al., 2020) and/or changes in mechanical properties (i.e., higher cortical tension from overlying blastocyst cavity versus ICM) (Chan et al., 2019; De Belly et al., 2019). Alternatively, non-cell autonomous effects, namely, the availability and distance from source of FGF4 ligand may drive the difference in ERK activity. To differentiate between these possibilities, we assessed the response of polar and mural TE cells by tracking TE cells in control (untreated) and FGF-treated embryo experiments (Figure 3H). In control embryos, there was a significant difference in ERK activity between mural and polar TE cells. Upon FGF treatment, however, mural TE cells responded to FGF at comparable levels to polar TE cells, and the association of ERK activity with distance was diminished (Figures 3H and S3E). These data argue that differences in ERK activities across the TE at this stage are not cell intrinsic and that mural TE are able to respond to exogenous ligand. Since FGF ligands are expressed exclusively in the ICM and not the TE (Nowotschin et al., 2019; Guo et al., 2010), these experiments suggest that the ICM spatially and temporally patterns ERK activities in TE cells. This patterning begins in the early blastocyst stage, preceding the expression of markers of polar and mural TE differentiation.

As FGF/ERK signaling is required for maintain *Cdx2* expression in trophoblast stem cells and peri-implantation TE (Christodoulou et al., 2019; Nichols et al., 2009; Yang et al., 2006; Tanaka et al., 1998), we suspected the early TE ERK gradient may also affect levels of *CDX2* in pre-implantation embryos. Therefore, we analyzed 3D spatial information of published *CDX2* immunostained wild-type embryos (Saiz et al., 2016b). We discovered a spatial *CDX2* protein gradient in the TE along the embryonic-abembryonic axis from 120-cell late-blastocyst-stage embryos (Figure S3H). Intriguingly, these data reveal that a *CDX2* gradient earlier than the distribution of high-*Cdx2* and low-*Cdx2* corresponding to polar and mural trophectoderm at peri-implantation (Christodoulou et al., 2019). This suggests a link between the gradient of ERK activity and the spatial gradient of *Cdx2* activity along the embryonic-abembryonic axis in pre-implantation embryos.

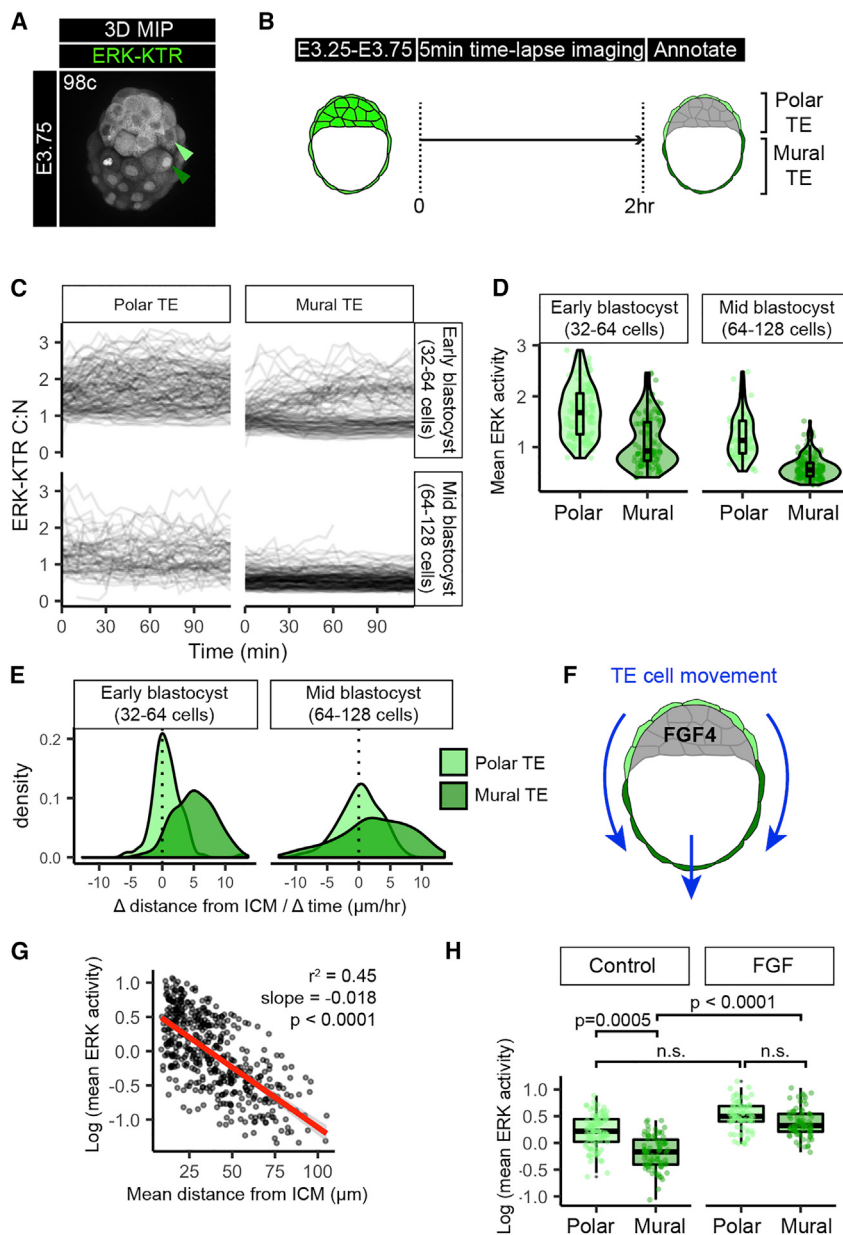


Figure 3. The Trophectoderm Is Patterned by a Spatial and Temporal ERK Activity Gradient

(A) Confocal image of hemizygous *Hprt^{ERK-KTR}* and homozygous *R26^{NLS-mKate2}* embryos during pre-implantation development. Embryonic day (E) and cell number are indicated for staging. Reproduced from panel within Figure 1D.

(B) Schematic of time-lapse confocal imaging where polar versus mural TE identity was annotated by position at the end of the video.

(C) Traces of single-cell ERK-KTR C:N values over the course of the 2-h time-lapse imaging. Single-cell traces are shown in black, for polar TE, and mural TE. Stage of embryo at the beginning of the video is indicated, early blastocyst (32–64 cell) stage and mid-blastocyst (64–128 cell) stage.

(D) Violin and boxplots showing mean ERK activities in individual TE cells over the 2-h time-lapse imaging. Mean ERK-KTR C:N values per cell are shown for every cell as individual points. Polar TE, light green; mural TE, dark green. Cell stages at the beginning of time-lapse are indicated. Early blastocyst stage: $n = 14$, mid-blastocyst stage: $n = 10$ embryos.

(E) Density plots showing the change (Δ) in distance from the nearest ICM cell over the duration of the time-lapse for individual polar TE and mural TE cells. (F) Schematic of TE movement along the embryonic-abembryonic axis away from the ICM (gray) derived FGF4 signals.

(G) Scatterplot comparing the mean distance over the lifetime of that cell to its nearest ICM neighbor in each frame, with the mean ERK activity of that cell. Linear regression line of best fit shown in red. Slope, adjusted r^2 goodness of fit, and p value are indicated.

(H) Boxplot showing mean ERK activities in individual ICM cells over the 2-h time-lapse imaging. Mean ERK-KTR C:N values per cell are shown for every cell as individual points. Color coding for TE subtypes as indicated before. Control: $n = 10$, FGF: $n = 8$ embryos (as in Figure 2). p values for statistical significance of pairwise comparisons are shown (Wilcoxon test), horizontal lines indicate groups compared. Not significant (n.s.) pairwise comparisons indicated where $p > 0.05$.

Signaling Heterogeneity between ICM Cells Corresponds to Lineage Identity

Endogenous FGF signaling is required for the emergence and distribution of PrE and EPI progenitors within the ICM population (Kang et al., 2013; Krawchuk et al., 2013). To address whether there is differential transduction of an FGF signal in ICM lineages, we focused on ICM cells in our time-lapse videos of early (32–64 cell)- and mid (64–128 cell)-blastocyst-stage embryos (Figure 4A). We tracked ICM cells at 5-min intervals throughout 2-h time-lapse periods (Figures 4A and S4A). We immediately fixed and immunostained embryos to assign lineage identities after the time-lapse and were able to identify terminal ICM cell fates. Embryos were processed individually to keep track of their origin, and ICM lineage identities were assigned based on

NANOG and GATA6 staining as described previously (Saiz et al., 2016a; Saiz et al., 2016b; Schrode et al., 2014; Plusa et al., 2008) (Figures 4A and 4B). Fixed embryos were then manually registered to the final frame of the time-lapse video to retrospectively assign ICM lineage identities to tracked cells (Figures 4A and 4C; Videos S4 and S5, see STAR Methods).

Individual cell traces revealed a range of baseline ERK activities and temporal variation within cells of the same identity (Figure 4D). In early blastocyst-stage embryos ERK activity in individual cells gradually increased (+18%), which was not seen in mid-blastocyst-stage embryos (+0.6%) (Figure 4D), consistent with increasing levels of *Fgf4* mRNA between E3.25 (~32 cells) and E3.5 (~64 cells) (Ohnishi et al., 2014). Thresholding to discriminate intervals of high and low ERK activity revealed

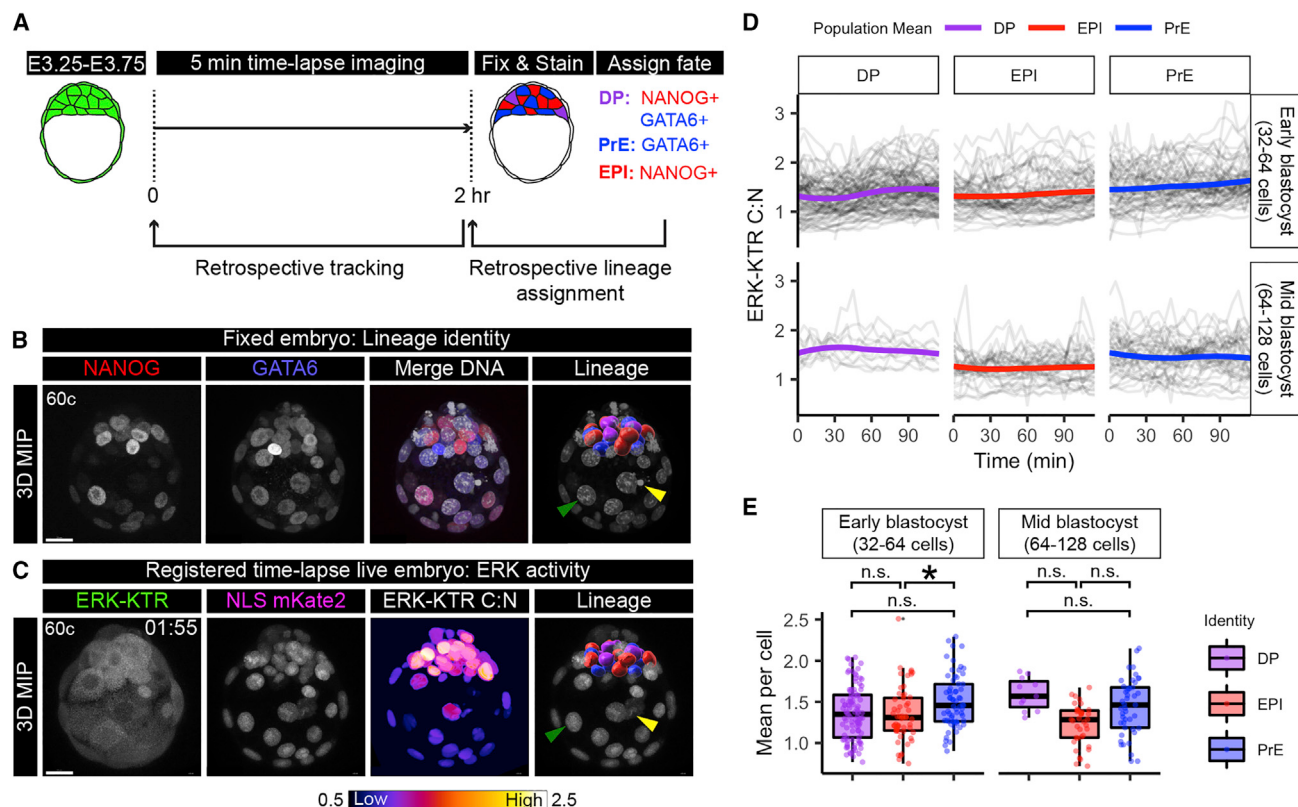


Figure 4. Signaling Heterogeneity between ICM Cells Corresponds to Lineage Identity

(A) Schematic of time-lapse confocal imaging as in (Figure 3C). Embryos were fixed immediately after the end of the time-lapse and stained to retrospectively identify ICM lineages. ICM progenitors are DP for NANOG and GATA6. DP cells are specified to PrE GATA6+ cells, and epiblast (EPI) NANOG+ cells. (B) Max intensity projection (MIP) confocal image of an embryo stained for NANOG (red) and GATA6 (blue). Merge with Hoechst (gray) to label nuclei. Lineage includes 3D rendering in Imaris (Bitplane) corresponding to a cell's lineage identity after k-means clustering of relative NANOG and GATA6 levels (Saiz et al., 2016b). DP: purple, PrE: blue, EPI: red. Merged with Hoechst (gray) to show nuclei. Arrows indicate landmarks within TE used for manual image registration with final time frame of time-lapse. Yellow: apoptotic cell, green: mural TE. Scale bars, 20 μ m. (C) Max intensity projection of hemizygous *Hprt^{ERK-KTR}* and homozygous *R26^{NLS-mKate2}* embryos in final time frame of 2-h time-lapse video. ERK-KTR C:N heatmap showing level of ERK activity. Retrospective ICM lineage identification when manually registered to fixed and stained (B) arrows indicate the same landmarks. Lineage is a 3D rendering in IMARIS colored corresponding to retrospective lineage identification (DP: purple, PrE: blue, EPI: red) merged with NLS mKate2 (gray) to show nuclei. (D) Traces of single-cell ERK-KTR C:N values over the course of the 2-h time-lapse imaging. Single-cell traces are shown in black. Population means are overlaid in color corresponding to lineage identity. Stage of embryo at the beginning of the video is indicated, early blastocyst (32–64 cell) stage and mid-blastocyst (64–128 cell) stage. (E) Boxplot showing integrated ERK activities in individual ICM cells over the 2-h time-lapse imaging period. Mean ERK-KTR C:N values per cell are shown for every cell as individual points. Color coding for lineage as indicated before. Asterisk indicates statistical significance (linear mixed effects model, post-hoc Tukey pairwise test, see STAR Methods). Early blastocyst stage EPI-PrE $p = 0.0271$, $n = 14$ embryos. Mid-blastocyst stage: $n = 8$ embryos. Not significant (n.s.) pairwise comparisons indicated where $p > 0.05$.

that all ICM lineages contained differing proportions of cells with periods of high signaling activity (Figure S4B). This heterogeneous pan-ICM activation of FGF/ERK is in line with observations made using p-ERK staining, as well as a *Spry4^{H2B-GFP}* transcriptional reporter (Azami et al., 2019; Morgani et al., 2018). Together, these data indicate that all ICM cells, irrespective of lineage identity, transduce FGF/ERK signals at early and mid-blastocyst stages.

Recent work on ERK signaling dynamics *in vitro* suggests that in single cells mean ERK activity is the strongest predictor of lineage-specific gene induction (Gillies et al., 2017). We, therefore, assessed whether the mean ERK activity per cell is associated with lineage outcomes within the ICM. From early blastocyst

stage, those cells that had adopted a PrE identity on average had the highest mean ERK activity (Figure 4E, left). At the mid-blastocyst stage although there were no statistically significant differences between lineages when performing pairwise comparisons, PrE cells showed a higher mean ERK activity than EPI cells (Figure 4E, right). These lineage differences were also apparent in between individual embryos. There was marked embryo-to-embryo variability, and in many, but not all embryos, PrE cells had higher mean ERK activity than EPI cells (Figures S4C and S4D).

Given that EPI cells express high levels of *Fgf4*, we wondered if their proportion within the ICM might influence ERK activity across the entire ICM population. We calculated the percentage

of EPI cells within the ICM for each embryo using the fixed immunostained time-lapse endpoints (Figure 4B) and compared this to the mean ERK activity within lineages for each embryo (Figure S4E). There was no correlation between the proportion of EPI cells on the mean ERK activity in double positive (DP) progenitors and EPI cells. By contrast, there was a positive association between the percentage of EPI cells and mean ERK activity in PrE cells. Therefore, ERK activity in cells that express *Fgf4*, DP (moderate levels) and EPI (high levels) (Nowotschin et al., 2019), which can signal in an autocrine (from the same cell) or paracrine (from neighboring cells) manner, seemingly are not correlated with global changes in FGF4. Whereas, ERK activity in cells specified as PrE by the end of the time-lapse are greatly influenced by population-based changes in FGF4, via paracrine signaling. This agrees with models of FGF4 as a dynamic readout of lineage size to determine cell fate specification as a means of tissue size control (Saiz et al., 2020).

Given that ERK signaling dynamics can span different time-scales (Conlon et al., 2016; Ryu et al., 2016), we also analyzed long-term signaling trends in the ICM. We imaged ERK-KTR in early blastocysts for a total of 12 h with 15-min time-lapse intervals (Figure S4F). Cells specified to PrE by the 12-h endpoint had significantly higher activity than EPI cells, and DP progenitor cells had intermediate activity (Figure S4G). These long-term trends were consistent with our results from short-term time-lapse imaging (Figures 4D and 4E). In the subset of cells that divided over the time course, ERK activity following cell division was initially low, then gradually increased over the remainder of the cell cycle for all ICM lineages (Figure S4H). Within ICM lineages, PrE cells exhibited elevated activity relative to EPI cells across the cell cycle (Figure S4I).

Lineage-Specific Dynamics of ERK Activity in ICM Cells

Integrated signaling activity is dependent on the strength and duration of the response. We noticed temporal variation in ERK activity within ICM cells in our short-term time-lapse imaging. Over the 2-h time-lapse ICM cells spent a varying percentage of time in a high versus low signaling state (Figures 4D and S4B). To characterize this temporal ERK activity, we classified peaks to extract dynamic information from single-cell traces. The peak classification parameters were optimized to identify local maxima of ERK activity for visually identifiable peaks in different cell types tracked across the ICM in 3D + *t* every 5 min for 2 h (Figures 5A–5D). To further validate this approach, we assessed peaks in our different treatment regimens of embryos cultured in MEKi or FGF (Figure 2). Using these settings only 3% of ICM cells were called with one or more peaks in MEKi-treated embryos (Figure S5A). By contrast, treatment with FGF increased the peak number and fraction of ICM cells with peaks as compared with untreated embryos (Figure S5B).

To assess differences between ICM lineages at early and mid-blastocyst stages, we compared the maximum signal, prominence (height relative to nearest minimum), and duration (width at half prominence) of each peak (Figure 5D). The maximum signal and duration of peaks were comparable between ICM lineages (Figures 5E and 5F). The peak prominence, however, was significantly higher in EPI cells than in PrE cells (Figure 5G). Given that the maximum signal was similar between EPI and PrE cells, we hypothesized that the difference in prominence was due to a

higher baseline ERK activity in PrE cells caused by sequential or sustained ERK activation. By comparing the nearest minimum (maximum signal—prominence) as an approximation of baseline levels, PrE cells indeed were elevated compared with EPI cells (Figure S5G). Furthermore, PrE cells had a higher proportion of cells with one or more peaks, relative to both EPI cells and DP progenitor cells (Figure 5H).

The peak duration was not significantly different between control (untreated) and FGF-treated embryos (Figures S5E and S5F). However, the maximum signal and nearest minimum (approximation of baseline) were elevated, while the peak prominence was reduced in cells of FGF-treated embryos compared with cells in controls (Figures S5D and S5G), indicating that the similar maximum signal of peaks in ICM lineages in untreated conditions (Figure 5E) was not due to saturation of the ERK-KTR biosensor but limited by the concentration of endogenous FGF ligand.

In conclusion, these data demonstrate that the ERK-KTR reporter enables the study of temporal changes in ERK activity during ICM lineage specification. ERK activity is dynamic with activation profiles that differ between cells of distinct lineage identities: EPI have lower baseline signaling with sporadic pulses of activity, while PrE cells have elevated baseline activity.

DISCUSSION

Genetic and pharmacological perturbation of FGF/ERK signaling has demonstrated its critical role for the proper patterning and proliferation of cells of the blastocyst-stage embryo prior to implantation into the maternal uterus. FGF/ERK signaling impacts all three blastocyst lineages; it drives PrE specification, regulates EPI maturation, and has been suggested as the mitogen which promotes TE proliferation (Christodoulou et al., 2019; Kurowski et al., 2019; Kang et al., 2017, 2013; Molotkov et al., 2017; Saiz et al., 2016b; Krawchuk et al., 2013; Yamanaka et al., 2010; Nichols et al., 2009; Chazaud et al., 2006; Yang et al., 2006; Goldin and Papaioannou, 2003; Chai et al., 1998; Tanaka et al., 1998; Feldman et al., 1995). To directly and quantitatively visualize the ERK signaling activities of individual cells within the entire blastocyst population, we developed an ERK-KTR-targeted mouse reporter line. Modulation of the FGF/ERK pathway in embryos revealed that our reporter was acutely responsive to these perturbations, thus, constituting a faithful readout of ERK activity. Although several ligands could potentially feed into the Raf/MEK/ERK cascade during pre-implantation stages, the absence of p-ERK staining and the complete loss of PrE specification in *Fgf4*^{−/−} embryos suggests that in the context of the blastocyst, ERK is the main effector of FGF signaling, and therefore, ERK-KTR primarily detects FGF4-driven ERK activity at this stage (Azami et al., 2019; Kang et al., 2013; Krawchuk et al., 2013; Chazaud et al., 2006).

By visualizing ERK signaling activities live in the TE, we quantified a spatiotemporal ERK activity gradient within the TE beginning from the early blastocyst stage, which was not possible with a previous transcriptional reporter of FGF signaling (Morgani et al., 2018). We observed decreasing ERK activity along the growing embryonic to abembryonic axis. Although FGF ligands may be freely diffusible (Yu et al., 2009), these data suggest that there is no substantial luminal accumulation of FGF4 in the blastocyst cavity as has been observed in other systems (Ryan

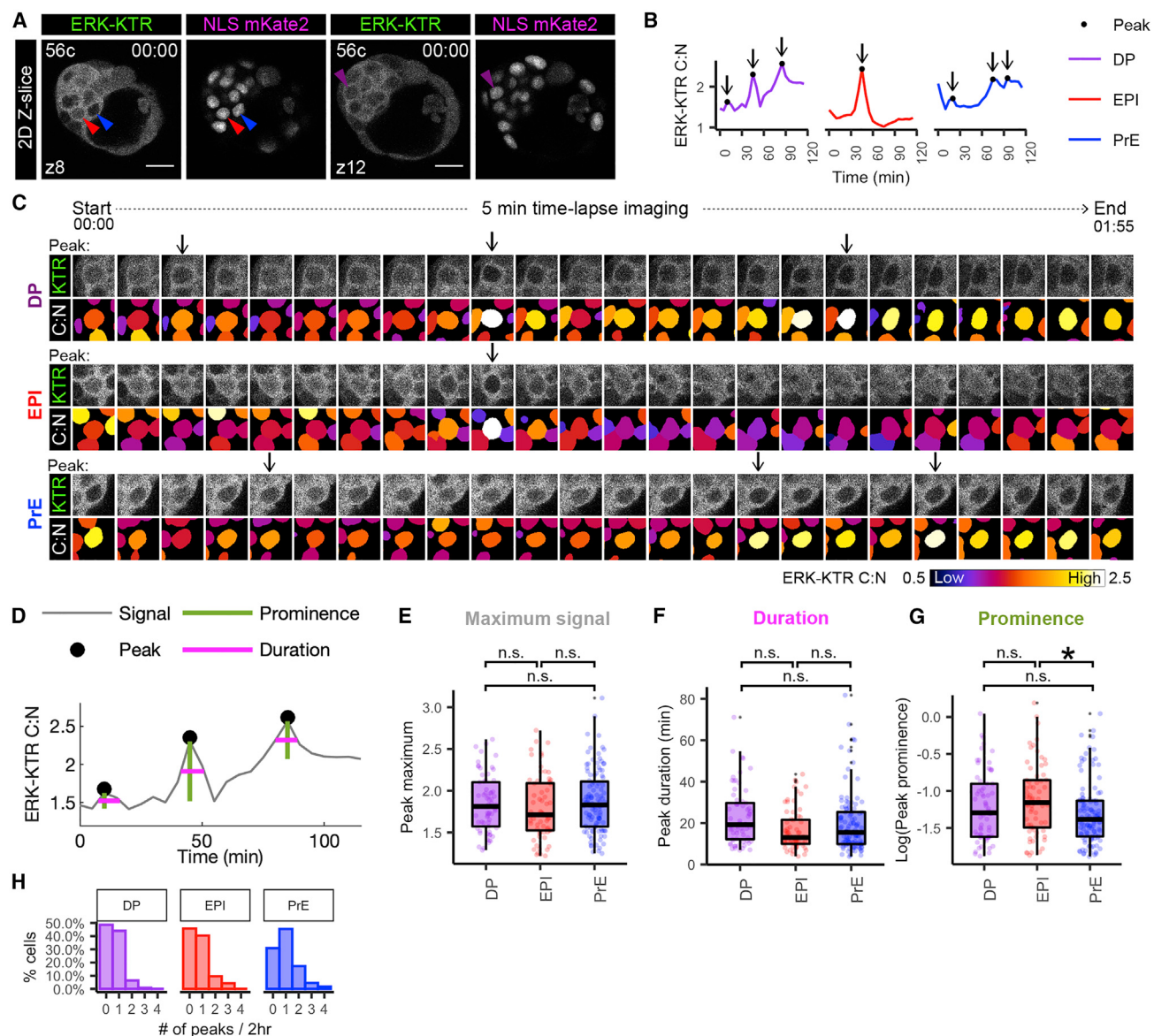


Figure 5. Lineage-Specific Dynamics of ERK Activity in ICM Cells

(A) Single Z slice of hemizygous *Hprt^{ERK-KTR}* and homozygous *R26^{NLS-mKate2}* embryos at beginning of 2-h 5-min time-lapse video (Figure 4A). Cell number and position of Z-slice is shown. Arrowheads indicate cells shown in traces (B) and sequential frames (C). Scale bars, 20 μ m.

(B) Single-cell traces of ERK-KTR C:N over 2-h time-lapse. Color coding indicate lineage as shown. Arrows indicate peaks.

(C) Sequential frames of example DP, EPI, and PrE cells from the same embryo shown in (A). ERK activity illustrated by C:N heatmap. Arrows indicate peaks.

(D) Peak calling (of the example DP trace from the embryo shown in A) to extract dynamic information from individual traces. Peaks identified as local maxima. Prominence is peak height relative to nearest minima. Duration is width at half prominence.

(E–G) Boxplot showing dynamic information extracted from peak calling (D). Each peak is shown as individual points. Color coding for lineage as indicated before. Asterisk indicates statistical significance. Prominence EPI versus PrE $p = 0.016$ (Wilcoxon test, Bonferroni adjustment). $n = 22$ embryos. Not significant (n.s.) pairwise comparisons indicated where $p > 0.05$. (H) Histogram showing the number of peaks detected within each cell as a percentage of cell in that lineage.

et al., 2019; Durdu et al., 2014). Alternatively, the presence of extracellular matrix components, such as heparin sulfate (HS), might act to concentrate ICM progenitor and EPI-derived ligands in the immediate vicinity of the ICM (Matsuo and Kimura-Yoshida, 2014; Ornitz, 2000). At early post-implantation stages, HS-deficient *Ext2* embryos show defective extra-embryonic ectoderm (of polar TE origin) development, due to a requirement for HS-chains in retaining locally high concentrations of FGF ligands

(Shimokawa et al., 2011). In support of the short-ranging FGF action, a recent study in mouse ESCs revealed that paracrine FGF4 signals act over distances of ~ 1 -cell diameter (Raina et al., 2020). We found that signaling within the mural TE increased upon FGF treatment, indicating that ligand availability can shape the spatiotemporal ERK activity gradient within the TE. *In vivo* and *in vitro* studies have revealed that FGF4 maintains trophoblast stem cell identity (Nichols et al., 2009; Goldin and Papaioannou,

2003; Chai et al., 1998; Tanaka et al., 1998). Competition within the TE for locally concentrated FGF4 mitogens emanating from the ICM niche could therefore act as a mechanism to balance the ratio of stem cell and differentiated progenitors, as seen in other systems (Kitadate et al., 2019).

FGF signaling via ERK is required within the ICM for PrE specification, and EPI maturation, which is abrogated upon MEK inhibition (Nichols et al., 2009). We observed some ERK activity in all ICM cells, in agreement with observations made using p-ERK staining, *Spry4*^{H2B-GFP} transcriptional reporter and pan-ICM expression of *Fgfr1* and its functional requirement in both PrE and EPI lineages (Azami et al., 2019; Morgani et al., 2018; Kang et al., 2017; Molotkov et al., 2017). ERK signaling was highest in those cells that had acquired a PrE identity by the end of the time-lapse videos. This differential ERK signaling could be explained by the local signaling environment where higher concentrations of the FGF4 ligand leads to signaling via FGFR1/ERK on progenitor cells to induce a PrE fate (Kang et al., 2017; Molotkov et al., 2017). Or additionally, by upregulation of RTK receptors FGFR2 and PDGFRA or modifiers of the pathway upon PrE specification (Azami et al., 2019; Kang et al., 2017; Molotkov et al., 2017). Although, as both FGFR2 and PDGFRA act via the PI3K axis for PrE-survival (Bessonard et al., 2019; Molotkov and Soriano, 2018; Artus et al., 2013), ERK activity is likely relayed by FGFR1, which is the predominant receptor for PrE specification (Kang et al., 2017; Molotkov et al., 2017). Therefore, it remains possible that, even though they are subtle, the higher ERK activities seen here in the PrE might be instructive for lineage acquisition. However, since we relied on endpoint analyses for assignment of lineage identities, we could not assign the exact point at which individual cells commit to a particular fate. Lineage-specific expression changes to FGF pathway components may also lead to differential ERK activity between lineages. Therefore, a limitation of this analysis is our inability to attribute causality between ERK activities and cell fate specification. Further study involving multiple spectrally distinct lineage-specific (PrE and EPI) reporters (Xenopoulos et al., 2015; Plusa et al., 2008) alongside the ERT-KTR would be needed to delineate the causality and timing of ERK activities and eventual fates.

Transient and pulsatile modes of ERK activity have been described in diverse systems including cultured mammalian cells, mouse epidermis, *Drosophila*, *C. elegans* embryos, and yeast through single-cell time-lapse imaging using ERK FRET, phase separation, or KTR-based biosensors (Deathridge et al., 2019; Moreno et al., 2019; Zhang et al., 2018; de la Cova et al., 2017; Gillies et al., 2017; Conlon et al., 2016; Ryu et al., 2016; Hiratsuka et al., 2015; Regot et al., 2014). Here, we quantified short-term temporal dynamics operating within ICM cells of the mouse blastocyst-stage embryo. PrE cells exhibit sustained ERK activity, whereas EPI cells exhibit infrequent pulses while maintaining lower baseline ERK activity, reminiscent of the steady-state in ESCs (Figures S1A and S1B). We were limited in the total length of time we could image short-term ERK dynamics, as we sought to reduce the overall light exposure time to reduce phototoxicity in embryos imaged by confocal microscopy. Moving forward, the use of alternative imaging modalities, for example selective plane illumination microscopy (SPIM), which require lower levels of light exposure should enable *in toto* embryo imaging at high

temporal resolution throughout the entire ICM cell fate specification process (~48 h) (Strnad et al., 2016).

How cells decode signaling dynamics has been studied in other systems (Patel and Shvartsman, 2018; Levine et al., 2013; Purvis and Lahav, 2013) but been a challenge to investigate within mammalian embryos. Reflecting on our observations with PrE fate acquisition, integrated ERK activity over time is associated with cell fate determination in multiple cell types (Gillies et al., 2017; Albeck et al., 2013; Aoki et al., 2013; Santos et al., 2007; Murphy et al., 2002). We hypothesize that ICM lineage identity is encoded by pulsatile versus sustained signaling dynamics, as has been shown for ERK pathway in neurogenesis (PC-12 cells) (Santos et al., 2007), and the Notch pathway during myogenesis (C2C12 cells) (Nandagopal et al., 2018). In these cultured mammalian cell lines, differential dynamics are elicited by distinct ligands, but it is an open question how differential dynamics are established in response to a single ligand within the ICM of the mouse blastocyst. Treatment of blastocysts with exogenous FGF4 lead to a sustained signaling response, due to increased baseline ERK activity, as well as a moderate increase in the frequency of pulses. Thus, differential activity of PrE and EPI cells may be indicative of heterogeneous FGF4 concentrations across the ICM. In addition, differential activity could also be due to the expression of distinct negative feedback regulators (EPI: ETV5, DUSP1/6, PrE: DUSP4) or receptors (EPI: FGFR1, PrE: FGFR1, FGFR2, PDGFRA) upon specification to EPI versus PrE identities (Azami et al., 2019; Nowotschin et al., 2019; Kang et al., 2017; Ohnishi et al., 2014; Guo et al., 2010). Alternatively, ICM cell fate decisions may operate through amplitude modulation (Li and Elowitz, 2019), as has been shown for p53 regulation of cell-cycle transitions (Reyes et al., 2018). Taken together, our results constitute direct visualization of dynamic signaling events underlying mammalian pre-implantation development, leading us to uncover spatial and temporal lineage-specific dynamics. The approach and tools we have developed will be broadly applicable to study signaling dynamics in other *in vivo* contexts using the mouse model.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2020.09.030>.

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AUTHOR CONTRIBUTIONS

Conceptualization, Writing, and Funding Acquisition, C.S.S. and A.-K.H.; Methodology, Investigation, and Analysis, C.S.S.; Data Curation, S.R. and C.S.S.; Resources, D.R. and C.S.; Supervision, A.-K.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-NANOG	Reprocell	Cat# RCAB001P; RRID: AB_1962694
Goat anti-GATA6	R&D Systems	Cat# AF1700; RRID: AB_2108901
Donkey anti-goat A568	Invitrogen	Cat# A-11057; RRID:AB_2534104
Donkey anti-rabbit A647	Invitrogen	Cat# A-10042; RRID: AB_2534017
Chemicals, Peptides, and Recombinant Proteins		
Hoechst 33342	Invitrogen	Cat# H3570
FGF4	R&D Systems	Cat# 235-f4
Heparin	Sigma	Cat# H3149
PD0325901	Reprocell	Cat# 04-0006-10
EmbryoMax KSOM (+AA w/o PhenolRed)	Sigma	Cat# MR-106-D
Mineral oil	Sigma	Cat# M5310
FHM	Millipore	Cat# MR-025-D
Proteinase K	Roche	Cat# 03115801001
PFA	Electron microscopy sciences	Cat# 15710
Acid Tyrode's	Millipore	Cat# MR-004-D
Glycine	Sigma	Cat# G7403
Triton X-100	Sigma	Cat# X100
Horse Serum	Sigma	Cat# H0146
DMEM	Life technologies	Cat# 11995073
NEAA	Life technologies	Cat# 11140-050
Glutamine	Life technologies	Cat# 25030164
Glutamax	Life Technologies	Cat # 35050038
Sodium Pyruvate	Life technologies	Cat# 11360070
2-mercaptoethanol	Life technologies	Cat# 21985023
Fetal Calf Serum	VWR	Cat# 97068-085
HAT supplement	Life technologies	Cat# 21060017
0.25% Trypsin EDTA	Life technologies	Cat# 25200114
Fibronectin	Millipore	Cat# FC010
Gelatin	Millipore	Cat# 104070
Penicillin/Streptomycin	Life technologies	Cat# 15140163
Mitomycin C	Sigma	Cat# M4287
Deposited Data		
CellProfiler pipelines, ERK-KTR C:N measurements from 3D time-lapse videos, R and Matlab scripts for data analysis	<i>This paper</i>	https://github.com/therealkatlab/Simon2020
Confocal microscopy images	<i>This paper</i>	https://doi.org/10.6084/m9.figshare.12794810
Experimental Models: Cell Lines		
Mouse: Embryonic stem cell line <i>Hprt^{ERK-KTR-mClover}</i>	<i>This paper</i>	N/A
Experimental Models: Organisms/Strains		
Mouse: <i>R26^{nls-mKate2}</i>	(Susaki, et al., 2014)	N/A
Mouse: <i>Hprt^{ERK-KTR-mClover}</i>	<i>This paper</i>	JAX#035333
Mouse: CD1	Charles River Laboratory	Cat# 022

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
mKate2_F: 5'- AGATCTGGTACTCGTATG GTGAGCGAGCTGATT-3'	(Susaki, et al., 2014)	N/A
mKate2_R: 5'-TGTGCCTGTAAGCTTTCT TCTGTGCCCCAGTTTGCT-3'	(Susaki, et al., 2014)	N/A
Software and Algorithms		
Imaris 9.1.2	Bitplane (Oxford Instruments)	https://imaris.oxinst.com/
ImageJ	(Schneider, et al., 2012)	https://imagej.nih.gov/ij/
R Studio 1.0.143	RStudio	https://rstudio.com/
Matlab R2014a/R2018a	MathWorks	https://www.mathworks.com/products/matlab.html
CellProfiler 2.1	(Lamprecht, et al., 2007)	https://cellprofiler.org/
MINS	(Lou, et al., 2014)	http://katlab-tools.org/
ZEN	Carl Zeiss Microsystems	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html
Other		
Glass-bottom dish	MakTek	Cat# P35G-1.5-14-C

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anna-Katerina Hadjantonakis (hadj@mskcc.org)

Materials Availability

Mouse lines generated in this study have been deposited to Jackson Labs, JAX#035333.

Data and Code Availability

The dataset and code generated and used in this study are available at Github, <https://github.com/therealkatlab/Simon2020>. Time-lapse microscopy images generated in this study are available at Figshare, [<https://doi.org/10.6084/m9.figshare.12794810>].

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

Mouse embryonic stem cell (ESC) line *Hprt*^{CAG:ERK-KTR-mClover} (abbreviated as ERK-KTR) was targeted as follows. The CAGGS enhancer/promoter sequence and a rabbit beta-globin poly-A sequence were PCR amplified from pCX-H2B-EGFP (Hadjantonakis and Papaioannou, 2004). The ERK-KTR-Clover coding sequence was amplified from pENTR-ERKTRClover (Addgene plasmid #59138) (Regot et al., 2014). A targeting vector containing homology arms to the *Hprt* locus (Doetschman et al., 1987) as well as a DTA cassette was linearized with AclI (NEB), and the PCR fragments were inserted via Gibson assembly. A sequence-verified targeting vector was linearized with Sall (NEB) and electroporated into E14tg2a ESCs (Hooper et al., 1987). ESCs were plated onto HPRT+ mouse embryonic feeder cells in DMEM (Life Technologies) supplemented with 15% FBS (Sigma), 10 ng/ml recombinant leukemia inhibitory factor (LIF) (Department of Biochemistry, University of Cambridge, and protein expression facility, MPI Dortmund), 0.1mM 2-mercaptoethanol, glutamax and non-essential amino acids (NEAA) (all Life Technologies). One day after electroporation, correctly targeted clones were selected by addition of 100 μ M hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine (HAT medium, Life Technologies) (Doetschman et al., 1987). All surviving clones evenly expressed the fluorescent sensor. We selected three independent clones for karyotyping (Nagy et al., 2008), all of which had a modal chromosome count of $n = 40$. After targeting, ESCs were maintained on 0.1% gelatin (Millipore) coated tissue-culture grade plates in a humidified 37°C incubator with 5% CO₂. Cells were grown in DMEM, supplemented with 2mM L-glutamine, 0.1mM MEM NEAA, 1mM sodium pyruvate, 100U/ml penicillin and 100 μ g/ml streptomycin, 0.1mM 2-mercaptoethanol (all from Life Technologies), 15% FBS (VWR), and 1000U/ml LIF. ESCs were maintained on a layer of Mitomycin C inactivated mouse embryonic fibroblast (MEF) for the generation of chimeric mice and maintained without feeders for live imaging analysis.

Mouse Strains and Husbandry

All animal work was approved by MSKCC Institutional Animal Care and Use Committee (IACUC). Animals were housed in a pathogen free-facility under a 12 hour light cycle. Mouse strains used in this study were *Hprt*^{ERK-KTR} (this study), *R26*^{CAG:3xNLS-mKate2} (abbreviated as NLS-mKate2) (Susaki et al., 2014) and wild-type CD1 (Charles River). *Hprt*^{ERK-KTR} mice were generated by the Mouse Genetics Core at MSKCC. Briefly, *Hprt*^{ERK-KTR} ESCs derived from E14Tg2a 129Ola mouse strain were injected into C57Bl6 host blastocyst. Resulting high contribution male germline chimeras were mated to wild-type CD1 females. Animals were maintained on a mixed (CD1/Bl6/129) strain background. Mice were genotyped by PCR and fluorescence of ubiquitously expressed fluorescent protein reporters. The *Hprt*^{CAG-ERK-KTR-mClover} mouse line will be available from The Jackson Laboratory as JAX stock no. 035333

METHOD DETAILS

Embryo Culture

Male mice homozygous for the nuclear reporter *R26*^{NLS-mKate2} were mated with female mice homozygous for the nuclear reporter *R26*^{NLS-mKate2} and heterozygous for the ERK reporter *Hprt*^{ERK-KTR/+}. Embryos for this study were obtained from natural matings of females 5 – 12 weeks of age. The sex of embryos was not determined. E3.0 – E4.5 blastocysts were flushed from uterine horns with flushing and holding medium (FHM, Millipore) as described (Behringer et al., 2014). Zona pellucidae were removed by brief incubation in acid Tyrode's solution (Sigma). Embryos were washed three times, and then cultured in 5μl in KSOM-AA medium without phenol red (Millipore) overlaid with mineral oil (Sigma), with approximately 5 embryos per droplet. Embryos expressing the ERK-KTR were identified by epifluorescence microscopy, cultured in 35mm glass-bottomed dishes (Maktek) and imaged on a Zeiss LSM880 confocal microscope in a humidified incubation chamber with 5% CO₂ at 37°C for 2 or 12 hours. Negative littermate embryos were cultured in 35mm dishes, in a humidified 37°C incubator with 5% CO₂ as a control for the same duration as their live-imaged littermates. For ERK signaling perturbation experiments embryos were cultured in the same way, and littermate embryos were randomly assigned in even sized groups between Control: KSOM and either FGF stimulation: 1μg/ml FGF4 (R&D Systems) and 1μg/ml Heparin (Sigma) in KSOM or ERK inhibition: 1μM PD0325901 (MEK inhibitor, Reprocell) in KSOM.

Live Image Data Acquisition

Time-lapse imaging of embryos was performed using Zeiss LSM880 laser-scanning confocal microscope. Images were acquired using an oil-immersion Zeiss EC Plan-Neofluar 40x/NA1.3 with a 0.17mm working distance. Laser power was measured at the beginning of each session; mClover: 488nm Argon laser at 30μW and mKate2: 561nm DPSS 561-10 laser at 80μW. Both colors were acquired simultaneously using 0.85μs pixel dwell, bi-directional line scanning, 2-line averaging, 1.5x zoom and 1 airy unit pinhole. To limit light exposure, the inner cell mass was centered in a 60μm stack, with a 2μm step, generating 12-bit range 512x512px images of 0.277μm/px resolution acquired in ~30s per embryo and time point. Embryos were imaged with a time-interval of 5 min for a total of 2 hours, or 15 min for a total of 12 hours, as indicated. Before each time-lapse video an image stack encompassing the entire embryo was acquired in only mKate2 to allow for an initial cell-count for staging, and time (~30 min) for the embryos to settle after placement on the scope to minimize movement during time-lapse acquisition. After time-lapse imaging a second complete overview stack was taken to allow for manual image registration to the corresponding fixed immunofluorescence image. Time-lapse images of cells were acquired using the same set-up in mClover only and a single z-slice, with a time-interval of 3 min for a total of 90 min,

Immunofluorescence

Immunofluorescence of embryos was carried out as previously described (Simon et al., 2018b). Briefly, live-imaged embryos (processed individually) and cultured littermate controls were collected immediately after time-lapse videos were complete and fixed in 4% PFA (Electron Microscopy Sciences) for 10 min at room temperature. Fixation quenched endogenous mKate2 fluorescence. Fixed embryos were stored in PBS and prior to staining washed in PBX; 0.1% TritonX-100 (Sigma) in PBS, permeabilized for 5 min in 0.5% TritonX-100, 100mM glycine (Sigma) in PBS, and washed in PBX. Embryos were blocked in blocking buffer, 2% horse serum (Sigma) in PBS, for 40 min at room temperature and incubated overnight at 4°C with goat anti-GATA6 (R&D, 1:100) and rabbit anti-NANOG (Reprocell, 1:500) in blocking buffer (see Key Resources Table). Then, embryos were washed 3x in PBX, blocked again in blocking buffer for 40 min at room temperature, and incubated with secondary antibodies donkey anti-rabbit A647 and donkey anti-goat A568 (both Invitrogen, 1:500) in blocking buffer for 1 hour at 4°C. Embryos were washed 2x in PBX, then incubated for at least 1 hour in 5μg/ml Hoechst 33342 (Invitrogen) in PBS prior to imaging.

Image Acquisition of Fixed Samples

Fixed immunostained samples were imaged on a Zeiss LSM880 laser scanning confocal microscope. Embryos were mounted in microdroplets of 5μg/ml Hoechst 33342 in PBS on glass-bottomed dishes (Maktek) coated with mineral oil (Sigma). Embryos were imaged along the entire z-axis with 1μm step using an oil-immersion Zeiss EC Plan-Neofluar 40x/NA1.3 with a 0.17mm working distance. Laser power was measured prior to each imaging session and adjusted to maintain consistent power output across experiments to reduce technical variability.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image Processing and Quantification

Quantification of the ERK-KTR is outlined in Figure 2A. The ERK-KTR is a ratiometric reporter which shuttles in and out of the nucleus, where the relative intensity between the cytoplasm and nucleus is a read-out of ERK activity. Raw images were converted to single files for each z-plane, time-point and channel. Segmentation and intensity measurements were performed in CellProfiler (Lamprecht et al., 2007). Image processing, segmentation and calculation of C:N ratio was performed independently for each z-plane and time-point i.e. in 2D. First, the embryo was identified in the ER-KTR channel and used as a mask for objects to ensure no background is included in cytoplasm and nuclear objects, and to measure background mean fluorescent intensity of ERK-KTR. Next, the NLS-mKate2 channel was processed (rescaled intensity, enhance features, gaussian blur), and nuclear segmentation carried out using a three-class Otsu thresholding method. The initial nuclear segmentation was shrunk by 2px and this area used to measure the mean nuclear fluorescent intensity of the raw ERK-KTR. For the cytoplasm, a ring of 6px width around the nucleus with a 1px buffer from the initial nuclear segmentation was used to measure the mean cytoplasmic fluorescent intensity of the raw ERK-KTR. The cytoplasmic ring was restricted to prevent overlap with neighboring cells, and to avoid detection of background pixels at the edge of the embryo. The C:N (cytoplasmic to nuclear ratio) was calculated for each nuclei by dividing the mean background-subtracted cytoplasmic ERK-KTR intensity by the mean background-subtracted nuclear intensity ERK-KTR. This ratio normalizes for the fluorescence decay along the z-axis. The C:N value was calculated per nuclei at each z-plane and time point. To generate a 3D+time image representation, each nuclear object was color-coded by its C:N value as a 16-bit image, and then each z-plane and time-point concatenated into a TIFF stack of C:N value images. In rare cases, there is under-segmentation of dense ICM cells. However, these clumped cells are automatically filtered out based on a size threshold for objects. For these under segmented cells, a second CellProfiler pipeline using two-class Otsu thresholding method, which better segments dense ICM cells, was used for C:N measurements. Calculations and image processing of C:N was performed using CellProfiler outputs in Matlab R2018a (Mathworks) and ImageJ (Schneider et al., 2012).

Cell-tracking of embryos was performed manually in Imaris (Bitplane) on the mKate2 channel using the Spots function. 3D+time images of C:N values were imported into Imaris to perform measurements. Spots of diameter 5 μ m were placed inside each tracked nucleus to sample at least two Z-slices (2 μ m step). Not all ICM cells could be tracked in each embryo due to cells moving out of frame, or difficulty tracking daughter cells after loss of nuclear NLS-mKate2 upon nuclear envelope breakdown at mitosis.

Time-lapse videos and fixed immunostained images of the same embryo were manually registered in Imaris. mitotic, apoptotic, distinctly shaped or sized cells, and abutting mural TE cells were annotated and used as landmarks to aid manual registration and orient embryos in 3D.

To perform cell-counts of live-imaged embryos and lineage assignment of fixed immunostained embryos, a Matlab based 3D nuclear segmentation software tool, MINS was used as described previously (Saiz et al., 2016a; Lou et al., 2014). Correction for fluorescence decay along the z-axis was performed by fitting linear regressions to the log fluorescence values as a function of the z -axis and correcting using an empirical Bayes method, as detailed in (Saiz et al., 2016b).

Lineage identities were assigned as follows. Outer TE cells were manually annotated in Imaris by their position; overlying the cavity as mural TE and overlying ICM as polar TE. ICM in fixed images were assigned based on NANOG and GATA6 expression using k-means clustering method, used previously (Saiz et al., 2016b), where GATA6+NANOG+ marks DP progenitors, NANOG+ marks EPI and GATA6+ marks PrE. Lineage identities of fixed immunostained embryos were then used to annotate corresponding cells in registered final frame in the time-lapse video of the same embryo. Any ambiguous ICM cells (inc. double negative cells) that could not be registered with the corresponding fixed image were not assigned a lineage identity and excluded from analysis.

To generate supplemental videos of time-lapse imaged embryos, images were registered between time points to compensate for embryo movement during the experiment. For this, we used the descriptor based series registration plug in in ImageJ (Preibisch et al., 2010), with NLS mKate2 as the registration channel.

Data Processing

All plots were generated using R Studio version 1.0.143 and Matlab R2018a. During mitosis there is breakdown of the nuclear envelope and thus, no shuttling of KTR. Therefore, time-points when a cell was undergoing mitosis were manually labelled based on NLS-mKate2 localization, and excluded from analysis. Apoptotic cells were also removed, and only cell tracks of length equal to or greater than 5 frames were included in the analysis. Parent and daughter cells before and after mitosis were treated independently.

Statistical power calculations were not used to pre-determine sample size. Figure legends and panels provide statistical details for each experiment, including p-values, the exact value and representation of n, definition of data points, and details of data representation. For comparison of ERK-KTR C:N values between different lineages (Figures 4F and 4D), we used a linear mixed effects model to account for embryo-fixed effects. If there was a significant effect ($p < 0.05$, ANOVA test) of lineage in the linear mixed effects model, we performed and reported p-values for post-hoc Tukey test for pairwise comparisons between lineages. For other pairwise comparisons, we used the non-parametric Wilcoxon test to compare between the means for each embryo (n reported in figure legends). When multiple groups were tested, we applied a Bonferroni correction for multiple comparisons. Boxplots in all figures are as follow: top and bottom edges of boxes represent third and first quartiles, middle lines mark the median, top and bottom whiskers extend from first and third quartile to lowest and highest value within 1.5 * inter-quartile range.

To identify a threshold ERK-KTR C:N value to assign high versus low ERK activity, we compared ICM cells from MEKi and FGF treated embryos. We iteratively tested thresholds at 0.01 steps across the entire range of the dataset to classify values as high (FGF treated) and low (MEKi treated) ERK activity. The best threshold (C:N value = 1.2142) was determined as the median of thresholds most accurately classifying cells as MEKi and FGF treated.

Peaks of ERK activity were classified in Matlab R2018a. Traces were smoothed using `smoothdat` with a Gaussian weighted moving average filter with window length 3 to reduce noise for peak calling. Peaks were identified using `findpeaks`. The threshold for the peak prominence was determined empirically based on visually identifiable peaks, and the threshold for the peak maximum was the same as assigning high-low ERK activity (described above).