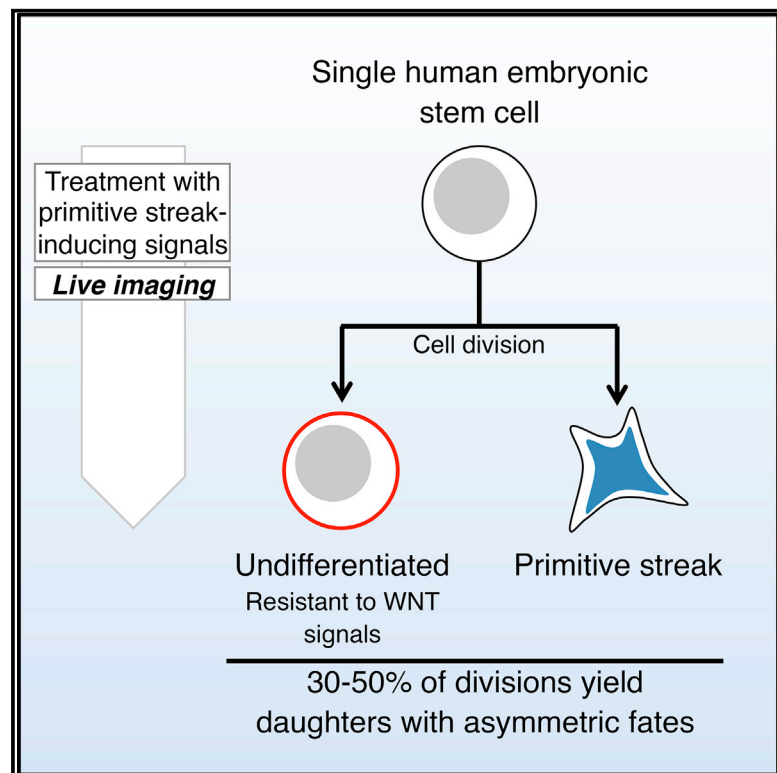


Cell Reports

Live Imaging Reveals that the First Division of Differentiating Human Embryonic Stem Cells Often Yields Asymmetric Fates

Graphical Abstract



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In Brief

Brown et al. show that 30%–50% of the time, the first division of differentiating human embryonic stem cells generates one differentiated and one undifferentiated daughter cell. These asymmetric fate outcomes have implications for the efficiency of stem cell differentiation protocols and the mechanisms underlying lineage commitment.

Highlights

- Live imaging of the first division of differentiating hESCs
- 30%–50% of divisions yield one non-primitive streak and one primitive streak daughter
- In asymmetric cell pairs, the non-primitive streak cell cannot respond to WNT
- Heterogeneous responses to WNT can induce asynchronous differentiation



Live Imaging Reveals that the First Division of Differentiating Human Embryonic Stem Cells Often Yields Asymmetric Fates

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SUMMARY

How do stem cells respond to signals to initiate differentiation? Here, we show that, despite uniform exposure to differentiation-inducing extracellular signals, individual human embryonic stem cells (hESCs) respond heterogeneously. To track how hESCs incipiently exit pluripotency, we established a system to differentiate hESCs as single cells and conducted live imaging to track their very first cell division. We followed the fate of their earliest daughters as they remained undifferentiated or differentiated toward the primitive streak (the earliest descendants of pluripotent cells). About 30%–50% of the time, hESCs divided to yield one primitive streak and one undifferentiated daughter. The undifferentiated daughter cell was innately resistant to WNT signaling and could not respond to this primitive-streak-specifying differentiation signal. Hence, the first division of differentiating hESCs sometimes yields daughters with diverging fates, with implications for the efficiency of directed differentiation protocols and the underlying rules of lineage commitment.

INTRODUCTION

Differentiation of stem cells into downstream cell types *in vitro* has traditionally been accomplished by *en masse* treatment of bulk stem cell populations with extracellular signals. However, individual stem cells differ in the lineages they commit to and the timing with which they differentiate (Okano and Temple, 2009). Hence there is a pressing need to follow stem cell differentiation at a single-cell level (Etzrodt et al., 2014). Despite progress in differentiating human embryonic stem cells (hESCs) into increasingly homogeneous populations for regenerative medicine, we have an incomplete understanding of how single stem cells commit to differentiate. To this end, it has not been trivial to propagate or differentiate hESCs as single cells in culture;

this is because individual hESCs invariably apoptose (Chen et al., 2010). By genetically manipulating hESCs in such a way that they survive differentiation as single cells and by exploiting reporters for lineage differentiation, we have re-examined how hESCs at the exit point from pluripotency incipiently commit to differentiate using live imaging.

RESULTS

The first lineage option that pluripotent hESCs face is to differentiate into ectoderm or the primitive streak (Murry and Keller, 2008) (Figure 1A). Primitive streak progenitors are the precursors to the endoderm and mesoderm germ layers; they are specified by extracellular signals including ACTIVIN/TGF- β , BMP, FGF, and WNT, which consequently lead to expression of key mesodermal transcription factors including BRACHYURY, MIXL1, and EOMES (Blauwkamp et al., 2012; Davidson et al., 2012; Davis et al., 2008; Gadue et al., 2006; Loh et al., 2014) (Figure 1A).

To track how hESCs incipiently exit pluripotency and commit to the primitive streak lineage, we exploited a simple, chemically defined differentiation system. This system—which entails simultaneous treatment with ACTIVIN/TGF- β , BMP, FGF, and WNT agonists (where the WNT agonist is either the GSK3 inhibitor CHIR99021 or recombinant WNT3A protein)—generates a >98% pure MIXL1⁺ primitive streak population within 24 hr of differentiation (Loh et al., 2016), as assayed by a *MIXL1-GFP* knockin reporter hESC line (Davis et al., 2008). When produced in these conditions, MIXL1⁺ primitive streak is competent to further differentiate into lateral mesoderm and heart cell types, as shown previously (Loh et al., 2016). We confirmed that primitive streak specification in this system was completely dependent on WNT signaling (Blauwkamp et al., 2012; Davidson et al., 2012; Gadue et al., 2006; Loh et al., 2014) (Figure 1A). In the absence of an exogenous WNT agonist, primitive streak formation was reduced, and it could further be completely inhibited by an inhibitor of endogenous WNT protein production, known as C59 (Proffitt et al., 2012) (Figure S1A). WNT-inhibited cells continued to express SOX2 (suggesting that they remained largely undifferentiated) and lacked expression of primitive streak markers BRACHYURY or MIXL1-GFP (Figures S1B and S1C). This underscores the absolute requirement of WNT for

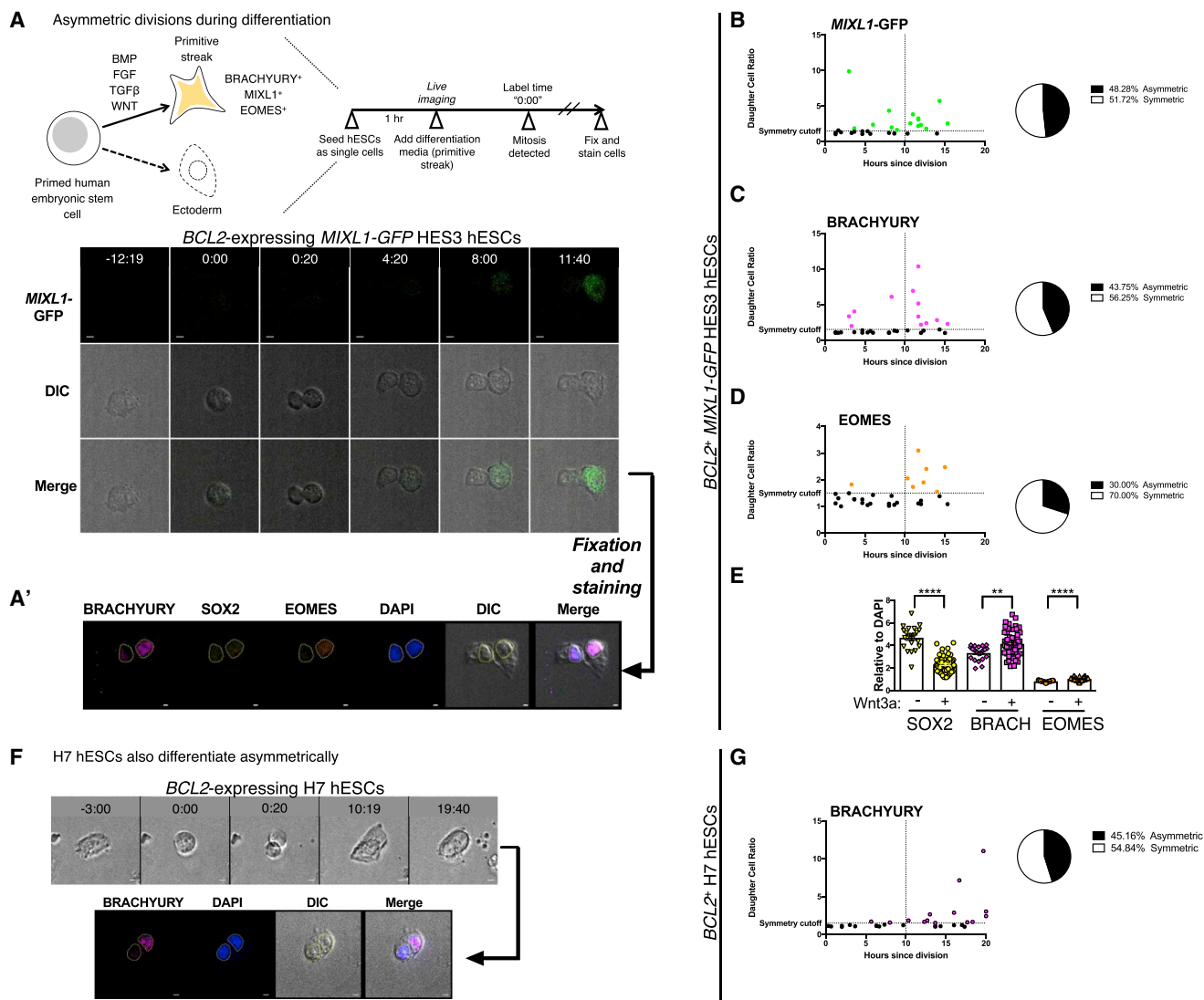


Figure 1. Incipient Differentiation of hESCs into Primitive Streak Often Generates Daughter Cell Pairs with Asymmetric Fates

(A) BCL2⁺ MIXL1-GFP HES3 hESCs were treated with primitive streak medium (containing ACTIVIN, BMP4, FGF2, and WNT3A; see [Experimental Procedures](#)) and monitored by live imaging, with still images shown. The time of mitosis was set to "0:00." Scale bar represents 10 μm.

(A') BRACHYURY, SOX2, and EOMES protein expression in fixed cells; same cells as in (A). Scale bar represents 5 μm.

(B–D) Ratio of MIXL1-GFP (B), BRACHYURY (C), or EOMES (D) expression in pairs of daughter cells, plotted against the number of hours since the mother cell divided prior to the end of live imaging. Pie charts enumerate the overall percentage of asymmetric versus symmetric daughter cell pairs observed. Data are representative of three to five independent experiments.

(E) Expression of SOX2, BRACHYURY, and EOMES proteins in hESCs exposed to primitive streak differentiation media (ACTIVIN, BMP4, and FGF2, with the inclusion of C59 to block endogenous WNT), in the presence of WNT3A; protein staining normalized to the intensity of DAPI staining. Error bars represent SD for this figure and all subsequent figures.

(F) BCL2⁺ H7 hESCs were treated with primitive streak medium (ACTIVIN, BMP4, FGF2, and WNT3A) and monitored by live imaging, with still images shown (top). The time of mitosis was set to "0:00." Fixed cells expressed BRACHYURY protein in an asymmetric fashion (bottom).

(G) Ratio of BRACHYURY expression in pairs of daughter cells, plotted against the number of hours since the mother cell divided prior to the end of live imaging. The pie chart enumerates the overall percentage of asymmetric versus symmetric daughter cell pairs observed.

primitive streak specification. Indeed, C59-induced inhibition of primitive streak formation could be rescued by addition of exogenous WNT3A ([Figure S1A](#)).

Interrogating whether hESCs divide and differentiate into symmetric or asymmetric pairs of primitive streak daughters is best done with single stem cells in isolation to accurately track the

fate of each daughter cell. However, single hESCs bereft of their neighbors invariably apoptose ([Chen et al., 2010](#)), which impeded studies of how single hESCs divide and differentiate. To circumvent this limitation, we overexpressed the anti-apoptotic protein BCL2, which enables hESCs to withstand single-cell dissociation without influencing cell cycle or

differentiation ability (Ardehali et al., 2011). Hence, to study individual isolated hESCs differentiating into primitive streak, we constructed a doubly transgenic *BCL2*-expressing *MIXL1-GFP* hESC reporter line with improved cell survival (Figure S1D).

Strikingly, live cell imaging showed that differentiating *MIXL1-GFP* reporter hESCs exposed to primitive-streak-inducing signals often divided to yield asymmetric daughter fates, leading to daughter cells with different *MIXL1-GFP* intensities (Figure 1A). Hereafter we denote such divisions as “asymmetric cell divisions” in reference to the phenotypic outcomes of these divisions—namely, the production of two daughter cells of distinct fates—without referring to the act of cell division itself as innately asymmetric or implying that it is caused by asymmetric inheritance of lineage determinants (Morrison and Kimble, 2006). Endogenous detection of *BRACHYURY* and *EOMES* proteins in fixed cells after live imaging also revealed asymmetric expression (Figure 1A'), indicating this was not an artifact of the *MIXL1-GFP* knockin reporter.

Differentiation-induced divisions sometimes, although not always, led to asymmetric fate outcomes, as evinced by heterogeneous expression of *MIXL1-GFP* (in 48.3% of cell divisions), *BRACHYURY* (42.8%), and *EOMES* (30%) (Figures 1B–1D and S1E). *BRACHYURY* was more strongly induced than *EOMES*, potentially consistent with earlier onset of *BRACHYURY* expression (Izumi et al., 2007) or different regions of the primitive streak heterogeneous for *EOMES* expression (Costello et al., 2011) (Figure 1E). Although primitive streak markers showed asymmetric expression in certain daughter cell pairs, *OCT4* was nonetheless symmetrically expressed in both cells, consistent with how it is expressed in both pluripotent and primitive streak states (Hoffman et al., 2013; Loh and Lim, 2011; Teo et al., 2011) (Figures S1F and S1G). Additionally, in daughter cell pairs with asymmetric primitive streak marker expression, pluripotency transcription factor *SOX2* was nonetheless suppressed in both cells (Figure 1A'), and the low remaining levels of *SOX2* did not show evidence of asymmetry between both daughters (Figure S1G). The exact identity of the “non-primitive streak” daughter in these asymmetric pairs is unknown. Given that it is *SOX2*^{lox/-}, it is neither pluripotent nor ectoderm. Presumably it has exited pluripotency but has not yet turned on primitive streak markers. Finally, nuclear volume was not significantly different between daughter cells (Figure S1H). In sum, ~30%–50% of the time, differentiating hESCs divided to yield one primitive streak and one non-primitive streak daughter.

Interestingly, the lineage outcomes of daughter cell pairs were contingent on the timing of cell division: hESCs that divided early (within the first ~1–10 hr) tended to generate asymmetric lineage outcomes, whereas hESCs that divided late (after 10 hr) tended to generate more symmetric lineage outcomes (Figures 1B–1D). This therefore underscores the importance of following the first cell divisions in hESC differentiation in real time by contrast to bulk population analyses of cells at later time points (i.e., 24 hr), by which they had become rather homogeneous (Loh et al., 2016).

However, our experiments up to this point exploited HES3 *MIXL1-GFP* hESCs that might display idiosyncratic primitive streak differentiation as one *MIXL1* allele had been replaced with *GFP* (Davis et al., 2008). As such, we set out to establish

whether hESCs with two intact *MIXL1* alleles also frequently differentiated into asymmetric daughter cells.

A second, distinct hESC line (H7) also divided to yield asymmetric daughter cell pairs upon primitive streak differentiation. To enable single-cell differentiation experiments, we overexpressed *BCL2* in H7 hESCs by way of integration into the *AAVS1* (otherwise known as *PPP1R12C*) safe harbor locus (Figure S1I). The resultant hESCs were still competent to differentiate into primitive streak (Figure S1J). Again, upon exposure to primitive-streak-inducing signals, H7 hESCs divided 45.2% of the time to generate daughter cells with asymmetric *BRACHYURY* expression (Figures 1F and 1G), similar to what was observed with the *MIXL1-GFP* hESCs. Additionally, early-dividing hESCs tended to generate asymmetric progeny whereas late-dividing hESCs tended to yield more symmetric daughters (Figure 1G).

Given that differentiating hESCs divide asymmetrically ~30%–50% of the time, we sought to determine the underlying mechanism(s). First we investigated the PI3K pathway. Asymmetric PI3K signaling in immune daughter cell pairs has been suggested to underpin lineage asymmetries (Lin et al., 2015), and indeed, PI3K inhibition enhanced primitive streak specification from hESCs (Figure S2A), as previously reported (Loh et al., 2014; Singh et al., 2012). Nonetheless even in the presence of a PI3K inhibitor, asymmetric division outcomes were still observed (Figures S2B and S2C). Next we considered that primitive streak specification relies on the concerted activation of BMP, FGF, TGF- β /NODAL, and WNT pathways. Hence the heterogeneous or spatially asymmetric responsiveness of cells to any of these signals could presumably lead to the generation of asymmetric fate outcomes from differentiating hESCs, among other possible mechanisms. Here we specifically focused on WNT signaling (Figure 2A).

Although single differentiated hESCs were uniformly exposed to extracellular WNT agonists during primitive streak differentiation, unexpectedly, their daughter cells sometimes demonstrated asymmetric WNT/ β -catenin transcriptional activity within their respective nuclei. LEF1 is a transcriptional target of WNT/ β -catenin signaling (Hovanes et al., 2001) and, as expected, was upregulated in primitive streak in response to WNT signaling (Figure S2D). Primitive streak differentiation of H7 hESCs in WNT3A-containing media revealed that 37.8% of the time, they divided to form daughter cell pairs that showed asymmetric LEF1 expression (Figures 2B and 2C). To more directly test whether WNT/ β -catenin transcriptional activity indeed differed within the nuclei of the daughter cells, we constructed a β -catenin transcriptional reporter (Figure 2D). The reporter contains seven Tcf/Lef-binding sites that when activated drive expression of a nuclear-localized, short-lived TurboGFP fluorescent protein containing a d2PEST sequence that enables its rapid degradation (Figure 2D). This *Tcf/Lef-TurboGreen-NLS-d2PEST* cassette therefore reports on WNT/ β -catenin transcriptional activity with temporal precision. Similar to the primitive streak markers and LEF1, the WNT/ β -catenin transcriptional reporter showed asymmetric activity between daughter cells in 46.7% of cell divisions (Figures 2D and 2E). Taken together, despite uniform exposure to extracellular WNT agonists, hESCs can divide asymmetrically to form

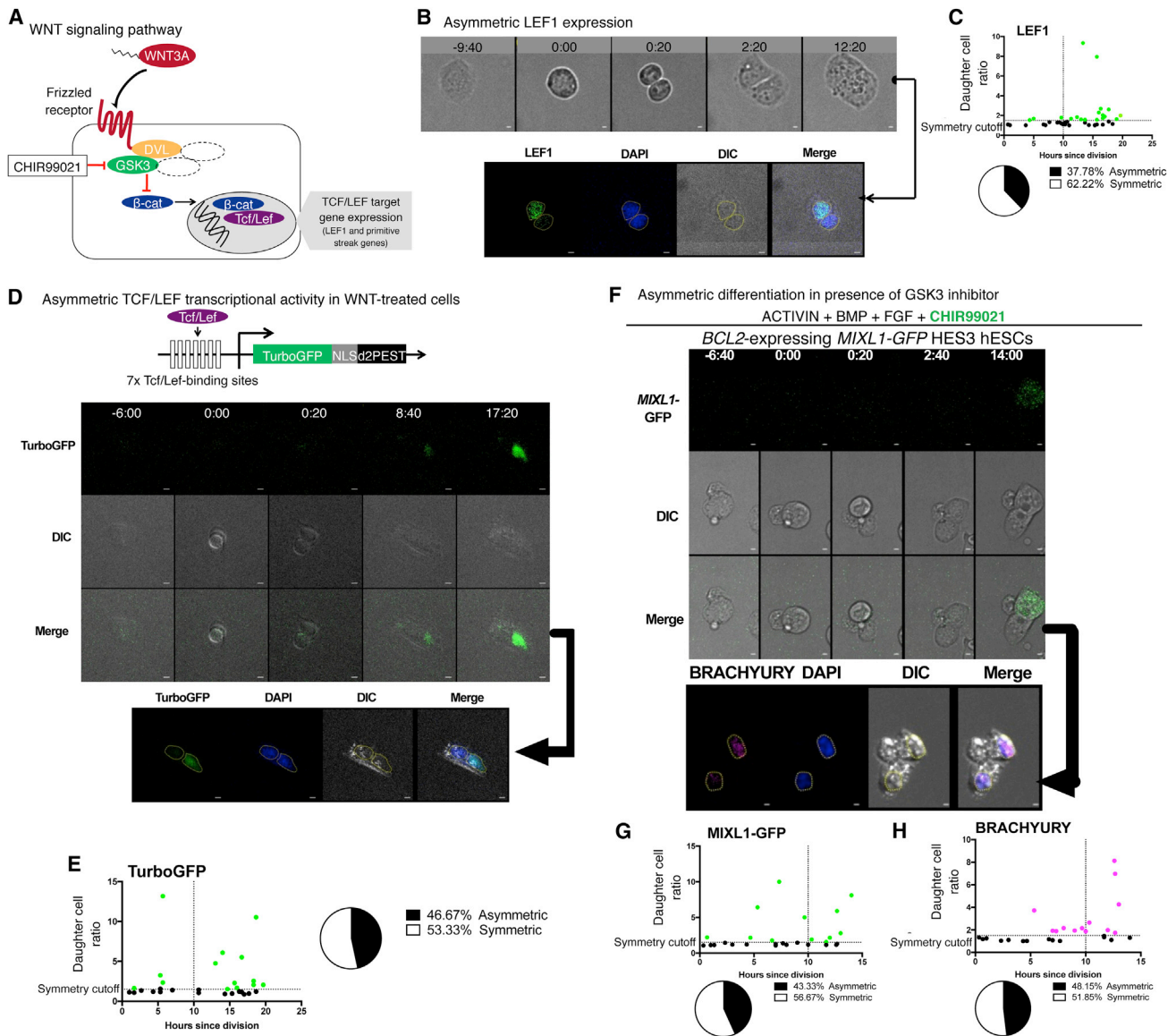


Figure 2. Innate Resistance to WNT Signaling in One Daughter Cell Leads to Asymmetric Primitive Streak Specification in Daughter Cell Pairs

(A) Overview of the WNT pathway.

(B) Stills taken from live imaging of hESCs differentiating toward primitive streak (top); staining of fixed cells reveals asymmetric LEF1 protein expression (bottom). Scale bar represents 10 μ m.

(C) Ratio of LEF1 protein expression in pairs of daughter cells derived from hESCs exposed to primitive streak differentiation media (ACTIVIN, BMP4, FGF2, and WNT3A); the ratio of LEF1 expression is plotted against the number of hours since the mother cell divided prior to the end of live imaging. Pie charts enumerate the overall percentage of asymmetric versus symmetric daughter cell pairs.

(D) Live imaging of hESCs carrying a *Tcf/Lef-TurboGFP:NLS:d2PEST* reporter allele revealed that upon treatment with primitive streak differentiation media (ACTIVIN, BMP4, FGF2, and WNT3A), WNT/ β -catenin signaling is often only activated in one cell in a daughter cell pair. Scale bar represents 10 μ m.

(E) Ratio of Tcf/Lef-TurboGFP fluorescence in pairs of daughter cells, plotted against the number of hours since the mother cell divided prior to the end of live imaging. Pie charts enumerate the overall percentage of asymmetric versus symmetric daughter cell pairs.

(F) *MIXL1-GFP* hESCs differentiated in primitive streak differentiation media (ACTIVIN, BMP4, FGF2) containing the GSK3 inhibitor CHIR99021 (3 μ M) still differentiated through asymmetric divisions, as shown by still images from live imaging. Immunostaining of fixed cells revealed that the *MIXL1-GFP*⁺ daughter also expressed BRACHYURY protein. Scale bar represents 5 μ m.

(G and H) Ratio of *MIXL1-GFP* (G) and BRACHYURY (H) protein expression in pairs of CHIR99021-treated daughter cells, plotted against the number of hours since the mother cell divided prior to the end of live imaging. Pie charts enumerate the overall percentage of asymmetric versus symmetric divisions. Data are representative of three to five independent experiments.

two cells with differing levels of WNT/ β -catenin transcriptional signaling.

Asymmetric responsiveness of daughter cell pairs to WNT signaling did not seem to be caused by differing levels of WNT receptors (Frizzled proteins) in the daughter cells (Momose and Houliston, 2007). When the WNT pathway was directly activated downstream of Frizzled receptors using the GSK3 inhibitor CHIR99021 (Ring et al., 2003) (which disables the destruction complex to stabilize β -catenin; Figure 2A), hESCs differentiating toward primitive streak still divided to yield asymmetric lineage outcomes as determined by MIXL1-GFP (43.3%) and BRACHYURY (48.1%) expression (Figures 2F–2H). Again, the probability of asymmetric fate outcomes was temporally correlated with when the mother cell divided (Figures 2G and 2H). Hence it seems that within certain daughter cell pairs, one of the two daughters is innately recalcitrant to WNT signaling. The inability of one daughter to respond to WNT, even upon GSK3 inhibition, manifests as an asymmetric cell fate choice between the two daughter cells.

Finally we considered whether the asymmetric cell-fate outcomes observed after WNT3A or CHIR99021 treatment might be an artifactual consequence of uniform exposure to WNT agonists, as WNT signals are typically presented in a focal manner in vivo (Clevers et al., 2014). To provide a focal source of WNT ligands, we exposed hESCs to WNT3A-conjugated beads (Habib et al., 2013). Strikingly even after exposure to WNT3A-conjugated beads, hESCs could still divide to yield asymmetric daughter cell pairs comprising one primitive streak and one non-primitive streak daughter (Figure S2D).

DISCUSSION

Here we show that, when uniformly exposed to primitive-streak-inducing extracellular signals, individual hESCs show cell-to-cell variability in their response. About 30%–50% of the time, their first cell division yields asymmetric lineage outcomes. Mechanistically, within these asymmetric daughter cell pairs, one cell is often refractory to WNT signaling and consequently fails to upregulate primitive streak markers; this manifests as an asymmetric lineage choice between the two cells. Hence, among a population of differentiating hESCs, not all cells are equally competent to respond to the same extracellular signal. This cell-to-cell variability in WNT responsiveness leads to asynchronous commitment to a primitive streak fate across a population of differentiating hESCs. If individual stem cells are unable to transduce various extracellular signals to initiate differentiation, this has consequences for whether stem cells can be efficiently differentiated toward desired lineages. Put more simply, the propensity of hESCs to generate daughter cells of two distinct fates could interfere with efforts to generate homogeneous populations of desired lineages.

It is surprising that hESCs uniformly exposed to an extracellular signal (e.g., WNT) should respond heterogeneously to divide asymmetrically. Although it has been known that cultured mammalian cells show cell-to-cell variability in their ability to perceive an extracellular signal, this was previously overcome by supplying saturating levels of the extracellular signal (Tay et al., 2010). By contrast, here we show that among certain

daughter cell pairs of differentiating hESCs, one cell is innately resistant to WNT signaling, despite provision of WNT3A or an intracellular WNT agonist (a GSK3 inhibitor). Unexpectedly, the fate of daughter cells correlated with when their mother stem cell divided: hESCs that divided earlier tended to generate daughters with asymmetric fates. However, it remains unclear why certain daughter cells should be innately resistant to WNT signaling.

The present findings also emphasize the value of using live imaging to track how single stem cells incipiently leave the stem cell state and execute differentiation. Although the very first cell division of differentiating hESCs can yield asymmetric lineage outcomes (as shown here), when primitive streak populations are analyzed at a later time point (24 hr), the differentiated cells are >98% homogeneous for MIXL1 and BRACHYURY expression (Loh et al., 2014, 2016). Taken together, it is clear that differing insights are gleaned from continuous live imaging (as exemplified by the present work) versus traditional saltatory time point analyses. Thus there is a pressing need to continuously follow differentiation at the single-cell level to track the fates of the earliest daughter cells as they exit the stem cell state and commit to lineage specification.

Finally, our in vitro system to differentiate single hESCs into primitive streak is advantageous in that it affords precise control over the extrinsic cues they are exposed to, enables facile tracking of the two daughter cells and their fates, and exploits how single hESCs (as opposed to larger colonies) are more sensitive to differentiation-inducing cues (Nemashkalo et al., 2017). However, pluripotent cells do not differentiate into primitive streak in vivo as single, isolated cells. Rather the primitive streak forms among a complex community of migrating cells wherein intercellular signals between adjacent cells, coordinated cellular movements, and polarized cell divisions are all observed (Voiculescu et al., 2014). The next challenge will be to understand how cell division and differentiation are spatiotemporally correlated in the native context of the mammalian embryo, as opposed to in culture.

EXPERIMENTAL PROCEDURES

Cell Culture

MIXL1-GFP reporter HES3 hESCs (Davis et al., 2008) and H7 hESCs were maintained in an undifferentiated state using mTeSR1 (STEMCELL Technologies) on Geltrex (A1569601; Thermo Fisher Scientific)-coated cell culture plates as previously described (Loh et al., 2016). hESCs were passaged when they reached ~70%–80% confluency by dissociation using Accutase (A1110501; Thermo Fisher Scientific) to obtain a single-cell suspension. For routine maintenance, hESCs were then plated in mTeSR1 supplemented with the ROCK inhibitor Thiazovivin (2 μ M; Tocris) for the first 24 hr after plating to enhance cell survival. hESC experiments were conducted under the cognizance of the Stanford Stem Cell Research Oversight (SCRO) Committee.

Differentiation and Live Imaging

For live imaging experiments, Accutase-dissociated *BCL2*-expressing hESCs were plated at single-cell density ($\sim 1 \times 10^5$ cells/well) in mTeSR1 + Thiazovivin on chambered four-well coverslips (155382; Thermo Fisher Scientific) that were pre-coated using recombinant human vitronectin (Thermo Fisher Scientific). Individual hESCs were allowed to settle and adhere for 1–3 hr before being switched to primitive streak differentiation media.

Primitive streak differentiation was conducted in fully defined, serum-free conditions. Middle primitive streak differentiation media was made as

previously described (Loh et al., 2016), except with the following modifications. For the basal medium (CDM2), phenol red-free IMDM was used for live imaging in lieu of phenol red-containing basal media. The extracellular signals used for differentiation were Activin A (30 ng/mL; R&D Systems), BMP4 (40 ng/mL; R&D Systems), FGF2 (10 ng/mL; R&D Systems) and C59 (1 μ M; Cayman Chemicals); C59 was added to block endogenous WNT protein production (Proffitt et al., 2012) in order to evaluate the effects of exogenous WNT agonists. Atop this minimal primitive streak induction condition, WNT3A protein (made in house from WNT3A-producing L cells), RSPO2 protein (10–20 ng/mL; R&D Systems), and/or the small-molecule WNT agonist CHIR99021 (3 μ M; Tocris) were added as indicated to potentiate WNT signaling and to drive primitive streak specification. To induce differentiation, the initial plating medium (mTeSR1 + Thiazovivin) was aspirated and differentiation media was added without intervening washes.

Two-dimensional (2D) live cell imaging of differentiating hESCs was performed on a Zeiss spinning disk confocal microscope with a 37°C incubated live imaging chamber, filled with humidified air supplemented with 5% CO₂. Multiple position tiling was performed in order to capture multiple division events during any given experiment. Images were captured every 20 min, with a 20 \times objective used to obtain bright-field images (using differential interference contrast [DIC]) and GFP signals (illuminated by a 488 nm laser).

Imaging of Fixed Cells

After live imaging differentiation experiments, cells were fixed by adding 16% paraformaldehyde (PFA) directly to cell media to a final concentration of 2% PFA for 10 min at room temperature. The media/PFA mixture was then replaced with 4% PFA in Dulbecco's PBS (DPBS) and incubated for another 10 min. Cells were washed three times with DPBS (5 min per wash) and then simultaneously blocked and permeabilized for 1 hr at room temperature in blocking solution (DPBS + 5% normal donkey serum + 0.2% Triton X-100). Primary antibodies were diluted in fresh blocking solution and added to the fixed cells overnight at 4°C. Cells were washed three times for 5 min each with DPBS and then incubated with the relevant fluorescent secondary antibody in blocking solution for 1 hr at room temperature, in the dark. Cells were washed three times with DPBS for 5 min each and then stained with DAPI (200 ng/mL) in DPBS before imaging.

Three-dimensional (3D) imaging was then performed on immunofluorescently labeled fixed cells using the Zeiss spinning disk confocal microscope. Cells were imaged using the 20 \times objective with 13 z stacks, each stack having 1.2 μ m slice thickness. Multiple-position tiling from the live imaging was reused to capture the same positions after fixation and antibody staining. Antibodies and concentrations used are listed in Supplemental Experimental Procedures.

Image Analysis

Images, either of immunostained cells (3D) or stills from live movies (2D), were analyzed using Imaris (Bitplane).

Three-dimensional images of nuclear transcription factors (BRACHYURY, EOMES, SOX2, etc.) were quantified for fluorescence content by creating a surface object on the DAPI (nuclear) channel, analyzing fluorescence from various channels within the object, and normalizing values to DAPI fluorescence. Normalized nuclear protein expression (e.g., that of BRACHYURY and others) was then compared between daughter cells. If a protein showed a >1.5-fold difference between a pair of daughter cells, it was regarded asymmetrically expressed. Images of fixed cells are shown as maximal projection from z stack.

MIXL1-GFP expression in 2D still images was quantified by assessing average GFP intensity over the entire area of the cell. The perimeter of each cell was delineated using manually drawn ROIs (regions of interest). If MIXL1-GFP fluorescent showed a >1.5-fold difference between two daughter cells, it was regarded as asymmetrically expressed.

Across all experiments, we defined "asymmetric" daughter cell pairs as ones in which there was a >1.5-fold difference between the two daughter cells. "Symmetric" daughter cell pairs were defined as cells in which there was less than a 1.5-fold difference in fluorescent intensity. Under this conservative criterion, pairs in which both cells were marker positive or both cells were marker negative were all regarded as "symmetric" daughter cell pairs. We chose a cutoff of a 1.5-fold difference to define "asymmetric" cell pairs because in

primitive streak cells, levels of pluripotency markers OCT4 and SOX2 were quite consistent across daughter cells, and were largely within 1- to 1.2-fold of each other (Figure S1G). Given that OCT4 and SOX2 levels showed limited variability (being within 1- to 1.2-fold of each other) among primitive streak cells, we conservatively defined any marker with a >1.5-fold difference as "asymmetric."

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.09.044>.

AUTHOR CONTRIBUTIONS

Conceptualization, K.B., K.M.L., and R.N.; Methodology, K.B. and K.M.L.; Investigation, K.B.; Resources, K.B. and K.M.L.; Writing – Original Draft, K.B. and K.M.L.; Writing – Review & Editing, K.B., K.M.L., and R.N.; Funding Acquisition, R.N.; Supervision, R.N.

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REFERENCES

- Ardehali, R., Inlay, M.A., Ali, S.R., Tang, C., Drukker, M., and Weissman, I.L. (2011). Overexpression of BCL2 enhances survival of human embryonic stem cells during stress and obviates the requirement for serum factors. *Proc. Natl. Acad. Sci. U S A* 108, 3282–3287.
- Blauwkamp, T.A., Nigam, S., Ardehali, R., Weissman, I.L., and Nusse, R. (2012). Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nat. Commun.* 3, 1070.
- Chen, G., Hou, Z., Gulbranson, D.R., and Thomson, J.A. (2010). Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell* 7, 240–248.
- Clevers, H., Loh, K.M., and Nusse, R. (2014). Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* 346, 1248012.
- Costello, I., Pimeisl, I.-M., Dräger, S., Bikoff, E.K., Robertson, E.J., and Arnold, S.J. (2011). The T-box transcription factor Eomesodermin acts upstream of *Mesp1* to specify cardiac mesoderm during mouse gastrulation. *Nat. Cell Biol.* 13, 1084–1091.
- Davidson, K.C., Adams, A.M., Goodson, J.M., McDonald, C.E., Potter, J.C., Berndt, J.D., Biechele, T.L., Taylor, R.J., and Moon, R.T. (2012). Wnt/ β -catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. *Proc. Natl. Acad. Sci. U S A* 109, 4485–4490.
- Davis, R.P., Ng, E.S., Costa, M., Mossman, A.K., Sourris, K., Elefanty, A.G., and Stanley, E.G. (2008). Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. *Blood* 111, 1876–1884.

- Etzrodt, M., Endeled, M., and Schroeder, T. (2014). Quantitative single-cell approaches to stem cell research. *Cell Stem Cell* 15, 546–558.
- Gadue, P., Huber, T.L., Paddison, P.J., and Keller, G.M. (2006). Wnt and TGF- β signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc. Natl. Acad. Sci. U S A* 103, 16806–16811.
- Habib, S.J., Chen, B.-C., Tsai, F.-C., Anastassiadis, K., Meyer, T., Betzig, E., and Nusse, R. (2013). A localized Wnt signal orients asymmetric stem cell division in vitro. *Science* 339, 1445–1448.
- Hoffman, J.A., Wu, C.-I., and Merrill, B.J. (2013). Tcf7l1 prepares epiblast cells in the gastrulating mouse embryo for lineage specification. *Development* 140, 1665–1675.
- Hovanes, K., Li, T.W., Munguia, J.E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R.F., and Waterman, M.L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nat. Genet.* 28, 53–57.
- Izumi, N., Era, T., Akimaru, H., Yasunaga, M., and Nishikawa, S. (2007). Dissecting the molecular hierarchy for mesendoderm differentiation through a combination of embryonic stem cell culture and RNA interference. *Stem Cells* 25, 1664–1674.
- Lin, W.-H.W., Adams, W.C., Nish, S.A., Chen, Y.-H., Yen, B., Rothman, N.J., Kratchmarov, R., Okada, T., Klein, U., and Reiner, S.L. (2015). Asymmetric PI3K signaling driving developmental and regenerative cell fate bifurcation. *Cell Rep.* 13, 2203–2218.
- Loh, K.M., and Lim, B. (2011). A precarious balance: pluripotency factors as lineage specifiers. *Cell Stem Cell* 8, 363–369.
- Loh, K.M., Ang, L.T., Zhang, J., Kumar, V., Ang, J., Auyeong, J.Q., Lee, K.L., Choo, S.H., Lim, C.Y.Y., Nichane, M., et al. (2014). Efficient endoderm induction from human pluripotent stem cells by logically directing signals controlling lineage bifurcations. *Cell Stem Cell* 14, 237–252.
- Loh, K.M., Chen, A., Koh, P.W., Deng, T.Z., Sinha, R., Tsai, J.M., Barkal, A.A., Shen, K.Y., Jain, R., Morganti, R.M., et al. (2016). Mapping the pairwise choices leading from pluripotency to human bone, heart, and other mesoderm cell types. *Cell* 166, 451–467.
- Momose, T., and Houlston, E. (2007). Two oppositely localised frizzled RNAs as axis determinants in a cnidarian embryo. *PLoS Biol.* 5, e70.
- Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* 441, 1068–1074.
- Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132, 661–680.
- Nemashkalo, A., Ruzo, A., Heemskerck, I., and Warmflash, A. (2017). Morphogen and community effects determine cell fates in response to BMP4 signaling in human embryonic stem cells. *Development* 144, 3042–3053.
- Okano, H., and Temple, S. (2009). Cell types to order: temporal specification of CNS stem cells. *Curr. Opin. Neurobiol.* 19, 112–119.
- Proffitt, K.D., Madan, B., Ke, Z., Pendharkar, V., Ding, L., Lee, M.A., Hannoush, R.N., and Virshup, D.M. (2012). Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. *Cancer Res.* 73, 502–507.
- Ring, D.B., Johnson, K.W., Henriksen, E.J., Nuss, J.M., Goff, D., Kinnick, T.R., Ma, S.T., Reeder, J.W., Samuels, I., Slabiak, T., et al. (2003). Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes* 52, 588–595.
- Singh, A.M., Reynolds, D., Cliff, T., Ohtsuka, S., Mattheyses, A.L., Sun, Y., Menendez, L., Kulik, M., and Dalton, S. (2012). Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. *Cell Stem Cell* 10, 312–326.
- Tay, S., Hughey, J.J., Lee, T.K., Lipniacki, T., Quake, S.R., and Covert, M.W. (2010). Single-cell NF-kappaB dynamics reveal digital activation and analogue information processing. *Nature* 466, 267–271.
- Teo, A.K.K., Arnold, S.J., Trotter, M.W.B., Brown, S., Ang, L.T., Chng, Z., Robertson, E.J., Dunn, N.R., and Vallier, L. (2011). Pluripotency factors regulate definitive endoderm specification through eomesodermin. *Genes Dev.* 25, 238–250.
- Voiculescu, O., Bodenstern, L., Lau, I.-J., and Stern, C.D. (2014). Local cell interactions and self-amplifying individual cell ingressions drive amniote gastrulation. *eLife* 3, e01817.