SOX17 links gut endoderm morphogenesis and germ layer segregation

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Gastrulation leads to three germ layers—ectoderm, mesoderm and endoderm—that are separated by two basement membranes. In the mouse embryo, the emergent gut endoderm results from the widespread intercalation of cells of two distinct origins: pluripotent epiblast-derived definitive endoderm (DE) and extra-embryonic visceral endoderm (VE). Here we image the trajectory of prospective DE cells before intercalating into the VE epithelium. We show that the transcription factor SOX17, which is activated in prospective DE cells before intercalation, is necessary for gut endoderm morphogenesis and the assembly of the basement membrane that separates gut endoderm from mesoderm. Our results mechanistically link gut endoderm morphogenesis and germ layer segregation, two central and conserved features of gastrulation.

Gastrulation is a choreographed sequence of cell fate specification, proliferation and movement that results in the generation of the three embryonic germ layers: ectoderm, mesoderm and definitive endoderm. During gastrulation, pluripotent epiblast cells ingress through the transient primitive streak and undergo an epithelial-to-mesenchymal transition (EMT). Ingressing cells emerge as mesoderm and definitive endoderm$^1$ (DE). These cells collectively migrate in the space between the adjacent epithelia of the epiblast and visceral endoderm (VE). As a paradigm for tissue growth and remodelling, gastrulation in amniotes transforms the embryo comprising the epiblast and adjacent VE into a three-layered configuration comprising epiblast/ectoderm, mesoderm and gut endoderm$^{1,3}$. The gut endoderm arises as an epithelium on the embryo’s surface. It gives rise to the multipotent progenitors of the respiratory and digestive tracts, and their associated organs$^4$. Our previous work revealed that in the mouse, emergent gut endoderm comprises cells of two distinct origins, DE and VE, arising from the widespread intercalation of these two cell populations$^{2,7}$. The cell behaviours associated with this morphogenetic event are not well understood.

Here we have investigated the molecular programs and behaviours of DE and VE cells during mouse gut endoderm morphogenesis. Using three-dimensional (3D) time-lapse imaging we tracked presumptive DE progenitors from the primitive streak into the mesoderm layer and onto the embryo surface where they intermingled with embryonic VE (emVE) cells$^8$. By analyzing different mutants exhibiting gastrulation and endoderm defects, we demonstrate that DE cells must polarize and modulate extracellular matrix (ECM) components, undergoing a mesenchymal-to-epithelial transition (MET) to insert into the emVE epithelium. The Sry-related HMG-box-containing transcription factor SOX17 is a key orchestrator of this egression. To facilitate the egression of prospective DE cells, the emVE epithelium must coordinately and transiently modulate their apico-basal polarity, cell–cell junctions and basement membrane (BM) composition. Together, our observations reveal an association between gut endoderm morphogenesis and BM assembly, two cardinal features of gastrulation, and implicate SOX17 in a genetic program coordinating these events.

RESULTS
Live imaging and tracking of DE progenitors from the primitive streak to the embryo’s surface
To follow the trajectories and behaviour of DE progenitors from their origin within the primitive streak to their destination in the gut endoderm, we combined live imaging with transient and transgenic fluorescent cell labelling. We electroporated a plasmid driving widespread expression of a red fluorescent protein (RFP) into the posterior epiblast of Afp::GFP transgenic embryos (Fig. 1a). The Afp::GFP reporter permitted visualization of VE cells$^{6,9}$. Embryos were cultured after electroporation and those exhibiting normal morphology with detectable RFP expression at the primitive streak were 3D time-lapse imaged (Fig. 1a–e and Supplementary Video 1). Over time, RFP-positive cells were identified in an anterior-ward stream (Fig. 1c–e and Supplementary Video 2). Close inspection of RFP-positive cells suggested that they underwent an EMT. Surface
Figure 1 DE cells originate in the posterior epiblast and migrate with the wings of mesoderm before egressing into the emVE epithelium. (a) Schematic depicting the electroporation and time-lapse imaging procedure. (b–e) Interior rendered views from a time-lapse video. (b′–e′) Surface rendered views from a time-lapse video (b–e). (f–i) Afp::GFP VE-reporter embryos showing progression of emVE dispersal from pre-dispersal (PS stage, E6.25) to late/completed dispersal (LB/EHF stage, E7.5) stage. (f–i) Transverse sections through Afp::GFP embryos in f,i.

renderings revealed an initially uniform GFP-positive layer. Over time, GFP-negative regions appeared, with a subset being RFP-positive (Fig. 1b′–e′ and Supplementary Video 3). Tracking identified trajectories adopted by prospective DE cells during gastrulation: DE progenitors initially reside in the posterior epiblast, ingress through the primitive streak, and emerge onto the embryo surface by multifocally inserting into the emVE (Supplementary Videos 1–5).

Cells egress into the VE from within the wings of mesoderm

We next imaged sequentially staged embryos expressing the pan-VE Afp::GFP reporter before, during and after emVE dispersal. At the pre-streak stage (embryonic day (E) 6.25), a uniform GFP distribution was observed on the embryo surface, indicating that emVE dispersal had not commenced (Fig. 1f). Transverse sections through the embryonic region identified two epithelia: a columnar epithelium comprised of the inner epiblast and a squamous epithelium comprised of the outer emVE (Fig. 1g). By the late streak stage (E7.0), surface renderings revealed a few GFP-negative areas present within the GFP-positive emVE layer, presumably representing the first DE cell cohort that egressed onto the embryo’s surface (Fig. 1g′). Transverse sections identified mesoderm positioned between the epiblast and outer emVE (Fig. 1g′, leading-edge of mesoderm, orange asterisk). A subset of GFP-negative cells, which aligned with the mesoderm located adjacent to the emVE, were indenting into the overlying GFP-positive emVE layer (Fig. 1g′, inset, white arrowheads), probably representing DE progenitors in the process of egression.
Notably, egressing cells, defined either as GFP-negative regions on the embryo’s surface in 3D renderings or regions of indentations in the GFP-positive layer in transverse sections, were not observed anterior to the mesoderm’s leading-edge, suggesting that DE progenitors are incorporated within or travel alongside the mesoderm. By the no bud stage (E7.25), embryos exhibited extensive emVE dispersal (Fig. 1h). Sections revealed that some GFP-negative cells already embedded in the surface epithelium (red arrowheads), whereas others were in the process of egressing, still enveloped by GFP-positive areas (Fig. 1h’, inset, white arrowheads). By the late bud (LB)/early head-fold (EHF) stage (E7.5), when emVE dispersal was complete, GFP-positive regions comprised isolated cells (Fig. 1i). Transverse sections confirmed that, at this time, the mesoderm had completed its migration, and the embryo’s surface was composed of both GFP-positive emVE descendants and GFP-negative epiblast-derived DE cells (Fig. 1i’).

Gastrulation mutants do not undergo VE dispersion
To analyse the genetic control of egression, we assessed emVE dispersal in embryos exhibiting defects in gastrulation. Mutations in FGF signalling components, including FGF8 or FGFR1, specified mesoderm, but cells failed to migrate away from the primitive streak10–12. Before gastrulation, Fgf8 or Fgfr1 mutant embryos were indistinguishable from wild-type littermates. However, by the no bud stage (E7.25), when emVE dispersal was underway in wild-type embryos, Fgf8 or Fgfr1 mutants exhibited a complete failure in emVE dispersal. Sections confirmed a failure in mesoderm migration (Fig. 1j,j’ and Supplementary Fig. 1a,a’ and b,b’). The T-box transcription factor EOMESODERMIN (EOMES) plays a critical role in both anterior visceral and definitive endoderm specification13,14. Its ablation in the epiblast (EomesGFP/wt) leads to cell accumulations at the primitive streak and defects in mesodermal migration and endoderm specification15. Like FGF signalling mutants, EomesGFP/wt embryos exhibited a failure of emVE dispersal (Supplementary Fig. 1c,c’), confirming that proper migration of mesoderm cells away from the primitive streak is necessary for VE dispersal and DE formation.

SOX17 and FOXA2 mark definitive endoderm cells before and during egression
We next investigated whether DE progenitors were molecularly distinct from neighbouring mesoderm cells before their egression onto the embryo’s surface. Analysis of SOX17, an evolutionarily conserved factor critical for endoderm specification16,17, revealed it to be expressed by DE cells before, during and after egression. Before emVE dispersal had started, SOX17 was detected at low levels throughout the emVE (Supplementary Fig. 2a–b’). During early stages of emVE dispersal (E7.0), high SOX17 levels were detected in a subset of cells present within the mesoderm layer, making contact with the emVE (Fig. 2a–c and Supplementary Fig. 2g), suggesting that these could be presumptive DE cells in the process of egression. By completion of emVE dispersal (Fig. 2e,f insets) SOX17 was detected at equivalently high levels in both DE and emVE-derived cells (Fig. 2d–f and Supplementary Fig. 2g).

The distribution of FOXA2, another conserved factor important in the specification and formation of endoderm18, was comparable to SOX17 at these stages and in this region of the embryo (Supplementary Fig. 2h–n). SOX17 and FOXA2 co-localization exhibited a strong correlation (Supplementary Fig. 2o–v). In contrast to SOX17, FOXA2 was, however, expressed at lower levels in some posterior epiblast cells before their ingression through the primitive streak (Supplementary Fig. 2y), and was robustly expressed later in midline structures7 (Supplementary Fig. 2w,x).

SOX17 is required for definitive endoderm cell egression
We investigated gut endoderm morphogenesis in Sox17 mutants as embryos lacking Sox17 are deficient in the midgut and hindgut DE, with endoderm cells having been reported as exhibiting a VE-like morphology16. At the late streak stage (E7.0), when DE cells have normally started to egress, Sox17 mutant embryos exhibited a uniform layer of GFP-positive cells on their surface, suggesting that DE progenitors had not egressed (Fig. 2g). By the LB/EHF stage (E7.5), when emVE dispersal is normally complete, the emVE appeared as a uniform, GFP-positive epithelial sheet in Sox17 mutants (Fig. 2h), except the prospective foregut region (Fig. 2h, white arrowhead). Sections through Sox17 mutants confirmed that gastrulation had occurred, as the embryo comprised three tissue layers, with the surface layer exclusively comprised of emVE (Fig. 2i,j).

Definitive endoderm cell egression occurs in the absence of FOXA2
Gut endoderm morphogenesis was also analysed in Foxa2 mutants, which exhibited an overall growth retardation with failure to form midline structures (Fig. 3a–d, see orange arrowhead). In contrast to Sox17 mutants, dispersal of the emVE appeared to occur in the absence of FOXA2 (Fig. 3b, see white arrowhead). Sox17 was expressed in Foxa2 mutants (Fig. 3c,d). FOXA2 expression was also unperturbed in Sox17 mutants, with mesodermal cells adjacent to the emVE exhibiting high levels of FOXA2, as in wild-type embryos (Supplementary Fig. 2s–v’).

Cells failing to execute a program of definitive endoderm differentiation are retained within the mesoderm layer
To investigate the fate of DE progenitors in Sox17 mutants, we used a Sox17GFP tracer. In LB/EHF stage Sox17GFP/+ embryos, GFP was detected in a belt of cells on the embryo surface, DE and emVE-derived cells comprising the gut endoderm (Fig. 4a–d). In contrast, in Sox17GFP/GFP mutants, an additional population of GFP-positive cells was present within the mesoderm layer (Fig. 4a’–d’). The SOX17–GFP-positive cells embedded within the mesoderm probably arose because of a failure in egression and in the DE program. These ‘trapped’ non-egressed cells did not express FOXA2, suggesting that they did not acquire a DE identity (Fig. 4c’). However, FOXA2 was expressed at earlier stages in Sox17 mutants (Supplementary Fig. 2u’), suggesting that when DE cells failed to egress, they did not maintain expression of endoderm markers.

We noted that trapped SOX17–GFP-positive cells exhibited low levels of E-CADHERIN, which normally marks epithelial cells, suggesting that cell polarization was initiated (Fig. 4e–h’, orange arrowheads). The presence of N-CADHERIN, which is exclusively localized in mesodermal cells, indicated that the trapped cells had acquired the mesenchymal character of their mesoderm neighbours (Fig. 4i–l’, orange arrowheads, and Supplementary Fig. 3a–c).
Figure 2 SOX17 marks DE cells before, during and after egression and is required for cell egression. (a) Whole-mount image of immunofluorescence for SOX17 in an Afp::GFP VE-reporter embryo at the early emVE dispersal stage. (b,c) Sections through the embryo in a indicate two different expression levels of SOX17 during early emVE dispersal. EmVE cells exhibit low levels of SOX17 (pink arrowheads), and DE cells in the process of egressing exhibit high levels of SOX17 (orange arrowheads). Orange asterisks mark the leading edge of the mesoderm. (d) Whole-mount image of immunofluorescence for SOX17 in an Afp::GFP VE-reporter embryo at the late/completed emVE dispersal stage. (e,f) Sections through the embryo in d indicate equal levels of SOX17 expression in all cells of the gut endoderm layer, regardless of emVE-derived (GFP-positive) or DE lineage (GFP-negative). (g) A Sox17 mutant Afp::GFP VE-reporter embryo at the LS stage exhibits a solid layer of GFP-positive cells on its distal surface. (h) A Sox17 mutant Afp::GFP VE-reporter embryo at the LB/EHF stage showing a uniform layer of GFP-positive cells on its distal surface, suggesting continuous failure of DE-cell egression. Limited egression of DE cells into the emVE has occurred anteriorly in the area around the prospective foregut invagination (white arrowhead). (i,j) Low and high magnifications of sections through the Sox17 mutant embryo in h show wings of mesoderm that have completed anterior migration and a solid GFP-positive layer on the surface of the embryo, representing the undispersed emVE epithelium. ps, primitive streak; end, endoderm; epi, epiblast; mes, mesoderm; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; LS, late streak; LB, late bud; EHF, early head-fold. Scale bars, 100 μm. See also Supplementary Fig. 2.

Gastrulation involves a transition from one to two BMs

Epithelia commonly reside on ECM proteins that collectively form a BM. BMs provide rigidity and separation between adjacent tissue layers. The acquisition of three germ layers at gastrulation is accompanied by a transition from a single BM present at the epiblast–emVE interface, to two BMs, one positioned at the epiblast–mesoderm interface, and another at the mesoderm–endoderm interface. If DE cells were traversing layers, they would need to remodel the BM at the mesoderm–endoderm interface. Hence, an absence of ECM proteins could be expected in their vicinity. We analysed ECM protein distribution at the time of DE cell egression. At the mid-emVE dispersal stage FIBRONECTIN-1 (FN-1), a critical BM factor, was localized in two belts at the epiblast–mesoderm and the mesoderm–endoderm interfaces (Fig. 5g–i). GFP-negative DE cells and GFP-positive emVE cells were positioned on the same side of the BM. As we were unable to reconcile these observations with a model of BM breakdown by egressing DE cells, we determined ECM factor distribution during successive stages of DE cell egression and emVE dispersal.
At the early streak stage (E6.75), before emVE dispersal, FN-1 was detected along the interface between epiblast and emVE, as a single belt separating these two tissue layers (Fig. 5a–c). During early emVE dispersal (E7.0), when the wings of mesoderm were migrating anteriorly between the epiblast and emVE layers, one continuous belt of FN-1 was detected at the epiblast–mesoderm interface (Fig. 5d–f). This BM was uninterrupted at the anterior exterimities of the mesoderm and was contiguous with the BM lying anterior at the epiblast–emVE interface. FN-1 was also detected in patches near the mesoderm–endoderm interface, even though at this stage the separation between these two tissue layers was not evident (Fig. 5f, f’).

At the mid-emVE dispersal stage (E7.25), FN-1 localization at the epiblast–mesoderm interface remained unchanged, whereas at the mesoderm–endoderm interface it became uninterrupted (Fig. 5g–i’). At the late/complete emVE dispersal stage (E7.5) when mesoderm migration was complete, FN-1 localization was observed as two distinct, continuous belts: one between epiblast–mesoderm, and one between mesoderm–endoderm (Fig. 5j–l’). LAMININ-1 (LAMA-1), another ECM protein (FN-1 was detected along the interface of the epiblast and emVE, Supplementary Fig. 4a). Notably, the distribution of FN-1 and LAMA-1 in Fgf8, Foxa2, and Foxa2 mutant embryos, as well as in epiblast-ablated Eomes embryos, invariably revealed a single BM between epiblast and emVE (Supplementary Fig. 4b). Together, these data reveal that mouse gastrulation involves a transition from one to two BMs. This must be coordinated with the migration of cells out of the primitive streak and generation of a new tissue layer.

During DE egression, we observed FN-1 and LAMA-1 localized at the prospective baso-lateral side of SOX17-positive DE cells, where they interfaced with other mesoderm cells, but not where they contacted emVE cells (Fig. 5m–p and Supplementary Fig. 4c–e). This suggested that egressing DE cells had acquired cell polarity and that de novo assembly of BM and/or remodelling of ECM was occurring baso-laterally in egressing DE cells.

Three-tissue layer configuration but only one BM in Sox17 mutants

As in wild type, Sox17 mutant embryos exhibited a continuous band of each of four ECM proteins (FN-1, LAMA-1, LAMB-1, and COLLAGEN IV (COLL-IV)) at the epiblast–mesoderm interface (Fig. 5q–z and Supplementary Fig. 4f,g and Video 6). Even though three tissue layers were present in Sox17 mutants, we failed to detect a BM at the mesoderm–endoderm interface, indicating that the formation of this BM requires SOX17.

To understand how the BM at the mesoderm–endoderm interface forms, we analysed the expression of genes encoding ECM proteins by in situ hybridization. As gastrulation proceeded, Fn1 was expressed by cells emanating from the primitive streak, and the emVE cells overlying them, but not in emVE anterior to the mesoderm, nor in the epiblast (Supplementary Fig. 4h). Lama1 exhibited a comparable expression pattern (data not shown). These data suggest that transcription of genes encoding ECM factors occurs concomitantly with cell migration from the primitive streak. As previous studies have reported a role for SOX17 in ECM transcriptional regulation, we determined whether gene expression might be perturbed in Sox17 mutants and found it to be unaffected (Supplementary Fig. 4i). This ruled out the direct transcriptional regulation of ECM proteins by SOX17 as a dominant mechanism causing the absence of a BM at the mesoderm–endoderm interface in Sox17 embryos.

Egressing cells form E-CADHERIN-rich cell–cell contacts with overlying emVE cells as an early step of epithelialization

The observation that non-egressed DE cells failed to epithelialize and remained within the mesodermal tissue layer in the absence of SOX17 raised the question of whether SOX17 might regulate the MET of DE cells. We analysed the localization of E-CADHERIN, which is commonly present at adherens junctions at the interfaces of neighbouring epithelial cells. In wild-type embryos before DE cell egression, E-CADHERIN localized at the interfaces of neighbouring VE cells as well as epiblast cells (Supplementary Fig. 5a–b6). At mid-emVE dispersal (E7.25), cells exiting the primitive streak and positioned within the mesoderm had downregulated E-cadherin (Fig. 6a–f). Gressing DE cells exhibited robust E-CADHERIN at their interface with emVE cells (Fig. 6a–f), indicating that they had acquired apico-basal polarity. Concomitant with DE cell egression, emVE cells had redistributed their membrane-localized E-CADHERIN from their interface with neighbouring emVE cells to their interface with egressing DE cells. E-CADHERIN was also present in cells of the epiblast and emVE anterior to the leading edges of the mesoderm, as well as in ingressing cells within the primitive streak (Supplementary Fig. 5c–c6). Once gut endoderm morphogenesis was complete, E-CADHERIN localized at the interfaces of all cells within the gut endoderm epithelium regardless of their origin (Fig. 6g–l).
Figure 4 Cells failing to egress remain within the mesodermal wings. (a–d) Low and high magnifications of sections through an LB/EHF stage (E7.5) Sox17<sup>+/+</sup> embryo with immunofluorescence for the endoderm marker FOXA2, indicating a single layer of GFP-positive cells on the surface of the embryo (all cells of the gut endoderm), uniformly expressing FOXA2. (a′–d′) A Sox17<sup>+/−</sup> embryo additionally exhibits some GFP-positive cells embedded within the mesodermal layer and does not express FOXA2 (orange arrowheads). (e–h) Low- and high-magnification views of sections through a Sox17<sup>+/+</sup> embryo depicting immunofluorescence for the epithelial marker E-CADHERIN (E-CAD). Fluorescent signal is present between cells in the epiblast layer and in the gut endoderm, which is GFP-positive. (e′–h′) A Sox17<sup>+/−</sup> embryo additionally exhibits some GFP-positive cells within the wings of mesoderm. These ‘non-egressed’ cells show low levels of cytoplasmic E-CAD (orange arrowheads). (i–l) Low and high magnifications of sections through a Sox17<sup>+/+</sup> embryo depicting immunofluorescence for the mesenchymal marker N-CADHERIN (N-CAD). N-CAD stain is present between cells of the wings of mesoderm, and absent from cells of the epiblast or the gut endoderm, which is GFP-positive. (i′–l′) A Sox17<sup>+/−</sup> embryo additionally exhibits some GFP-positive cells within the wings of mesoderm. These ‘non-egressed’ cells show low levels of cytoplasmic E-CAD (orange arrowheads). ps, primitive streak; emVE, embryonic visceral endoderm; end, endoderm; epi, epiblast; exVE, extra-embryonic visceral endoderm; mes, mesoderm; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; LB, late bud; EHF, early head-fold. Scale bars, 100 μm. See also Supplementary Fig. 3.
Figure 5 Formation of BM at the mesoderm–endoderm interface does not occur in Sox17 mutants. (a–l) Immunofluorescence for the BM protein FN-1 in Afp::GFP VE-reporter embryos. (a–c') At the ES stage (E6.75), shortly after the wings of mesoderm have begun their anterior migration (leading tips indicated by orange asterisks), a single continuous signal for FN-1 is present between epiblast and the GFP-positive emVE. (d–f) During early emVE dispersal, one BM is visible anterior to the leading tips of the wings of mesoderm (orange asterisks), and two BMs in regions where the wings of mesoderm are present. The BM between mesoderm and endoderm is heavily fenestrated. (g–i') FN-1 at mid-emVE dispersal identifies two BMs where the wings of mesoderm are present. (j–l') FN-1 at the late/completed emVE dispersal stage identifies two solid BMs separating germ layers. (m–p) Double stains in a Afp::GFP VE-reporter embryo show egressing DE cells with high levels of Sox17 in the process of inserting between emVE cells exhibiting FN-1 basally. EmVE cells never exhibit FN-1 at their interface with egressing DE cells. (q–t) Stained Afp::GFP VE-reporter wild-type embryo showing egressing DE cells (orange asterisks) always exhibit FN-1 basally. (q'–t') In the Sox17 mutant, the interface between mesoderm and emVE exhibits only faint punctate FN-1 fluorescent signal. (u,u') Digital quantification of FN-1 fluorescent signal indicating two peaks in the wild type and only one peak in the Sox17 mutant. (v–y) Stained Afp::GFP VE-reporter wild-type embryo showing egressing DE cells (orange asterisks) always exhibit LAMA-1 basally. (v'–y') In Sox17 mutants, the interface between mesoderm and emVE exhibits punctate LAMA-1 fluorescent signal, similarly to the puncta interspersed between cells of the wings of mesoderm of the wild type. (z,x') Digital quantification of fluorescent signal indicating two peaks for LAMA-1 in the wild type, and only one in the Sox17 mutant, ps, primitive streak; emVE, embryonic visceral endoderm; end, endoderm; epi, epiblast; mes, mesoderm; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; ES, early streak; LS, late streak; OB, no bud; LB, late bud; EHF, early head-fold. Scale bars, 20μm in m, 100μm in all other panels. See also Supplementary Fig. 4 and Video 6.

We quantified the levels of E-CADHERIN in egressing DE cells in wild type and Sox17 mutants. Measurement of fluorescent signal intensities in individual wild-type egressing cells revealed higher levels of E-CADHERIN on the region of their plasma membrane in proximity to the embryo’s surface (Fig. 7a–d,m). In Sox17 mutants, we failed to detect E-CADHERIN in cells within the mesodermal layer adjacent to the emVE (Fig. 7a–d,m) suggesting that in the absence of SOX17, these cells failed to express E-CADHERIN22. Further, in Sox17 mutants, emVE cells adjacent to the mesoderm maintained E-CADHERIN localization at their interfaces, with the epithelium generally appearing more organized than wild type (Fig. 7c,d').

Egressing cells modulate distribution of cell polarity markers

To further investigate how cell polarity, another hallmark of epithelial cells, was modulated in DE and emVE cell populations, we analysed the distribution of SCRIBBLE (SCRIB), which typically localizes to the baso-lateral domain of epithelial cells21. In wild-type embryos SCRIB exhibited an isotropic localization in inner cells of the mesodermal wings (Fig. 7e–h). In contrast, egressing cells exhibited SCRIB polarization along their prospective apico-basal axis (Fig. 7g,h orange asterisks). Quantification of signal confirmed that egressing cells had a higher degree of polarization compared to inner mesodermal cells (Fig. 7n). Interestingly, of 50 egressing cells analysed, only 29 showed enrichment of SCRIB on their prospective basal side. The rest exhibited enrichment of SCRIB on their prospective apical side. This suggested that, even though egressing cells distribute polarity markers in a biased way, the apical and basal domains can still change and are not irreversibly determined until cells are fully epithelialized. In Sox17 mutants, SCRIB was localized uniformly in inner cells of the mesoderm layer, and virtually all cells adjacent to the emVE exhibited enrichment of SCRIB at their interface with emVE cells (Fig. 7e–h,n). Hence, in Sox17 mutants the emVE retains its epithelial integrity, and its baso-lateral distribution of SCRIB at the interface with the mesoderm. These observations underscore the inability of DE progenitors within the mesodermal layer to modulate the distribution of polarity markers in the absence of SOX17.

Definitive endoderm cells modulate deposition of BM anchors during egression

Having noted that the absence of SOX17 did not affect ECM gene expression at a transcriptional level (Supplementary Fig. 4i), we investigated whether the absence of a BM at the mesoderm–gut endoderm
interface in *Sox17* mutants resulted from a failure in BM assembly, possibly caused by modulation of cell polarity in either DE, emVE or both. From our previous analysis of gene expression profiles of the embryonic region of E7.5 embryos, we noted INTEGRIN-A5 (ITGA5), the receptor for FIBRONECTIN-1 (FN-1), as one of the predominant integrins expressed at this stage (Supplementary Fig. 6a). *In situ* analyses showed that in wild-type embryos at mid-emVE dispersal stages (E7.25), all cells in the mesodermal wings expressed ITGA5 (Fig. 7i–l). Egressing DE cells preferentially localized ITGA5 on their prospective basal side (Fig. 7k,l,o orange asterisks; Supplementary Fig. 6b,c). In contrast, inner cells in the mesodermal wings exhibited a uniform surface localization of ITGA5. The polarized distribution of ITGA5 in DE cells suggested that it was being selectively trafficked and could account for ECM protein polymerization on the prospective basal side to form a BM. We also found that in wild-type embryos, dispersing emVE cells exhibited polarized, albeit downregulated localization of ITGA5 in comparison with emVE cells in *Sox17* mutants (Fig. 7k,l pink asterisk and Fig. 7k,l), consistent with a transient reduction of aspects of polarity within wild-type emVE cells during DE cell egression. In *Sox17* mutants, cells in the mes!ernal wings adjacent to the emVE did not localize ITGA5 on their prospective basal side (Fig. 7o,i l) Instead, these cells always exhibited enrichment on their
DISCUSSION

In contrast to the prevailing view of strict lineage segregation in mammals, our data reveal that the gut endoderm comprises cells of both embryonic and extra-embryonic origin. Here we demonstrate that cells that will form the DE originate within the primitive streak and migrate within, or aligned with the mesoderm before intercalating into the VE on the embryo’s surface. Our observations support the mechanism of endoderm morphogenesis proposed in the chick\(^{24}\) where DE precursors ingress at the primitive streak, migrate within the mesodermal layer, insert into the extra-embryonic endoderm layer, and transiently form a mosaic epithelium composed of embryonic and extra-embryonic cells.\(^{25,26}\) It has been suggested that chick extra-embryonic endoderm cells persist and contribute to the developing
Late bud (LB) stage ~E7.5
Visceral endoderm (VE)
Epiblast (epi)
Primitive streak derivatives: mesoderm (mes) and definitive endoderm (DE)
Basement membrane
Sox17-low to Sox17-high

3 tissue layers
2 basement membranes

ECM receptors (for example, integrin)
ECM gene expression
Basement membrane

Modulation of apico-basal polarity (facilitates intercalation, driven by egression of DE cells into VE)

ECM receptor clustering and baso-lateral BM assembly (separates gut endoderm from mesoderm)

Figure 8 Working model of cell behaviours during gut endoderm morphogenesis and germ layer segregation in mice. The gut endoderm forms by widespread intercalation between embryonic and extra-embryonic endoderm, which occurs concomitantly with the assembly of a BM at its interface with the mesoderm. Gastrulation transforms the embryo’s configuration from a two-layered tissue into a three-layered tissue. Epiblast cells ingress and undergo EMT at the primitive streak. They emerge as mesoderm or gut endoderm. SOX17 orchestrates a MET of DE cells at the interface with the VE, in which DE cells become polarized, enrich ECM receptors, and assemble BM components basally. The overlying VE layer transiently moderates its epithelial characteristics, facilitating DE cell egression. The emergent gut endoderm (composed of VE and DE cells) subsequently reinforces the underlying BM, segregating mesoderm from gut endoderm tissue layers.

Liver27. Lineage tracing studies will be needed to determine whether emVE descendants contribute to adult endodermal tissues in mice.

Elucidating the gene regulatory network controlling DE formation has important implications for regenerative medicine. The evolutionarily conserved transcription factors SOX17 and FOXA2 mark DE progenitors before their intercalation onto the embryo’s surface. We observed that some posterior epiblast cells express FOXA2, but not SOX17, suggesting that FOXA2 is the first marker of the DE lineage that is determined before ingress through the primitive streak. However, our in vivo studies revealed that DE cell egression requires SOX17, but not FOXA2. As Foxa2 mutants express SOX17, this refutes a strict lineage specification dependence on FOXA2. The additional observation that FOXA2 is expressed in Sox17 mutants suggests that SOX17 and FOXA2 probably act in parallel, but separate pathways.

Sox17 mutants exhibited limited emVE dispersal anteriorly, around the prospective foregut region (Fig. 2h). At later stages, Sox17 mutants exhibited defects in midgut and hindgut, leading to their developmental arrest and death at around E10.5 (ref. 7). Conversely, Foxa2 mutants exhibit a primary defect in foregut and midline structures28,29, with midgut and hindgut areas largely unaffected30. It is tempting to speculate that SOX17 controls gut endoderm
morphogenesis in the prospective midgut and hindgut areas, and that foregut morphogenesis is orchestrated by FOXA2.

Our data identify the transition from a single BM positioned at the epiblast–VE interface present before gastrulation, to two BMs positioned at the interfaces of epiblast–mesoderm and mesoderm–endoderm as gastrulation proceeds. Although Sox17 mutants exhibit a trilaminar structure comprised of epiblast–mesoderm–VE, they possess only one BM, at the epiblast–mesoderm interface. Thus, the BM at the mesoderm–endoderm interface is assembled at gastrulation, and its formation is associated with gut endoderm morphogenesis. Sox17 must have at least two critical, but not necessarily mutually exclusive, roles: one regulating an endoderm identity, and another regulating cell polarity, and by extension epithelialization. As the absence of Sox17 does not affect ECM gene expression (Supplementary Fig. 4i), the establishment of a BM at the mesoderm–endoderm interface probably results from post-transcriptionally regulated assembly, perhaps by baso-lateral clustering of proteins, for example, integrins.

Much is known about how cells exit epithelia, but limited attention has been paid to how cells enter them. For DE cells to egress into the emVE epithelium, DE and emVE cells must coordinate modulate their epithelial characteristics (Fig. 8). DE cells adopt an epithelial identity including acquiring cell polarity, forming cell–cell junctions and exhibiting a biased deposition of BM. Concomitantly, emVE cells transiently relax their polarity, redistribute junctions, and can temporarily be relocated away from an underlying BM so as to accommodate the incoming DE cell flux. After cell egression is complete, this mixed population of cells (the gut endoderm) reinforce their epithelial qualities with BM forming de novo underneath them, thereby facilitating their segregation from the mesoderm. It will be important to determine whether aspects of this cellular and molecular program control MET in the metastatic colonization of cancers of endoderm-derived tissues and organs.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.-K.H. conceived the project. M.V.N. and A.-K.H. designed the experiments and interpreted results. M.V. and S.N. carried out the experiments. M.V. and A.-K.H. wrote the manuscript with input from S.N.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

**AUTHOR DECLARATION**

The authors declare no competing financial interests.

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METHODS

Mouse husbandry and strain genotyping. Mouse strains used in this study were: Sox17mycRFP1 and Sox17mCherry (ref. 19), Fgfr1CreER (ref. 31), Fgfr1CreER (ref. 10), EomesCreERTO (ref. 32), Foxa2CreER (ref. 29), Sox2CreERTO (ref. 33), Afp::GFP (refs 6, 9) and wild-type CD-1 (Charles River). The Sox17mCherry was used to generate the null Sox17allele by crossing to the Sox2::Cre strain. PCR genotyping was performed as previously described. Mouse husbandry and embryo experiments were performed in accordance with Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee-approved protocols. For all experiments, males and females were used indiscriminately.

Embryo recovery, manipulation and ex utero culture. Mice were maintained under a 12-h light/dark cycle. Embryos were dissected in DMEM/F12 (Gibco) containing 5% fetal calf serum (Lonza) and staged according to ref. 34. For live imaging, embryos were cultured in 50% rat serum; 50% DMEM/F12 (ref. 35).

Embryo electroporation. Electroporation of DNA constructs was performed as previously described. CAG::mRFP1, CAG::myr-mRFP1 or CAG::myr-mCherry constructs were electroporated into the primitive streak region of MS (E6.75) stage Afp::GFP embryos. A solution of DMEM/F12 (Gibco) containing ~3 μg ml⁻¹ plasmid was micro-injected into the proamniotic cavity. Embryos were moved to pH 7.5 Tyrode Ringer’s solution and Tweezertrodes placed on either side of the embryo, with the anode positioned posteriorly. An ECM830 square wave electroporator (BTX Harvard Apparatus) was used to deliver 5 pulses of 1517 V charge. After electroporation, embryos were washed in DMEM/F12 and processed for live imaging.

In situ hybridization and immunofluorescence. For in situ hybridization, embryos were fixed in 4% PFA in PBS overnight at 4 °C, dehydrated in methanol and stored at ~20 °C. In situ hybridization was performed using antisense riboprobes as described previously. Primary antibodies were used: COLL-IV (1:300, Millipore), FN-1 (1:300, Rockland), FOXA2 (1:1,000, Abcam), LAMA-1 (1:300, Sigma), LAMB-1 (1:300, Abcam) and SOX17 (1:1,000, R&D Systems), ITGA5 (1:300, Santa Cruz), E-CAD (1:300, Sigma), N-CAD (1:300, Santa Cruz), and SCRIB (1:200, Santa Cruz). Secondary Alexa-Fluor-conjugated antibodies (Invitrogen) were used at a dilution of 1:1,000. DNA was visualized using Hoechst-33342 (5 μg ml⁻¹, Molecular Probes). For cryosections, fixed embryos were taken through a sucrose gradient, embedded in O.C.T. (Tissue-Tek) and sectioned at 12 μm on a cryostat (CM3050S, Leica).

Image data acquisition, processing and quantification. Wide-field images were collected with Zeiss Axiocam MRc/m CCD cameras mounted on a Leica MZ16FC microscope. Confocal images were acquired using a Zeiss LSM510META or LSM700 as described previously. Fluorescence was excited with a 405-nm diode laser (Hoechst-33342), a 488-nm argon laser (GFP), a 543-nm HeNe laser (Alexa Fluor-543/555) and a 633-nm HeNe laser (Alexa Fluor-633/647). Images were acquired using Plan-Apo ×20/NA.0.75 and Fluor ×5/NA.25 objectives, with 0.2–2 μm z-separation. For live imaging experiments, embryos were maintained in a temperature-controlled, humidified chamber with 5% CO₂ atmosphere as described previously. Raw data were processed using ZEN or Imaris software (Zeiss and Bitplane respectively) and assembled in Photoshop CS6 (Adobe). Digital quantification of immunofluorescent signal intensities was performed using ZEN software (see Supplementary Fig. 7 and Table 1). In all figures, representative images were selected from N > 6 embryos. No statistical method was used to predetermine sample size, no experiments were randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. No samples were excluded from the analysis.

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Supplementary Figure 1 Gastrulation mutants fail to disperse the emVE.
(a and a’) Whole mount and transverse section of Fgfr1 mutant expressing the Afp::GFP VE-reporter at PS stage, showing a solid emVE layer on the embryo’s surface. (b and b’) Whole mount and transverse section of Fgfr1 mutant expressing the Afp::GFPVE-reporter at OB stage, showing no mesodermal migration and no emVE dispersal. (c and c’) Whole mount and transverse section of epiblast-ablated Eomes mutant expressing the Afp::GFP VE-reporter at OB stage, showing thick epiblast layer, no wings of mesoderm migration, and no emVE dispersal. ps, primitive streak; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; PS, pre-streak; OB, no bud. Scale bars = 100 μm.
Supplementary Figure 2 Expression of SOX17 and FOXA2 in VE and DE cells. (a-f') Immuno-fluorescence for SOX17 in Afp::GFP VE-reporter embryos in progressive stages of gastrulation. (g) Quantitation of fluorescent intensity show high levels of SOX17 in egressing DE cells, and subsequent equalization of expression levels between DE cells and emVE cells. (h-m') Immuno-fluorescence for FOXA2 in Afp::GFP VE-reporter embryos in progressive stages of gastrulation. (n) Quantitation of fluorescent intensity show high levels of FOXA2 in egressing DE cells, and subsequent equalization of expression levels between DE cells and emVE cells. (o-v) Double immuno-fluorescence for FOXA2 and SOX17 in a mid-emVE dispersal Afp::GFP wild-type embryo. DE cells display high levels of SOX17 as well as FOXA2 (white arrowheads). Both SOX17 and FOXA2 are expressed at low levels in GFP-positive emVE cells (orange arrowheads). (s'-v') Sox17 mutants show absence of SOX17 but presence of FOXA2 in the cells of the mesodermal wings bordering the emVE. FOXA2 is also present at lower levels in GFP-positive emVE cells. (w-x) Anterior views of Afp::GFP embryos at the EHF/LHF stage (E7.75) with immuno-fluorescence for SOX17 or FOXA2. Node and anterior midline are devoid of SOX17 and show strong localization of FOXA2. (y) Double immuno-fluorescence staining for SOX17 and FOXA2 in sequentially staged Afp::GFP embryos. These data reveal low SOX17 expression in VE cells at pre-dispersal stages and upregulation once VE dispersal initiates. Strong SOX17 expression is present in egressing DE cells. Low levels of FOXA2 are detected in some epiblast cells overlying the region of the anterior primitive streak (white arrowheads) or in certain cells leaving the primitive streak. FOXA2 expression is high in egressing cells. Note, blue arrowhead marks FOXA2-positive cells during notochord formation. n, node; m, midline, ps, primitive streak; epi, epiblast; mes, mesoderm; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; PS, pre-streak; LS, late streak; OB, no bud; LB, late bud; EHF, early head-fold; LHF, late head-fold. Scale bars in a-x = 100 μm. Scale bars in y = 50 μm (whole mounts) and 20 μm (sections).
Supplementary Figure 3  N-CAD localizes between neighbouring cells that failed to egress in the absence of SOX17. (a-c) Section through LB/EHF (E7.5) Sox17GFP/GFP embryo, showing N-CAD at the interface (blue arrowheads) of neighbouring non-egressed DE cells (blue asterisks). Pink asterisks mark emVE cells at the surface of the embryo. end, endoderm; epi, epiblast; mes, mesoderm; LB, late bud; EHF, early head-fold. Scale bars = 20 μm.
Supplementary Figure 4 BM expression and localization dynamics in wild-type and Sox17 mutant embryos. (a) LAMA-1 immuno-fluorescence in Afp::GFP VE-reporter embryos. Note the single BM at early stages, and the progressive establishment of a second BM. Orange asterisks mark the anterior tips of the wings of mesoderm. (b) LAMA-1 localization in Fgf8 mutant Afp::GFP VE-reporter at the OB stage (E7.25) indicates a single BM. (c and d) High magnifications of FN-1 or LAMA-1 immuno-fluorescence in Afp::GFP embryos. Egressing DE cells (marked with pink asterisks) always display FN-1 and LAMA-1 basally, never at their apical interface with emVE cells. (e) Double immuno-fluorescence stains in Afp::GFP VE-reporter embryos show egressing DE cells with high SOX17 inserting between emVE cells, displaying FN-1 on their basal side. EmVE cells never display FN-1 at their interface with egressing cells. (f) Afp::GFP VE-reporter embryo stained for COLL-IV. In wild-types, egressing DE cells (orange asterisks) always display enriched LAMA-1 basally. EmVE cells never display FN-1 at their interface with egressing cells. Potential DE cells showed intense staining, suggesting high levels of transcription (white arrowheads). (g) Afp::GFP VE-reporter embryos stained for LAMB-1. In wild-types, egressing DE cells (orange asterisks) always display enriched LAMB-1 basally. In Sox17 mutants only displays one peak. (h) Afp::GFP VE-reporter embryos stained for LAMB-1. In wild-types, egressing DE cells (orange asterisks) always display enriched LAMB-1 basally. In Sox17 mutants, the interface between mesoderm and emVE only displays faint LAMB-1 fluorescent signal. Digital quantitation indicates two peaks for the wild-type LAMB-1 signal and only one peak in the Sox17 mutant. (i) Fn1 in Sox17 mutant embryos is present in all cells of the mesodermal wings and overlying emVE. Potential DE cells showed intense staining, suggesting high levels of transcription (white arrowheads).
**Supplementary Figure 5** Analysis of E-CADHERIN localization during gut endoderm morphogenesis. (a-e6) Immuno-fluorescence staining for E-CAD on Afp::GFPlox/lox embryos depicting changes of E-CAD localization from pre-through late stages of VE dispersal. White arrowheads depict E-CAD expression at interfaces between VE cells, pink arrowheads highlight expression at interfaces between VE and egressing cells during emVE dispersal, and blue arrowheads depict cell-cell junctions between egressing cells. White circles mark the junction between the extra-embryonic ectoderm and epiblast, namely the extra-embryonic/embryonic boundary, which coincides with the boundary of extra-embryonic (exVE) and embryonic visceral endoderm (emVE) only at LHF stages. ps, primitive streak; end, endoderm; epi, epiblast; mes, mesoderm; A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right; PS, pre-streak; ES, early streak; OB, no bud; HF, head-fold; LHF, late head-fold. Scale bars = 50 μm (whole mounts) and 20 μm (sections).
Supplementary Figure 6 Analysis of ITGA5 localization during gut endoderm morphogenesis. (a) Transcriptomic expression quantitation of integrins in E7.5 wild-type embryos. N=3 (3 distal regions of wild-type embryos), graph depicts data points (circles) and mean with SD (horizontal lines). The complete data set is deposited in NCBI’s Gene Expression Omnibus (GEO), where it is publicly accessible under the code GSE33353. (b and c) Transverse section through Afp::GFP VE-reporter wild-type embryo at the OB stage (E7.25) immuno-fluorescently stained for ITGA5. (b’ and c’) Magnifications of boxed regions in (b) and (c), showing isotropic distribution of ITGA5 in inner cells of the mesodermal wings (yellow asterisks), and polarized enrichment in egressing cells (orange asterisks) at the cell membrane section facing the embryo cavity, their prospective basal side. ps, primitive streak; end, endoderm; epi, epiblast; mes, mesoderm; A, anterior; L, left; P, posterior; R, right; OB, no bud. Scale bars = 20 μm.
Supplementary Figure 7 Method used for immuno-fluorescent digital signal intensity quantitation. (a) Example of image used for quantitation of immuno-fluorescent signal. Section through Afp::GFP VE-reporter transgenic embryo stained for E-CADHERIN and ACTIN at the OB stage (E7.25), showing GFP-negative DE cells inserting between GFP-positive VE cells (used in Fig. 7c,d). (b) High magnification of E-CADHERIN channel, indicating two regions of data acquisition for a single egressing cell (red arrows inside pink squares). One measurement is taken on the cell membrane section facing the surface of the embryo, and the other is taken on the cell membrane section facing the embryo cavity. (c) Measurement of the cell membrane section facing the surface of the embryo of an egressing cell, used to generate the intensity graph (d) and Supplementary Table 1. (d) Graph of immuno-fluorescent signal intensity for region defined in (c). (e and f) Example of two measurements for a single inner cell; one measurement is taken on the cell membrane section facing the surface of the embryo, and the other is taken on the cell membrane section facing the embryo cavity. ACTIN signal facilitates visualization of the cell membrane. See also Supplementary Table 1.
**Supplementary Table 1.**

Method used for immuno-fluorescent digital signal intensity quantitation.

Table of measurements pertaining to Supplementary Fig. 7c,d. The table lists point measurements for fluorescent intensities taken in set intervals along the defined line represented by red arrow in Supplementary Fig. 7c. The arithmetic mean for the set of values is calculated, resulting in a single value used to represent immuno-fluorescent signal intensity in the defined region.

| Distance [nm] | Fluorescent Intensity Red Channel [AU] |
|---------------|---------------------------------------|
| 0             | 12291                                 |
| 78            | 14455                                 |
| 156           | 56954                                 |
| 134           | 62683                                 |
| 313           | 56549                                 |
| 391           | 33245                                 |
| 469           | 23175                                 |
| 547           | 15382                                 |
| 625           | 10418                                 |
| 703           | 10418                                 |
| 781           | 9210                                  |
| 860           | 5167                                  |
Supplementary Video Legends

Supplementary Video 1
Selective labeling of cells within the primitive streak of a mouse embryo.
360 degree rotational views of a longitudinal half of an Afp::GFP VE-reporter embryo. The embryo was electroporated with an RFP-expressing plasmid. Cells of the posterior epiblast adjacent to the primitive streak express RFP, and display a columnar morphology. The entire surface of the embryo expresses GFP, indicating that emVE dispersal has not yet begun.
A, anterior; D, distal; P, posterior; Pr, proximal.

Supplementary Video 2
Time-lapse depicting migration of mesoderm and DE cells.
Internal views of the embryo in Video S1, imaged over 8 hours. The RFP-positive columnar cells at the primitive streak change morphology becoming mesenchymal and progressively migrate anteriorly.
A, anterior; D, distal; P, posterior; Pr, proximal.

Supplementary Video 3
Time-lapse displaying egression of DE cells into the emVE.
Surface views of embryo in Video S2, initially depicting a uniform GFP-positive layer (the emVE). The emVE is progressively dispersed, and over time an increasing number of RFP-expressing cells appear on the surface of the embryo (white arrowheads).
A, anterior; D, distal; L, left; P, posterior; Pr, proximal; R, right.

Supplementary Video 4
Time-lapse displaying migration and division of DE cells.
Surface view of Afp::GFP embryo electroporated with CAG::RFP. The second segment tracks a labeled cell migrating anteriorly, moving into a GFP-negative area of the VE, and dividing.

Supplementary Video 5
Time-lapse displaying migration of cells in the mesodermal wings
Afp::GFP embryo electroporated with CAG::RFP. The first segment shows green and red channels. Note that the GFP-positive VE layer remains uninterrupted for the duration of the time-lapse, suggesting that no egression has yet taken place. Second segment shows red channel and brightfield merge.

Supplementary Video 6
The wild-type OB stage (E7.25) embryo has two BMs, while the Sox17 mutant only has one.
High magnification views of LAMA-1 localization in wild-type and Sox17 mutant embryos expressing the Afp::GFP VE-reporter. The wild-type displays two BMs, one at the epiblast-mesoderm interface and another at the mesoderm-endoderm interface. The Sox17 mutant exhibits a single BM, at the epiblast-mesoderm interface.
end, endoderm; epi, epiblast; mes, mesoderm.