Developmental clock and mechanism of de novo polarization of the mouse embryo

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Embryo polarization is critical for mouse development; however, neither the regulatory clock nor the molecular trigger that it activates are known. Here, we show that the embryo polarization clock reflects the onset of zygotic genome activation, and we identify three factors required to trigger polarization. Advancing the timing of Tfap2c and Tead4 expression in the presence of activated RhoA induces precocious polarization as well as subsequent cell fate specification and morphogenesis. Tfap2c and Tead4 induce expression of actin regulators that control the recruitment of apical proteins on the membrane, whereas RhoA regulates their lateral mobility, allowing the emergence of the apical domain. Thus, Tfap2c, Tead4, and RhoA are regulators for the onset of polarization and cell fate segregation in the mouse.
**Introduction**

The totipotent mammalian zygote can produce any embryonic or extra-embryonic tissue but this becomes restricted in the first cell fate decision that generates distinct inner cell mass (ICM) and outer extra-embryonic trophectoderm (TE). The ICM will form epiblast (EPI) and extra-embryonic primitive endoderm (PE), generating the future fetus and the yolk sac, respectively. The TE will form the placenta. Formation of these three lineages by implantation is a prerequisite for successful pregnancy.

Embryo polarization is key to the segregation of the ICM and TE lineages (1, 2). In the mouse, this process happens at the 8-cell stage (2-4) when each blastomere acquires an apical domain, comprising the Par protein complex and ERM proteins (ezrin, radixin and moesin) enclosed by an actomyosin ring (5, 6). The apical domain enables expression of transcription factors such as Cdx2 and Gata3, which drive differentiation into TE, whereas apolar cells maintain pluripotency to become ICM (7, 8).

Mammalian embryo development is regulative and yet the timing of embryo polarization remains unchanged even if embryos are split into individual blastomeres, when cells are aggregated together, or when cell divisions are prevented. Thus, the polarization seems set to a strict developmental clock that is independent of cell number (9, 10). Here, we show that this clock reflects activation of the zygotic genome, and we identify three factors whose convergent activity triggers self-organization of the apical domain.
Critical threshold of transcripts required for embryo polarization

Polarization timing varies between species, reflecting the onset of zygotic-genome-activation (ZGA) (11, 12). In mouse, the major wave of ZGA occurs at the 2-cell stage but an additional transcriptional wave occurs at the early 8-cell stage just before polarization (13). To determine whether the latter transcriptional wave is associated with polarization, we first treated embryos from the early 8-cell stage with two transcription inhibitors: 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside DRB and Triptolide. Either drug prevented apical localization of the polarity marker Pard6 (Fig.1A-D; Fig.S1A-G). Polarization was restored by washing-out the reversible transcription inhibitor DRB (Fig.S1H-J). Thus, transcription at the early 8-cell stage appears to be required for embryo polarization. As a second test, we reduced the cytoplasmic volume (Fig.1E), shown by others to increase the concentration of newly synthesized mRNA (14, 15). We injected zygotes with an apical marker (Ezrin-RFP mRNA) and resected 30-40% of cytoplasm from one 2- or 4-cell stage blastomere, using a technique that does not compromise development (16)(Fig.1F). Single-molecule fluorescence in situ hybridization confirmed that this increased the concentration of newly transcribed mRNAs (17)(Fig.S2A-E). Such blastomere resection brought forward embryo polarization by 2.1hr when performed at the 2-cell stage (Fig.1F-I; N=62 pairs; Movie S1) and by 3.3hr at the 4-cell stage (Fig.S2F-H; Fig.1J; N=76 pairs; Movie S2). Both experimental and control embryos had established all three lineages as blastocystS (Fig.S2I-K). Inhibiting transcription with a 3hr pulse of DRB led both resected and control blastomeres to polarize simultaneously (Fig.1K; Fig.S2L-N). These results indicate that de novo synthesis of transcripts and their accumulation to a critical threshold is required for embryo polarization.

Tfap2c and Tead4 are required for embryo polarization

We hypothesized that the requirement for 8-cell stage transcription could result from direct expression of cytoskeletal regulators of cell polarization or their indirect expression through a transcriptional hierarchy. We therefore interrogated published single-cell RNA-sequencing data (18) and selected genes for 118 cytoskeletal polarity regulators and 6 transcription factors that show increased expression by the 8-cell stage and hence are likely to be active according to ATAC-seq (19)(Fig.S3; Table
S1,2). We downregulated each of these 124 genes by RNAi (Fig.S3; Fig.S4A-B) and scored the timing of embryo polarization from time-lapse imaging of the distribution of Ezrin-RFP (Fig.2A-D). Only depletion of the transcription factors Tfap2c or Tead4 prevented embryo polarization (Fig.2A-C,E; Fig.S4A-E). Individual depletion of Tfap2c and Tead4 delayed polarization from the 8- to the 16-cell stage (Fig.2A-C, E-F) whereas polarization was entirely abolished by their co-depletion (Fig.2D-H). Depletion of Tfap2c and Tead4 also prevented precocious polarization resulting from blastomere resection (Fig.S4F-G).

To confirm the requirement for Tfap2c and Tead4 in polarization, we deleted both genes by CRISPR-Cas9 mutagenesis. We designed three sgRNAs to target a single protein-coding exon of each gene (Fig.2I) and injected them into the zygote together with Cas9 mRNA and Ezrin-RFP mRNA as an apical marker. We categorized blastomeres based on whether they had undetectable, moderate, or wild-type levels of Tfap2c or Tead4 proteins at the 8-16-cell stage (Fig.S5A-B) and confirmed by DNA sequencing that blastomeres with undetectable Tfap2c or Tead4 were homozygous mutants (Fig.2J; Fig.S5C-D). Simultaneous deletion of Tfap2c and Tead4 completely abolished embryo polarization, contrasting with their individual deletions that were less severe (Fig.2J-L; Fig.S5E-F). Thus, zygotic expression of Tfap2c and Tead4 is required for embryo polarization at the 8-cell stage.

Previously Tead4 had been previously shown to function only downstream of polarization, following nuclear re-localization of its transcriptional co-activator Yap to induce TE transcription factor expression (7). To gain further insight into the earlier role of Tead4, we examined the localization of Yap. We found Yap localization in the nucleus before polarization at the 8-cell stage (20)(Fig.S6A-C) that was diminished by downregulation (Fig.S6D-E) and enhanced by upregulation of Tead4 (Fig.S6F-G). Thus, Tead4 affects the localization of Yap before polarization, indicating a previously undescribed, polarity-independent Tead4 function.

**Advancing expression of Tfap2c, Tead4, and Rho GTPase advances polarization timing**

As we found that Tfap2c and Tead4 are required for embryo polarization, we wished to determine whether advancing their expression would advance the timing of
polarization. We therefore injected Tfap2c and Tead4 mRNAs into one blastomere at the late 2-cell stage, to elevate their expression at the 4-cell stage (Fig.S7A-C). Advancing expression of Tead4 did not induce premature polarization (Fig.S7D-E, I-J). Advancing expression of Tfap2c led to formation of cell protrusions enriched in apical polarity proteins, including Pard6 and Ezrin at the 4-cell stage (Fig.S7F-I). Advancing the expression of Tfap2c and Tead4 together also induced premature formation of protrusions (Fig.S7G-I; Fig.3B-C; Movie S3-4). In all cases, the induced protrusions were smaller than the natural apical domain at the 8-cell stage (Fig.3B; Movie S3-4; Fig.S7H). These results suggested Tfap2c and Tead4 might be sufficient to trigger polarization of apical proteins but other factors are required for proper apical domain formation.

We previously found that actomyosin activation by PKC-Rho GTPase signaling was necessary but not sufficient to trigger apical domain formation (Fig.3B-C, Movie S5) (21). We therefore hypothesized that Rho GTPase activation might be required with Tfap2c and Tead4 to achieve complete polarization. To test this, we injected mRNAs for Tfap2c and Tead4 at the 2-cell stage (with Ezrin-RFP mRNA as apical marker) and mRNA for constitutively active RhoA-Q63L at the 4-cell stage (with LifeAct-GFP as injection marker)(Fig.3A; Fig.S7K). Expression of all three factors established complete apical domains at the 4-cell stage (Fig.3B-C; Movie S6). These induced apical domains were enriched with Ezrin and Pard6, strongly resembling apical domains that form normally only at the 8-cell stage (Fig.3B; Fig.S8B-C).

To confirm these results, we overexpressed these factors in just half the embryo and found that the targeted blastomeres polarized earlier than controls in the other embryo half (Fig.3D-F; Movie S7-8). We observed no differences in division timing between blastomeres suggesting induced 4-cell stage polarization is not caused by a cytokinesis delay (Fig.S8A). Together our results indicate that a transcriptional program triggered by Tfap2c and Tead4 alongside activation of actomyosin downstream of Rho GTPase signaling triggers polarization at a specific stage of embryogenesis.

Advancing Tfap2c, Tead4 and Rho GTPase expression advances morphogenesis and differentiation
Embryo polarization at the 8-cell stage is followed by the zippering of adjacent apical domains, which expand and seal their boundaries at the late 16-cell stage to enable blastocyst formation (22). To determine whether premature polarization could also advance the zippering process, we induced expression of Tfap2c/Tead4/RhoA-Q63L either in the whole embryo or in half of the embryo to trigger 4-cell stage polarization and followed subsequent development by time-lapse microscopy. The induced premature polarization resulted in zippered domains associated with the tight junction protein ZO-1, not at the 16-cell but at the 8-cell stage (Fig. S8B-F). Thus, embryo polarization is sufficient to advance the subsequent step of embryogenesis leading to blastocyst formation.

As polarization in the mouse embryo is followed by cell fate specification, we determined whether overexpressing Tfap2c/Tead4/RhoA-Q63L to induce polarization at the 4-cell stage would advance differentiation of cells inheriting an apical domain into TE. Premature polarization induced premature expression of the TE transcription factors Cdx2 and Gata3 in a cell-autonomous manner (Fig. S8G-K; Movie S9-10). Thus, the combined activities of Tfap2c, Tead4, and RhoA-Q63L are sufficient to advance the timing not only of polarization but also the differentiation program.

**Tfap2c/Tead4 are required for apical protein centralization**

To define the relative roles of RhoA, Tfap2c, and Tead4 in driving polarization, we visualized events leading to apical domain formation in living embryos expressing LifeAct-GFP and Ezrin-RFP from the mid- to late-8 cell-stages. During apical protein polarization, Ezrin-RFP first became concentrated at the center of the cell-contact free surface to form an apical patch concomitant with a local reduction of actin. We refer to this stage as “centralization” (Fig. 4A-B; Movie S11). This apical patch of Ezrin-GFP expanded and actin became concentrated in a ring around it. We refer to this phase as “expansion” (Fig. 4A,C; Movie S11). Downregulation of Tfap2c and Tead4 diminished the initial centralization of Ezrin-RFP (Fig. 4D; Fig. 2G), implying that centralization is required for apical domain formation.

**Tfap2c/Tead4 control polarized growth of apical protein clusters**

Imaging of apical domain centralization in mid- to late-8 cell stage embryos with higher temporal-spatial resolution (Movie S12) revealed Ezrin formed clusters that co-
localized with actin clusters when the embryo had just compacted but not yet polarized (Fig.4E, J). As polarization progressed, the Ezrin clusters grew; those clusters more distant grew faster than those near cell-cell contacts resulting in Ezrin enrichment towards the middle of cell-contact free surface (Fig.4E-F). The amount of membrane-associated Ezrin increased as the Ezrin clusters grew (Fig.4G) suggesting cluster growth is driven by Ezrin’s recruitment to the membrane.

Overexpression of Tfap2c/Tead4 led to an increase in membrane enrichment of Ezrin and precocious growth of both Ezrin and actin clusters at the late 4-cell stage (Fig.4H-I, L-N). By contrast, Tfap2c/Tead4 depletion decreased Ezrin’s membrane enrichment and prevented growth of Ezrin and actin clusters at the mid 8-cell stage (Fig.4J-N). Together, this suggests that Tfap2c/Tead4 are required for the growth of actin and apical protein clusters, recruitment of apical protein to the membrane, and centralization of apical protein.

**Tfap2c/Tead4 regulate actin dynamics to promote apical protein cluster growth**

Since our results suggested that Tfap2c/Tead4 could regulate actin dynamics to direct the growth of apical protein clusters, we next examined the cortical movements generated by the contractile actomyosin network during apical domain formation (23) (Movie S13). We first tested whether such cortical movements can drive asymmetric growth of apical protein clusters by tracking LifeAct and Ezrin clusters using Particle Image Velocimetry (PIV) (Movie S14; Methods). PIV did not detect any obvious movement toward the center of the cell-contact free surface (Fig.S9A-B) in contrast to actin flows of post-mitotic cells (22)(Fig.S9C). In accord, we found that inhibiting actin flows with Blebbistatin failed to prevent asymmetric Ezrin cluster growth (Fig.S9D-G; Movie S15). Thus, asymmetric clustering of Ezrin is not driven by the cortical flow mediated by actomyosin contractility.

Time-lapse observations revealed that Ezrin cluster growth occurred during the merging and splitting of actin clusters and was unimpeded by Blebbistatin (Fig.5A), suggesting that cortical actin remodeling may allow Ezrin’s recruitment to growing clusters. In accord, perturbing actin de-polymerization in 8-cell embryos with Jasplakinolide (JASP) prevented apical domain formation (Fig.S9H,K). Moreover, when we inhibited the Arp2/3 complex with CK666 to prevent actin nucleation, Ezrin
cluster growth (Fig.5B-C) and apical domain formation (Fig.S9I,K) were also blocked. By contrast, treatment of embryos with a Formin inhibitor (SMiFH2) did not affect apical domain formation (Fig.S9J, L). These observations indicate actin remodeling is required for Ezrin clustering and apical domain formation. We also found that CK666 abolished apical protein polarization induced by Tfap2c/Tead4 overexpression in 4-cell embryos (Fig.5D-E). Thus, Tfap2c/Tead4-dependent regulation of actin dynamics is required for the growth of Ezrin clusters and apical protein formation.

**Tfap2c/Tead4 control expression of actin regulators**

To view events downstream of Tfap2c/Tead4, we carried out RNA-sequencing of 8-cell stage embryos depleted of Tfap2c and/or Tead4. For each group of embryos (control GFP RNAi, Tfap2c RNAi, Tead4 RNAi, Tfap2c/Tead4 co-RNAi), two biological replicates were collected with 10 embryos per sample and experiments performed on two strains to eliminate genetic background effects (Fig.5F, Methods). The effect of Tfap2c and Tead4 depletion was highly reproducible between biological replicates and between genetic backgrounds (Fig.S10A). Depletion of Tfap2c led to the downregulation of 749 or 929 genes (with a 2-fold differential cut-off) depending on the strain, whereas Tead4 depletion led to downregulation of 242 or 314 genes (Fig. S10B-C). Their co-depletion led to an additional 135 or 95 genes being downregulated compared to single knockdown embryos, depending on the strain (Fig.S10B-C).

A significant proportion of downregulated genes in double-knockdown embryos had actin polymerization functions (Fig.5G; Table S3). These included known actin regulators for apical domain formation, such as Cdc42 effector protein family members (Borg)(24), and other actin regulators, whose functions have not been explored in the mouse embryo including the Arp2/3 complex component Arpc1b, the Tropomyosin protein Tpm4, Marcks and Marcksl1 proteins, and the FREM family member Ebp4.1l5. Expression of these actin regulators becomes upregulated between the 2-cell and 8-cell stages and correlates with the size increase of actin clusters during polarization (Fig.5G). Depletion of Tfap2c and Tead4 eliminated expression of actin regulators and accordingly led to a decreased actin cluster size (Fig.4H,J-M; Fig.5G).

To test whether these actin regulators participate in apical domain formation, we depleted Arpc1b, Tpm4, Marcksl1, or Ebp4.1l5 individually from 2-cell embryos and
determined the apical domain formation efficiency at the late 8-cell stage (Fig.5H-J; Fig.S11A-D). In accord with the effects of CK666, depletion of Arpc1b led to defective apical domain formation (Fig.5H-J) in natural 8-cell embryos and prevented Tfap2c/Tead4 induced apical protein polarization at the 4-cell stage (Fig.5K-L). Together, these results suggest Tfap2c and Tead4 control embryo polarization by activating expression of actin regulatory proteins.

**RhoA signaling reorganizes the actin network during polarization**

Knowing that not only Tfap2c/Tead4, but also RhoA-Q63L were required for apical protein clustering and apical domain formation in 4-cell embryos, we wished to determine how excess RhoA activity (by overexpressing RhoA-Q63L) or reduced RhoA activity (by treatment with RhoA inhibitor C3-transferase) at the mid 8-cell stage would affect Ezrin’s membrane distribution (Fig.S12A). Overexpression of RhoA-Q63L eliminated actin and Ezrin cluster formation resulting in the homogeneous distribution of actin and Ezrin on the membrane (Fig.S12B-C). By contrast, C3-transferase treatment resulted in the ectopic clustering of actin and Ezrin on the cell membrane (Fig.S12D-E) reminiscent of 4-cell embryos overexpressing Tfap2c/Tead4 but lacking RhoA activity (Fig.3B; Fig.S7G,H). Thus, RhoA signaling is required to reorganize the actin network in the embryo and thereby the clustering of apical proteins induced by Tfap2c/Tead4.

**Positive feedback and lateral mobility govern apical domain formation**

To gain biophysical understanding of how Tfap2c/Tead4/RhoA regulate the timing and pattern of apical domain formation, we measured the growth of Ezrin cluster size during apical protein centralization and found it increases exponentially (Fig.4F), suggesting involvement of a positive feedback mechanism. To gain understanding of this mechanism, we tagged Ezrin with green to red photo-activatable Dendra2 fluorescent protein to track Dendra2-Ezrin movement after blue light conversion. RFP signal dynamically dissipated within seconds when Dendra2 was photo-converted at the mid 8-cell stage either within or outside the nascent apical domain, suggesting rapid Ezrin membrane turnover (Fig.6A-D). Irrespective of the site of photo-conversion, Ezrin-Dendra2 relocated to the nascent domain in proportion to the concentration of Ezrin in this area, indicating positive feedback of Ezrin on its own
accumulation. This accords with previous measurements of cooperative recruitment of Ezrin to PIP2 membranes (25, 26).

To determine whether Ezrin dynamics can account for apical protein centralization, we constructed a model (Methods) based on four empirically grounded assumptions (Fig. 6E): 1) a cooperative increase in Ezrin binding rate ($k_{on}$) with increasing Ezrin concentration saturating above a critical concentration ($E_{crit}$) suggested by the positive feedback above (Fig. 6B,D) and known cooperative binding of Ezrin to membranes (25, 26); 2) a limit to Ezrin membrane loading by the finite pool of PIP2 ($P_{tot}$) based on co-localization of Ezrin with PIP2 in the apical domain (Fig. S12F) and prevention of apical domain formation by reduced PIP2 (21); 3) lateral motility of Ezrin along the membrane as observed (Fig. 6A-D; Fig. S12G), and that can be modelled as effective diffusion with diffusivity ($D_E$); 4) dissociation of Ezrin at a uniform rate from the membrane ($k_{off}$) (Fig. 6E).

We simulated Ezrin dynamics in one dimension and estimated parameter values for simulations best fitting our experimental measurements (Methods, Supplementary Modeling). Our simulations reproduced the dynamic changes of Ezrin distribution during centralization in vivo (Fig.6F). The model also recapitulated the ability of single cells to form centralized Ezrin domain in the absence of cell-cell contacts (Fig.S12H, Methods) and the ability of cell-cell contacts to constrain apical Ezrin to the center of cell-contact free surface (Fig.S12I). Thus, experimental observations and computational simulation together reveal positive feedback, lateral mobility, and competition for limiting PIP2 are sufficient to explain apical protein centralization step.

**Tfap2c/Tead4/RhoA control cooperative recruitment and lateral mobility of apical proteins**

Our model predicts that elevating the saturation threshold ($E_{crit}$) for cooperative recruitment of Ezrin would change the steady-state distribution of membrane-bound Ezrin, resulting in a narrowed peak resembling the Tfap2c/Tead4 overexpression phenotype (Fig.6G, Supplementary Modeling). This suggests Tfap2c/Tead4 regulates the kinetics of the cooperative Ezrin recruitment. To test this, we computed the rate of change in membrane-bound Ezrin concentration ($\Delta E/\Delta T$) as a function of the Ezrin concentration during apical centralization, for both control and Tfpa2c/Tead4
overexpressing embryos (Fig. S12J). This revealed that when the Ezrin concentration falls below a threshold value, local Ezrin subsequently decreases but, when the local concentration exceeds this value, more Ezrin gets recruited to the membrane (Fig. S12J).

Myosin motor activity within the actin cortex can affect the lateral mobility of membrane proteins that can negatively regulate the formation of membrane clusters (27) suggesting that RhoA might regulate the lateral mobility of Ezrin. To test this, we quantified the “spread” of photo-converted signal (Methods) and compared the difference between control and RhoA-Q63L overexpressing cells (Fig. S12K). Elevating RhoA activity increased the spreading effect of photo-converted Ezrin on the membrane, suggesting that RhoA positively regulates the lateral mobility of Ezrin (Fig. S12K). In accord, our simulations predict that increasing lateral mobility leads to homogeneously distributed Ezrin (Fig. 6G) and that insufficient lateral mobility results in multiple peaks of apical protein (Fig. S12L). These predictions are concordant with the phenotypes resulting from overexpression of RhoA-Q63L and depletion of RhoA in the 8-cell embryo (Fig. S12B-E).

Our model suggests that lateral mobility and the cooperative recruitment threshold have opposing effects on the shape of the apical patch (Fig. 6H; Fig. S12M). Specifically, normal apical domain will form only when both processes are activated at an appropriate level. This prediction provides a qualitative explanation for the concurrent requirements for Tfap2c/Tead4 and RhoA signaling in regulating apical domain formation (Fig. 3B; Fig. 6I).

Discussion

The importance of the first appearance of apical-basal cell polarity in mammalian development is evident from its requirement to trigger the first cell fate diversification event. Here, we show that zygotic expression of Tfap2c and Tead4 is a prerequisite for such polarization. We have been able to induce precocious embryo polarization and thereby advance subsequent embryogenesis by driving the ectopic expression of Tfap2c/Tead4 and activated Rho GTPase. Our findings help account for the temporal relationship between zygotic genome activation and the establishment of embryo polarization across multiple mammalian species (11, 12).
Embryo polarization at the 8-cell stage has been viewed as a model for epithelial polarization. However, formation of the apical domain is distinct from many other cell types as it can occur in the absence of external cues such as the extra-cellular matrix or cell adhesion. The mechanisms behind such unique spontaneous symmetry breaking properties have remained elusive. Here we show the initial step for symmetry breaking is the centralization of apical proteins through their two types of behavior on the membrane: actin-mediated cooperative recruitment and lateral mobility. These two processes act as opposing forces to regulate the shape of the apical domain; a cooperative recruitment mechanism enables symmetry breaking and concentration of the apical proteins whereas lateral mobility allows apical proteins to diffuse, thereby establishing a crescent-shape patch (Fig.6G-H; Fig.S12M). The balanced activity between the two processes ensures the proper shape of the apical domain because an excessive cooperative recruitment force would lead to small and often multiple domains, whereas excessive lateral mobility would lead to the uniform distribution of apical proteins and thereby inhibit symmetry breaking (Fig.6G; Fig.S12L).

Our results suggest that cooperative recruitment is regulated by actin remodeling controlled by Tfap2c and Tead4. Although the detailed mechanism is beyond the scope of this work, it is possible that Ezrin is preferentially recruited to the actin structure promoted by the Arp2/3 complex in a process similar to protein condensation (28). In such a case, the density of the branched actin network would positively impact on the saturation level of Ezrin in the cooperative recruitment process. It has been observed in vitro that the actin clusters formed by Arp2/3 activity are degraded by high levels of cortical myosin (29), which could explain the opposing effects between Tfap2c/Tead4 and RhoA in regulating the apical protein clustering and accordingly the apical domain shape.

The regulatory regime we describe is based on the behavior of Ezrin and it is likely to apply to other apical proteins such as the Par complex, whose polarization dynamics are highly similar and also require the actin network and membrane binding, the key conditions of the process we describe (21). Our work illustrates how the embryo establishes cell polarization at a specific developmental stage under the regulation of stage-dependent pathways. Our results also provide a biophysical explanation for how
polarization is established, indicating that positive feedback combined with lateral mobility are sufficient to drive this self-organization process. These results therefore provide insight into both the timing and mechanism of the establishment of *de novo* polarization in the mouse embryo, the critical event for the first cell fate specification.
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upon request from corresponding author. The RNA-sequencing data has been deposited on GEO database (accession number: GSE124755); the code for computation simulation has been deposited on https://jakesorel.github.io/Apical_Domain_2020/.

Summary of the method
This research followed regulations of the Animals (Scientific Procedures) Act 1986 - Amendment Regulations 2012 - reviewed by the University of Cambridge Animal Welfare and Ethical Review. Embryos were collected from superovulated F1 females (C57Bl6xCBA) crossed with F1 males. For embryo culture and inhibitor treatment, embryos were recovered at the zygote or 2-cell stage in M2 medium and transferred to KSOM medium for long-term culture. The inhibitors, or the same amount of vehicles (for control condition), were applied to the culture. The microinjection procedure, immunostaining, static imaging and image processing, and real-time qPCR were carried out as previously described (21). For photo-conversion experiment, region of interest (ROI) covered a rectangular region of length roughly 5μm and width roughly 2μm on the membrane of cells expressing Ezrin-Dendra2, using the mid-plane of the blastomeres as a reference. The ROI was illuminated at 405nm for 5s, after which converted proteins were imaged with a 568nm laser and at an emission wavelength between 580-620nm, in every 2s/frame for 5min. The converted scanning speed is 200Hz, and the normal scanning speed is 700Hz. For all imaging settings, the images have been recorded using the 1024x1024 pixel format. Particle Image Velocimetry (PIV) analysis was performed using PIVlab MATLAB algorithm (pivlab.blogspot.de). For statistics, the sample distribution as well as statistic tests were performed using Prism software (http://www.graphpad.com).

Details of the materials and methods, as well as the details for RNA-sequencing and modeling methods can be found in the supplementary materials.

Figure Legends
Figure 1. The dependency of polarization on nascent transcripts. (A) Scheme indicating inhibitor treatments. (B) DMSO (control)- or DRB-treated 8-16-cell embryos analyze F-actin, Pard6 and DNA. (C) Polarized cell number in DMSO (control) or DRB-
treated embryos. ****p<0.0001, Mann-Whitney U test. N=2 experiments. (D) Apical enrichment of Pard6 (Methods) in 8-16-cell stage cells treated with DMSO (control) or DRB. ****p<0.0001. Mann-Whitney U test. (E) Scheme of the hypothesis: newly synthesized factors important for polarization accumulate up to a point upon which polarization is induced at the 8-cell stage. Decreasing the cell size would elevate the concentration of such factors leading to an advance in polarization timing. (F) Scheme showing blastomere resection procedure. (G,H) Time-lapse of control or smaller sister blastomeres from experiment in F. Arrows indicate the apical domain. Yellow squares indicate the magnified regions (top). (I,J) Polarization time difference (Methods) between smaller and control sister blastomeres from F or Fig. S2F, each bar represents one comparison. Smaller cells polarize earlier in the significant majority of cases. N=13 experiments for I, N=6 experiments for J. ****p<0.0001, one-sample t-test, hypothetical mean=0. (K) Polarization time difference between control and smaller DRB-treated sister cells, from experiments in Fig. S2L. Each bar represents one comparison. Pulsed transcription inhibition prevents the early polarization of smaller cells. N=3 experiments. ns, not significant, one-sample t-test, hypothetical mean =0. Arrows indicate the apical domain. Scale bars, 15µm.

**Figure 2. Zygotic expression of Tfap2c and Tead4 is essential for polarization.** (A-D) Time-lapse of Ezrin-RFP localization in embryos with or without Tfap2c and/or Tead4. Tfap2c and Tead4 co-depletion causes polarization failure until the 16-cell stage. Arrows indicate apical domain. (E,F) Polarized cell number in different conditions and stages. Each dot represents an embryo. dsTT: dsTfap2c+dsTead4. *p=0.0306; ***p=0.0006; ****p<0.0001. Kruskal-Wallis test for E, one-way ANOVA test for F. (G) F-actin, Pard6, and Ezrin localization in late 8-cell stage embryos injected with dsGFP (control) or dsTT. (H) Quantification of Ezrin apical enrichment. ****p<0.0001, Student’s t-test. (I) CRISPR-Cas9 strategy to deplete Tfap2c and Tead4. (J) Tfap2c and Tead4 protein levels in wild-type (Cas9 mRNA, control); Tfap2c depleted (with Tfap2c sgRNAs); Tead4 depleted (with Tead4 sgRNAs); Tfap2c and Tead4 co-depleted (with sgRNAs targeting both Tfap2c and Tead4), Ezrin-RFP expressing embryos. (K) Proportions of polarized cells in different genotypes presented in J. Number of cells analyzed presented within each bar. ns, not significant; ****p<0.0001, Fisher’s exact test. N=2 experiments. Tfap2c and Tead4 co-depletion represses apical domain formation. (L) Quantifications of Ezrin apical enrichment.
Each dot represents a cell. **p=0.0012, ***p=0.0007, ****p<0.0001, Kruskal-Wallis test. N=4 experiments. Scale bars, 15µm.

**Figure 3.** Premature expression of Tfap2c, Tead4, and activated-RhoA is sufficient to advance polarization timing. (A) Scheme of Tfap2c, Tead4, and RhoA-Q63L overexpression. (B) Ezrin-Venus dynamics in late 4-cell or 8-cell stage control cells (Ezrin-Venus only); or late 4-cell stage cells overexpressing a)Tfap2c+Tead4(TT); b)RhoA-Q63L(RhoA) or c)Tfap2c+Tead4+RhoA-Q63L(TTRhoA). Tfap2c+Tead4+RhoA-Q63L overexpression induces premature, full apical domain. In all conditions cell divisions were not affected. Short arrows indicate Ezrin-Venus enrichment at the cell-contact free surface. Long arrows indicate apical domain expansion. Squares indicate the magnified regions. (C) Quantification of morphologies induced by conditions in B. Number of cells analyzed presented within each bar. (D) Scheme of Tfap2c, Tead4, and RhoA-Q63L overexpression. (E) Representative images of embryos overexpressed with Ezrin-RFP, LifeAct-GFP mRNA only (control) or with Tfap2c, Tead4 and RhoA-Q63L mRNA at 4- or 8-cell stage. The cells overexpressed with Tfap2c, Tead4 and RhoA-Q63L polarize significantly earlier than control cells in the same embryos, or cells in the control embryos. Arrows indicate the apical domain. (F) Polarization time difference between cells with or without LifeAct-GFP or Tfap2c+Tead4+RhoA-Q63L overexpression in the same embryo, or in control embryos. ns, not significant; ****p<0.0001, one-sample t-test. hypothetical mean = 0. Control group: N=13 embryos ; TTRhoA group: N=19 embryos . N=2 experiments. Scale bars, 15µm.

**Figure 4.** Tfap2c and Tead4 regulate apical domain centralization through regulating the apical protein clustering. (A) LifeAct- GFP and Ezrin-RFP dynamics during polarization. Squares denote magnified regions; yellow and grey arrows indicate apical protein or actin ring movements. The apical domain forms following centralization and expansion steps. N=7 embryos, N=4 experiments. (B,C) Ezrin-RFP signal at the cell-contact free surface during centralization or expansion steps in A. Ezrin signal is normalized against average membrane signal intensity. (D) Ezrin-RFP distribution on cell-contact free surface at the late 8-cell stage in dsGFP (control) or dsTfap2c+dsTead4 injected cells. Numbers indicate examined cells. N=2 experiments. (E) Ezrin-RFP distribution during apical centralization. (F) Ezrin cluster
size during polarization. More than 1500 clusters were analyzed for each time-point. Data shown as mean ± S.D. N=2 experiments. (G) Ezrin membrane enrichment during centralization. N=4 cells from 4 embryos. Data shown as mean ± S.E.M. (H-K) Localization of LifeAct-GFP and Ezrin-RFP in embryos injected with or without Tfp2c+Tead4 mRNA at late 4-cell stage, or embryos injected with or without dsTfp2c+dsTead4 at 8-cell stage. Yellow squares indicate magnified regions (right). (L,M) Size of actin (L) or Ezrin (M) clusters in embryos shown in (H-K). Data shown as mean ± S.E.M. ***p<0.001, ****p<0.0001, One-way ANOVA test. Numbers indicate examined cells. More than 500 clusters were measured in each condition. (N) Ezrin membrane enrichment in different conditions and stages. TT, Tfp2c+Tead4 overexpression; dsTT, dsTfp2c+dsTead4 knockdown. Numbers indicate examined cells. Data presented as mean ± S.E.M. Scale bars for magnified images in H-K, 5µm. All other scale bars, 15µm.

Figure 5. Clustering of apical proteins is regulated by local actin dynamics. (A) LifeAct-GFP and Ezrin-RFP dynamics with or without Blebbistatin treatment during polarization. Arrows indicate the merging of Ezrin clusters during actin polymerization. Scale bar, 1µm. N=5 cells for each condition. N=3 experiments. Blebbistatin treatment did not prevent the clustering of actin or Ezrin proteins. (B) LifeAct-GFP and Ezrin-RFP localization in mid 8-cell stage embryos treated with DMSO (control) and CK666. Scale bars, 5µm. (C) Ezrin cluster size in cells treated with DMSO or CK666 in B. ****p<0.0001, Mann-Whitney U test. More than 1500 clusters were measured in each condition, N=2 experiments. (D) F-actin and Ezrin-RFP localization in late 4-cell embryos expressing Ezrin-RFP only, or with Tfp2c+Tead4 treated with or without CK666. (E) Ezrin cluster size in D. ****p<0.0001, Kruskal-Wallis test. More than 1100 clusters were calculated in each condition, N=2 experiments. (F) Experimental strategy for RNA-sequencing. (G) Heatmap showing the expression of selective cytoskeleton regulators downstream of Tfp2c and Tead4. (H) LifeAct-GFP and Ezrin-RFP localization in dsGFP or dsArpc1b injected embryos. (I) Polarized cell numbers in dsGFP and dsArpc1b groups. Numbers represent cell number. ***p<0.001, Fisher’s exact test. (J) Ezrin-RFP apical enrichment in dsGFP or dsArpc1b cells. Each dot represents a cell. ***p<0.001, Student’s t-test. (K) LifeAct-GFP and Ezrin-RFP localization in different conditions. Squares indicate magnified regions (right). (L) Number of cells showing apical protrusions in K. Scale bars, 15µm.
Figure 6. Tfap2c/Tead4/RhoA regulate apical domain formation through a positive feedback and mobility system. (A,C) Ezrin-Dendra2 localization during and after photoconversion. Yellow squares indicate converted region, red and green squares indicate Ezrin-high or low regions measured in B and D. (B,D) Signal intensity of converted Ezrin-Dendra2 (experimental setting shown in A and C) in Ezrin-low and Ezrin-high regions within 6 min post-conversion. Scale bars, 15µm. (E) Structure of the biophysical model. (F) Kymographs comparing in silico and in vivo polarization dynamics. Same color corresponds to the same Ezrin intensity. (G) Time courses of simulated Ezrin apical distribution at different regions of parameter space. Colors corresponding to the elapsed simulation time. (H) Phase space of polarization shape within the monopolar regime. (I) Summary of the results.
Figure 2

A dsTfap2c Late 8-cell 8-16 cell Early 16-cell t=10 t=13 t=20
B dsTead4 Late 8-cell 8-16 cell Early 16-cell t=1 t=5 t=14
dsTfap2c+dsTead4 Late 8-cell 8-16 cell Early 16-cell t=22 t=28 t=13
dsGFP dsTfap2c dsTead4 dsTT

C
D
E Late 8-cell stage
Number of polarized cells
Non-polarized cell Polarized cell
F Early 16-cell stage
Number of polarized cells
Non-polarized cell Polarized cell
G F-actin Pard6 F-actin Pard6 3D Ezrin-RFP Ezrin-RFP 3D
H Ezrin apical enrichment
dsGFP dsTT
I
J Cas9 only Tfap2c-null Tead4-null Tfap2c-null Tead4-null
K Percentage (%) Polarized cell Non-polarized cell
L Ezrin apical enrichment
Cas9 only Tfap2c-null Tead4-null Tfap2c-null Tead4-null
Figure 4

Figure 4A: Time course of Ezrin-RFP and LifeAct-GFP signals.

Figure 4B: Centralization of Ezrin signal over time.

Figure 4C: Expansion of Ezrin signal over time.

Figure 4D: Ezrin cell-contact free signal intensity.

Figure 4E: Growth of Ezrin cluster over time.

Figure 4F: Size of Ezrin cluster.

Figure 4H: Control 4-cell stage.

Figure 4I: Tfp2c+Tead4 4-cell stage.

Figure 4J: Control 8-cell stage.

Figure 4K: dsTfp2c+dsTead4 8-cell stage.

Figure 4L: Ezrin cluster size.

Figure 4M: Actin cluster size.

Figure 4N: Ezrin membrane enrichment.

Figure 4O: LifeAct-GFP and Ezrin-RFP signals in different cell stages.
Figure 5

8-cell stage
Zygote
1. dsGFP
2. dsTead4
3. dsTfap2c
4. dsTfap2c+dsTead4

Genetic Background:

A. ♀ C57BL/6J x CBA/J x ♂ C57BL/6J x DBA/2J
B. ♀ C57BL/6J x DBA/2J x ♂ C57BL/6J x DBA/2J

D. F-actin Ezrin Ezrin
Ezrin onlyTfap2c+Tead4
Tfap2c+Tead4

G. Vangl1 Vav1 Trip10 Tpm4 Tmsb10 Tbc1d Tagln2 Tagln2 Plekhg2 Nck2 Mylfp Marcks1 Marcks Lcp1 Frmpd1 Frmd4b Fgfr2 Epb4.115 Cdc42ep4 Cdc42ep3 Cdc42ep1 Cdc42ep1 Arpc1b Arhgef19 Arhgef16 Arhgdib Arhgap9 Arhgap18 Anil Anil

H. dsGFP dsArpc1b

J. Apical Ezrin enrichment
Ezrin dsArpc1b

K. Ezrin only

L. Percentage (%)

Polarized cell Non-polarized cell

Percentage (%) B

Ezrin dsArpc1b

F. Zygote 8-cell stage
1. dsGFP
2. dsTead4
3. dsTfap2c
4. dsTfap2c+dsTead4

I. Percentage (%)

Ezrin dsArpc1b

0 20 40 60 80 100
0 100 200 300 400 500 600 700 800 900 1000

K. Ezrin only

2-cell stage 8-cell stage Tfap2c+Tead4 knockdown
Figure 6

A) Dendra Unconverted, Dendra Pre-conversion, Dendra Post-conversion

B) Inside apical domain conversion

C) Ezrin-low region

D) Outside apical domain conversion

E) Theoretical model

F) Theoretical vs Experimental

G) Intensity vs Distance

H) Heatmap

I) Diagram showing the relationship between Tfap2c+Tead4, RhoA, and Ezrin recruitment

- Ezrin-high region
- Ezrin-low region
- Normal apical domain
- Apical protrusion
- Expanded apical domain
- Cooperative Ezrin recruitment
- Actin remodeling
- RhoA
- Lateral mobility of Ezrin

1. Cooperative association ($k_{on}$, $E_{crit}$)
2. Limiting PIP2 pool ($P_F$)
3. Diffusion ($D_E$)
4. Dissociation ($k_{off}$)

- RhoA-Q63L
- Tfap2c+Tead4
- Tfap2c+Tead4+RhoA-Q63L

- Control
- RhoA-Q63L
- Tfap2c+Tead4
- Tfap2c+Tead4+RhoA-Q63L