Membrane potential drives the exit from pluripotency and cell fate commitment via calcium and mTOR

Transitioning from pluripotency to differentiated cell fates is fundamental to both embryonic development and adult tissue homeostasis. Improving our understanding of this transition would facilitate our ability to manipulate pluripotent cells into tissues for therapeutic use. Here, we show that membrane voltage ($V_m$) regulates the exit from pluripotency and the onset of germ layer differentiation in the embryo, a process that affects both gastrulation and left-right patterning. By examining candidate genes of congenital heart disease and heterotaxy, we identify $KCNH6$, a member of the ether-a-go-go class of potassium channels that hyperpolarizes the $V_m$ and thus limits the activation of voltage gated calcium channels, lowering intracellular calcium. In pluripotent embryonic cells, depletion of $kcnh6$ leads to membrane depolarization, elevation of intracellular calcium levels, and the maintenance of a pluripotent state at the expense of differentiation into ectodermal and myogenic lineages. Using high-resolution temporal transcriptome analysis, we identify the gene regulatory networks downstream of membrane depolarization and calcium signaling and discover that inhibition of the mTOR pathway transitions the pluripotent cell to a differentiated fate. By manipulating $V_m$ using a suite of tools, we establish a bioelectric pathway that regulates pluripotency in vertebrates, including human embryonic stem cells.

Action potentials are fundamental to the function of excitable cells, including neurons, cardiomyocytes, and pancreatic cells. They are produced through tightly orchestrated changes in the membrane potential ($V_m$). However, most animal cells, excitable or not, have a resting state $V_m$ (resting membrane potential) that depends on (a) the permeability of the plasma membrane for each ion ($p$ in the Goldman-Hodgkin-Katz (GHK) equation, Fig. 1a, indicating the number of active ion channels), and (b) the driving force for each ion across the plasma membrane, determined by its electrochemical gradient (e.g., $[K]_i$ vs. $[K]_o$ in GHK equation). Although molecules that influence membrane potential have established roles in excitable tissues, their functions in embryonic or adult “non-excitable” tissues are emerging. For example, ion channels and pumps appear to be crucial for the formation of the left-right (LR) body axis. While the vertebrate body plan may appear symmetrical across the LR axis, some of our internal organs, including the heart and gut, require asymmetry across the LR axis for proper

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formation or function. Chemical inhibition or overexpression of ion channels or pumps disrupt the proper alignment of internal organs along the left-right axis and affect global LR patterning. Notably, using voltage sensitive dyes, \( V_m \) appears to vary across the developing embryo suggesting it could play instructive roles. There is now a growing field that has implicated \( V_m \) in various embryonic contexts including Drosophila wing patterning, craniofacial morphogenesis, and chondrogenesis as well as the differentiation of excitable tissues such as muscle cells and neurons. A challenge in the field is connecting changes in \( V_m \) to voltage responsive effectors that lead to the gene expression changes that pattern the embryo.

In order to respond, voltage sensitive effector molecules depend on the magnitude in the change of \( V_m \). Quantitative \( V_m \) measurements in early embryos are rare but were performed in the 1960s and 1970s from 1-cell stage embryos through blastula stages in *Triturus* and *Xenopus* embryos. The blastula embryo has completed a series of rapid cell divisions (cleavages), has established germ-layer cell fates (ectoderm, mesoderm, and endoderm), and is poised to begin gastrulation, the process by which cell movements transform the embryo to acquire the adult body plan. Notably, while the \( V_m \) at early cleavage stages is depolarized (more positive \( V_m \) \( (V_{m,2-cell} = -19 \pm 10 \text{ mV}) \), it becomes progressively hyperpolarized (more negative) towards blastula stages \( (-50 \text{ mV}) \). The implications of this progressive \( V_m \) polarization during early development are unclear, as is a mechanism by which \( V_m \) could transduce a signal within embryonic cells or act complementary to signals transduced biochemically (i.e., ligand-receptor).

Here we show that the \( V_m \) established in the blastula is essential for LR patterning and the exit from pluripotency. Depolarization of \( V_m \) using a variety of approaches leads to loss of ectodermal and paraxial mesodermal cell fates due to a persistence of pluripotency in these tissues. Membrane depolarization leads to the opening of voltage-gated calcium channels elevating intracellular calcium and maintaining pluripotency. Using RNA-seq, we find that mTOR signaling is downstream of \( V_m \) and modulates the transition from pluripotency to differentiated cell fate. These results define an electrochemical signaling pathway that acts complementary to biochemical (ligand-receptor) signaling pathways that transition pluripotent embryonic stem cells to differentiated cell fates.

### Results

\( V_m \) of the blastula regulates LR patterning and gastrulation. To address the question of when \( V_m \) is critical for embryonic development, we employed barium ions to block K+ channels at different time points of embryonic development, since K+ conductance is paramount for determining \( V_m \). Because K+ conductance drives the membrane voltage to a negative (hyperpolarized) potential, blocking K+ channels depolarizes cells. In line with the previous electrophysiological evidence demonstrating that embryos first become polarized at the blastula stage, we found that Barium treatment affected embryonic development primarily when embryos were treated from blastula stages through gastrulation rather than at earlier cleavage stages (Fig. 1b, c, f, g, j, l). Embryonic development was affected in two ways: (1) \( 37 \pm 3 \% \) (SEM) of the embryos failed to complete gastrulation (compared to just \( 4.7 \pm 1 \% \) in control embryos) (Fig. 1b, c, j, k), and (2) \( 23 \pm 2 \% \) that completed gastrulation exhibited misplacement of their organs relative to the left-right axis (compared to just \( 4 \pm 2 \% \) in control embryos; Fig. 1f, g, j, l); these included abnormal heart looping to the left, an L-loop (vs a normal D-loop to the right), inverse gut rotation and misplacement of the gall bladder on the left (vs a normal positioning of the gall bladder on the right side of the body axis) (Fig. 1f, g). Because Barium can affect more than just K+ channels, we tested an alternative strategy for achieving membrane depolarization, namely manipulating \( V_m \) by increasing extracellular potassium ([K+]o in GHK eq. Fig. 1a). Increasing the extracellular potassium reduces the chemical driving force for potassium to leave the cell and decreases the potassium current which depolarizes the embryo (more positive \( V_m \)). Incubating embryos in high K+ at blastula/gastrula stages was sufficient to cause a) gastrulation failure in \( 32 \pm 6 \% \) of embryos (compared to just \( 6 \pm 1 \% \) in controls Fig. 1b, d, k) and b) defective gastrulation (see Fig. 1j).
organ situs at later stages in 28 ± 3% of embryos (compared to just 4 ± 2% in controls; Fig. If, h, l). Thus, our results suggest that establishing proper V_m at blastula stages is essential for both gastrulation and LR patterning, providing context to previous work showing that V_m varies during embryonic development by becoming steadily more polarized from egg to blastula.

**KCNH6 is essential for LR patterning and gastrulation**

Recent studies in patients with congenital heart disease identified a number of variants in KCNH ether-a-go-go (EAG) potassium channels (Table 1) as candidate disease genes. Many of these patients had heterotaxy, a disorder of LR development that can have a significant impact on the structure and function of the heart that can be life-threatening. While multiple ions can affect membrane potential, the flow of potassium down its electrochemical gradient (K^\text{o} = -K^\text{out}) has the largest impact on V_m because in most cell types cell membranes are most permeable to potassium. Since KCNH6 was the most common family member in a total of five patients with heterotaxy (Table 1), we began our studies by examining the CHD/Htx candidate gene, KCNH6.

In *Xenopus*, we found kcnh6 to be expressed in the prospective ectoderm and dorsal/paraxial mesoderm at gastrulation onset, suggesting that it could play a role during gastrulation (Fig. 2a–h). Additionally, high temporal resolution RNA-seq shows that the increase in kcnh6 expression is temporally and spatially consistent with the onset of gastrulation (Supplementary Fig. 1a). We thus tested a role for kcnh6 in gastrulation and LR defects (Fig. 3a and Supplementary Fig. 1f). Importantly, replacement of Na+ with equimolar choline does not affect the V_m. V_m, rather than KCNH6 per se, is essential for gastrulation. Depletion/inhibition of potassium channels or elevation of extracellular K^+ should lead to membrane depolarization. Thus, we reasoned that the inverse condition, namely hyperpolarizing by lowering extracellular K^+, should rescue kcnh6-depleted embryos. Lowering extracellular K^+ (K_m in GHK eq. Fig. 1a) increases the outward driving force for flow of K^+, provided that other K^+ channels are present. Indeed, lowering extracellular K^+ rescues the gastrulation defect in kcnh6-depleted embryos (CRISPR and MO) (Fig. 3a and Supplementary Fig. 3a). To test the significance of K^+ conductance independently of a specific K^+ channel, we employed valinomycin, a K^+ selective ionophore that inserts itself into the plasma membrane and mimics the ability of a K^+ channel to passively conduct K^+. Treatment of blastula/gastrula embryos (stage 8 to stage 12) with Ergtoxin, a scorpion peptide that specifically acts as a pore blocker of the KCNH channel family, also led to identical gastrulation and LR defects (Fig. 3a and Supplementary Fig. 1f). These results indicate that KCNH channels, and specifically Kcnh6, contribute to gastrulation and LR development, consistent with their identification in patients with Htx/CHD.

**Table 1 | KCNH gene variants identified in patients with CHD**

| Blinded ID | Gene   | Phenotype | Allele type | Class                   | AA change        |
|------------|--------|-----------|-------------|-------------------------|------------------|
| 1-09347    | KCNH1  | CTD/Htx   | LOF het     | splice                  | .                |
| 1-01004    | KCNH1  | CTD/Htx   | LOF het     | frameshift_deletion     | p.G149Fs         |
| 1-05070    | KCNH3  | LVO       | LOF het     | stopgain                | p.R139X          |
| 1-07611    | KCNH3  | CTD       | CmpHet      | misD/misD               | A911V/ S1021P     |
| 1-01856    | KCNH3  | LVO       | CmpHet      | misD misD               | A357T/F542L       |
| 1-05499    | KCNH3  | CTD       | de novo     | misD                    | p.E859D          |
| 1-02696    | KCNH5  | Htx/TGA   | de novo     | misD                    | p.N817S          |
| 1-05146    | KCNH6  | Htx       | LOF het     | stopgain                | p.S858X          |
| 1-07207    | KCNH6  | CTD       | LOF het     | stopgain                | p.E587X          |
| 1-02620    | KCNH6  | other     | LOF het     | stopgain                | p.Q487X          |
| 1-06077    | KCNH6  | Htx       | LOF het     | frameshift_insertion    | p.S671fs         |
| 1-02515    | KCNH6  | Htx       | de novo     | misD                    | p.T274M          |
| 1-01783    | KCNH7  | LVO       | LOF het     | stopgain                | p.Y1162X         |
| 1-12888    | KCNH7  | CTD       | LOF het     | stopgain                | p.E944X          |
| 1-06600    | KCNH8  | Htx/TGA   | LOF het     | frameshift_deletion     | p.D782fs         |
| 1-06579    | KCNH8  | other     | LOF het     | stopgain                | p.E576X          |

CTD constitutive defect, Htx heterotaxy, LVO left ventricular outflow tract obstruction, TGA transposition of the great arteries, LOF loss of function, CmpHet compound heterozygote, misD damaging missense mutation according to previously published criteria.
the normal membrane potential ($V_{m} = -41 \pm 4$ mV) (Supplementary Fig. 3b). Therefore, we tested 1/2 sodium replacement with choline on embryos and assayed for gastrulation which led to a remarkable rescue in $kcnh6$-depleted embryos (Fig. 3a). These data suggest that $V_{m}$, which is determined by the conductance of K+ through Kcnh6 and influenced by other K+ and Na+ channels, is key to gastrulation.

Finally, we sought to measure the change in $V_{m}$ when $kcnh6$ is depleted. Using intracellular electrodes in the animal pole of $kcnh6$ MO vs control MO-injected embryos at gastrulation onset, we recorded a $V_{m}$ of $-24 \pm 1.9$ mV in $kcnh6$ MO vs $-44 \pm 2.1$ mV in control MO embryos (Fig. 3b, c). Thus, $kcnh6$ contributes ~20 mV to the cell’s negative resting potential, and embryos lacking $kcnh6$ are abnormally depolarized compared to their control counterparts.

**Depolarized $V_{m}$ increases calcium levels**

We then asked how $V_{m}$ is transduced into a signal that affects embryonic development. There are a limited number of voltage responsive elements in a cell. We reasoned that depolarization ($V_{m} = -24$ mV) in $kcnh6$-depleted embryos could aberrantly activate voltage-gated Ca2+ channels (VGCCs), which facilitate inward Ca2+ flux. L-type VGCCs are present in the prospective ectoderm and dorsal mesoderm and can induce potent intracellular Ca2+ increases that can alter germ-layer patterning, yet upstream regulators of these calcium channels remain elusive. Interestingly, intracellular Ca2+ is elevated after fertilization and during early cleavage stages but declines as the embryo approaches gastrulation concomitant with the onset of membrane polarization. We argued that, if VGCCs are aberrantly activated due to an abnormally depolarized $V_{m}$, they should be able to detect changes in intracellular Ca2+ levels. To assess this, we microinjected the calcium indicator GCaMP6S mRNA together with mCherry mRNA (to enable ratiometric analysis) into control MO or $kcnh6$ MO embryos and performed calcium imaging in animal cells of early gastrula embryos. Within the animal pole of stage 10 control MO-injected embryos, we observed multiple intracellular calcium increases, signified by a pulse-like appearance of GCaMP6 fluorescence in isolated cells, which then propagated to adjacent cells (Supplementary Movie 1). These increases are well documented in Xenopus stage 8–12 gastrulae, i.e., last a few seconds, in which they spread to adjacent cells and then extinguish, are VGCC dependent and may contribute to neural induction. We confirmed the existence of Ca2+ transients at stage 10 by performing 20 s time lapse recordings, and additionally observed that they are of low intensity and typically do not simultaneously affect more than 16 ± 10% of the total animal pole area (Supplementary Movie 1 and Fig. 3d–f). Interestingly, the same transients were dramatically increased in stage 10 $kcnh6$ MO embryos both in intensity and area (Supplementary Movie 2 and Fig. 3d–f), affecting 71 ± 11% of the animal pole on average, with most embryos displaying simultaneous calcium increases in >90% of the animal pole. Thus, $kcnh6$ contributes to a hyperpolarized $V_{m}$ and is key for suppressing calcium levels at gastrulation onset, a signal that may facilitate correct gastrulation.

**Depolarization affects paraxial mesoderm and ectoderm**

For gastrulation to proceed normally, two steps are critical: first, the germ layers of the blastula embryo (ectoderm, mesoderm, and endoderm) must be patterned correctly and second, the embryo must undergo the cellular rearrangements that drive morphogenesis. Calcium has been previously identified to play a role in morphogenesis cell behaviors during gastrulation. Alternatively, calcium may play a role in patterning the mesoderm that also drives gastrulation cell movements. Patterning precedes morphogenesis, and morphogenesis can fail as a result of abnormal patterning. We, therefore, first examined if patterning is disrupted in $V_{m}$-depolarized embryos via marker
gene expression. Since the mesoderm is critical for gastrulation movements, we began with this germ layer. Markers of the dorsal (gsc, nodal3) and ventral mesoderm (vent2) appeared unaffected in kcnh6-depleted, barium and high K+ depolarized embryos (Supplementary Fig. 4e–i); however, the paraxial mesoderm fates appeared lost as marked by myoD, myf5, and tbxt (brachyury, xbra) (Fig. 4a–d and Supplementary Fig. 4a–d). In fact, absent patterning of paraxial mesoderm by myoD persisted into the Left-Right Organizer (Supplementary Fig. 5), a transient structure formed at the end of gastrulation where cilia driven flow is thought to pattern the LR axis. In the LRO, dand5 (coxo) is normally expressed in the paraxial mesoderm symmetrically until cilia driven flow suppresses dand5 expression on the left. However, consistent with a mispatterning of the paraxial mesoderm in the LRO, dand5 was also absent even before the occurrence of cilia driven flow (Supplementary Fig. 5i, j). A disruption in the LRO is further supported by defective ptx2c expression in the left lateral plate mesoderm at later stages (Supplementary Fig. 1k, l). Therefore, in kcnh6-depleted embryos, the paraxial LRO is mispatterning, and a defect in this tissue can be detected already at the onset of gastrulation.

When certain biochemical signaling factors are depleted, loss of one-cell fate (e.g., paraxial mesoderm) is often concomitant with gain of another cell fate. Since the dorsal or ventral mesoderm appeared unaffected (Supplementary Fig. 4e–j), we considered that the ectoderm or endoderm might be expanded into the mesodermal area of embryos with abnormally depolarized $V_m$. Interestingly, while the endoderm (vegT) and its border to the mesoderm (mixer) seemed unaffected (Fig. 4g–j), ectodermal fates (ectodermin and foxI1a) were lost similar to the paraxial mesoderm (Fig. 4e, f and Supplementary Fig. 4k, l). In fact, depletion of ectodermin (trim33) leads to developmental arrest midway through gastrulation, which corresponds to the arrested phenotype in a portion of depolarized embryos. These results indicate that $V_m$ has an effect on germ-layer differentiation, and specifically paraxial mesoderm and ectoderm, at gastrulation onset (Fig. 4a–j).

**Cacna1c responds to $V_m$ depolarization**

In depolarized embryos, we have established (1) changes in cell fate and (2) elevated intracellular calcium levels, so we next tested if these aberrant cell fates are dependent on voltage-gated calcium channels. To determine the specific embryonic VGCCs downstream of $V_m$, we reviewed our available high temporal resolution RNA-Seq data. *Xenopus* contains detectable transcripts of L- and T-type VGCCs between the 1-cell and gastrula stages, while other VGCC types (N-, R-, and P/Q) are less abundant. L-type VGCCs become activated at $V_m > -40$ mV (and then inactivated at $V_m > 10$ mV) and have been previously implicated in gastrula patterning, while T-type channels become inactivated at $V_m > -60$ mV and would be inactive both at physiological $V_m$ (−50 mV) and at more depolarized potentials. Therefore, we tested the L-type VGCC blocker nifedipine. This significantly ameliorated both ectodermin (ectoderm) and myf5 (paraxial mesoderm) expression losses in kcnh6 knockdown embryos (Fig. 5a–d). Specifically, myf5 was lost only in 16 ± 1% and 18 ± 5% of nifedipine-treated kcnh6 CR and MO embryos (vs 38 ± 5% and 35 ± 5% in kcnh6CR or MO embryos treated only with DMSO, Fig. 5b, d). Similarly, absent ectodermin was only observed in 16 ± 7% and 17 ± 3% of nifedipine-treated kcnh6 CR and MO embryos (vs 48 ± 2% and 51 ± 6% in kcnh6 CR or MO embryos treated with DMSO, Fig. 5a, c). Of the VGCCs identified in our RNA-seq data at blastula/gastrula stages, two genes cacna1c (Cav1.2, L-type) and cacna1d (Cav3.1, T-type) encode alpha (pore-forming) channel subunits, which are indispensable for channel function. Co-depletion of cacna1c in kcnh6-depleted embryos rescued expression of ectodermin and myf5, while co-depleting cacna1d (Fig. 5a–d) resulted in no rescue. Thus, Kcnh6, which sets a negative $V_m$, is essential to limit the
activation of L-type VGCCs and specifically Cacna1c, a critical step for ectodermal and paraxial mesodermal differentiation.

$V_m$ depolarization maintains pluripotency in gastrula embryos
The loss of some cell fates (ectoderm and paraxial mesoderm) without a concomitant expansion of other cell fates was puzzling given that most biochemical signaling factors (Wnt, BMP, Nodal) generally balance different cell fates in the early embryo. We speculated that these unspecified cells may simply lack the ability to assume any cell fate because they remain pluripotent abnormally. To test this hypothesis, we examined markers of pluripotency $\text{OCT4}$, $\text{NANOG}$, and $\text{SOX2}$. In $\text{Xenopus}$, there are three $\text{OCT4}$ homologs (pou5f3.1, 2, and 3, formerly oct91, oct25, and oct60)40, and the ventx1.2/2.2 factors, which have overlapping functions in maintaining differentiation competence and are thought to be structurally and functionally equivalent to mammalian Nanog41. Sox2, a core pluripotency factor in mammals, is highly...
they are pluripotent (Fig. 4o), an assay used for decades to test the activity of a host of differentiation factors. However, towards the end of gastrulation at stage 12, these animal cap cells are no longer pluripotent and when explanted will only differentiate into epidermis, even when stimulated with activin\(^{34,44}\) (Fig. 4o). Using this animal cap assay, we sought to test the role of \(kcnh6\) in determining pluripotency. We explanted stage 9 and stage 12 animal caps and assayed differentiation under three conditions: (1) no activin to examine spontaneous differentiation into epidermis (marked by cytokeratin), (2) low activin to stimulate differentiation into mesoderm (tbx5), and (3) high activin to stimulate differentiation into endoderm (sox17\(^{b}\)). Both control and \(kcnh6\) CR animal caps explanted from stage 9 embryos were capable of differentiating into all three germ layers, indicating full differentiation potential even when \(kcnh6\) is depleted (Fig. 4o). On the other hand, as expected, animal caps explanted from stage 12 control embryos differentiated into epidermal fate but not into meso- or endoderm despite activin administration (Fig. 4o). Strikingly, stage 12 animal caps explanted concurrently from \(kcnh6\)-depleted embryos were able to differentiate into cell fates of all three germ layers with activin administration (Fig. 4o). From these and the previous experiments, we conclude that V\(_m\) polarization via \(kcnh6\) enables the exit from pluripotency.

\textbf{V\(_m\) limits mTOR to allow for exit from pluripotency}

Our findings indicate that a polarized V\(_m\) limits voltage-gated calcium channels and intracellular calcium, a process that reduces the expression of pluripotency genes as germ-layer differentiation initiates. A critical question is what are the signaling pathways invoked when V\(_m\) is depolarized or intracellular calcium is elevated. To address this question in an unbiased manner, we temporally profiled gene expression via RNA-Seq in control and high K\(^+\) depolarized embryos by collecting embryos every 30 min from pre- to post-gastrula stages (stages 8–12; Supplementary Fig. 7a–d). We identified genes exhibiting temporal differential expression employing a Gaussian Process framework\(^{5}\); this determines genes whose expression trajectory differs between control and high K\(^+\) embryos over the time course. We found 4043 genes on average activated in high K\(^+\) over the time course, and 1101 genes on average repressed (Supplementary Fig. 7e). We further used k-means clustering to subdivide these into 8 clusters, 4 activated (A1–4, Fig. 6a, b) and 4 repressed (R1–4, Supplementary Fig. 7f, g). In both cases, the clustering segregated genes showing dysregulated gene expression prior to gastrulation (Clusters 1, 2) and during gastrulation (Clusters 3, 4) (Fig. 6a, Supplementary Fig. 7f, g). To assess the composition of these clusters, we performed gene set enrichment using Enrichr\(^{57}\). Comparing activated clusters to repressed clusters over seven different annotated gene set libraries, we found 1279 terms significantly associated with at least one of the 4 activated clusters, but only 44 terms significantly associated with repressed clusters. Therefore, given the total number of genes and associated terms, we focused our attention on the analysis of the activated genes.

We found a hierarchy of gene set enrichments from early to late in our time course, reflecting the changing response in the transcriptome (Fig. 6c). Notably, we found enrichment for the mTOR signaling pathway activated early with members including \(rictor\), \(depdc5\), \(pik3cb\), \(stk11\), arg13 in cluster A1 (Fig. 6e) and \(akt1s\), \(gsk3b\), \(lamtor1\) in cluster A2. The enrichment for mTOR members continues to span all activated clusters (clusters over seven different annotated gene set libraries, we found 1279 terms significantly associated with at least one of the 4 activated clusters). This enrichment is accompanied by pathways associated with mTOR, including autophagy, ubiquitin transferase activity, and ER response (Fig. 6c). The intermediate clusters A2 and A3 show the most prominent ER response enrichment together with significant upregulation of spliceosome machinery (including 12 snRNPs and 5 SRSF family members). In contrast, in later clusters, we find enrichments for terms explaining the sustained pluripotency and germ-layer defects, including pluripotency (\(pouf3.1, pouf3.2, ventx1.2\) and WNT signaling (\(fzd7,\)

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**Fig. 5** | The role of VGCCs in germ layer differentiation. a WMISH for ectodermin: lateral views with the animal pole to the top; asterisk marks the animal pole with arrowhead marks loss of expression; embryos with abnormal expression are quantified in c; CR CRISPR, Nfd nifedipine. b WMISH for myf5: vegetal views with dorsal to the top; myf5 expression; embryos with absent expression are quantified in d. c, d Percentages of embryos with abnormal ectoderm expression (c) and myf5 (d) expression. Graphs depict mean ± SEM; p-values are in c: (kcnh6 MO + DMSO vs DMSO) = 2.88e-006, (kcnh6 MO + Nfd vs kcnh6 MO + DMSO) = 4.59e-002, (kcnh6 MO + cacna1c CR vs kcnh6 MO) = 1.53e-002, (kcnh6 MO + cacna1c CR vs kcnh6 MO) = 9.99e-001 (ns), (kcnh6 CR vs DMSO) = 3.50e-007, (kcnh6 CR vs DMSO) = 1.20e-002; in d: p-values are (kcnh6 MO + DMSO vs DMSO) = 4.07e-013, (kcnh6 MO + Nfd vs kcnh6 MO + DMSO) = 8.92e-005, (kcnh6 MO + Nfd vs kcnh6 MO + DMSO) = 9.78e-005, (kcnh6 MO + cacna1c CR vs kcnh6 MO) = 2.24e-001 (ns), (kcnh6 CR vs DMSO) = 2.04e-014, (kcnh6 CR vs Nfd) = 1.45e-003; two-sided Fisher’s exact test; total embryo numbers (%) in the graphs are from at least 2 independent experiments; Key for asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, ns non-significant for p > 0.05. Source data are provided as a Source Data file.

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conserved in amphibians and also expressed at high levels prior to lineage commitment throughout the \textit{Xenopus} blastula\(^{34,45}\). We examined the prospective ectoderm of embryos, which is best characterized in its pluripotent properties, and confirmed that \(pouf3.1, pouf3.3, sox2\), and \(ventx1.2\) are robustly expressed at stage 9 prior to lineage commitment, but their transcripts are sharply reduced by stage 10 in control embryos (Fig. 4k–n). In contrast, \(kcnh6\) CR embryos retain robust expression of these factors well beyond stage 9 and into stage 10, a prolonged expression compared to wildtype embryos (Fig. 4k–n). This, in turn, is not due to a general delay in development, since \(kcnh6\) CR embryos were staged according to the physical progression of gastrulation, i.e. presence of blastopore lip. Moreover, in \(kcnh6\)-depleted late gastrula embryos, abnormal maintenance of \(pouf3.3\) and \(ventx1.2\) can be abolished by incubating the embryos in L-type VGCC blocker nifedipine (Supplementary Fig. 6b–d). These results suggest that \(kcnh6\) is upstream of V\(_m\), and VGCCs in promoting the exit from pluripotency, which takes place as gastrulation proceeds.

Based on this result, we sought to test the pluripotency of these \(kcnh6\)-depleted embryos. In the blastula (stage 9), the prospective ectoderm or “animal cap” contains cells that when explanted will differentiate into epidermis (Fig. 4o). Importantly, when stage 8–9 explanted animal cap cells are treated with activin, they can be differentiated into mesodermal and endodermal cell fates indicating that
in proximity to one of ETS1, CREB1, and CREM (FDR < 10^{-6})
remarkable enrichment accounts for 794/1520 (52.2%) of genes found
sites (Supplementary Fig. 7j); in the case of activated cluster A2 this
found in proximity to publicly available ETS1, CREB1 and CREM binding
intersection between activated gene clusters A1 and A2 and genes
elements are bound by calcium responsive family members, CREB1,
Supportive of these factors driving gene expression, we
respectively, Fisher Exact Test). Therefore, we
fi
layer resolution and this repression is removed in high Ca^{2+} embryos.
K+ conditions (Supplementary Fig. 7i). This suggests that the activity of
high levels at stage 8, and then are gradually downregulated to a

motifs in 500 bp upstream of cluster promoters, see also Supplementary Fig. 7g.
Bubble size reflects fold change over background and color is ~log10 Hypergeo-
metric right tail p-value for enrichment. e Expression of exemplar genes in control
and high K+. Central line and shaded region are transformed Gaussian process
median and 95% CI. Circle in top right hand corner gives cluster number. UC
(untreated control), High K+ (depolarizing conditions). Data analysis performed
from N = 13 samples/each 10 embryos over one biological replicate time course.

unt8a, tcf7l1) (Fig. 6c, e). Key members of the Xenopus pluripotency
network are also found activated early, in cluster A1 including foxh1,
sox3, and pouf3.3 (Fig. 6c, e).

To build the underlying gene regulatory networks, we examined transcription factor motif enrichment in the promoters of each of
these activated gene clusters. Mirroring the gene set enrichments, we
found motif enrichments segregated between early (A1,2) and later
(A3,4) gene clusters (Fig. 6d, Supplementary Fig. 7h). We find strong
enrichment for SP1, ETS, YY1, RFX, and CRE/ATF1 motifs driving gene
expression changes in clusters A1 and A2. Interestingly, both ETS and
CRE motifs are bound by factors responsive to calcium. Calcium
induced phosphorylation of ETS1 inhibits binding activity46, and CRE
motifs are bound by factors responsive to calcium. Calcium

pluripotency TFs whose transcripts are activated in cluster A1 (Fig. 6e).
In our data, each of these factors is expressed at high levels at stage 8, and then are gradually downregulated to a
minimum at stage 12. Of note, their mRNAs are not upregulated in high
K+ conditions (Supplementary Fig. 7i). This suggests that the activity of
these factors is post-translationally modified in depolarized embryos
experiencing high Ca^{2+} to drive gene expression changes. In the case of
ETS1, this factor may act to repress gene expression in normal germ-
layer resolution and this repression is removed in high Ca^{2+} embryos.
Supportive of these factors driving gene expression, we find a large
intersection between activated gene clusters A1 and A2 and genes
found in proximity to publicly available ETS1, CREB1 and CREM binding
sites (Supplementary Fig. 7j); in the case of activated cluster A2 this
remarkable enrichment accounts for 794/1520 (52.2%) of genes found
in proximity to one of ETS1, CREB1, and CREM (FDR < 10^{-6}, 10^{-3}, 10^{-5},
respectively, Fisher Exact Test). Therefore, we find that initial
transcriptional responses captured by activated gene clusters A1 and
A2 appear to be largely driven directly by calcium response. This
includes the activation of mTOR and pluripotency genes in high K+
conditions; mTOR genes show an enrichment in CRE sites in their
promoters across all clusters (p < 0.0072, Odds Ratio 2.46, Fisher’s Exact Test), pluripotency genes show enrichment for CRE sites in
cluster A1 and A2 (p < 0.017, Odds Ratio 3.08, Fisher’s Exact Test), and
ETS sites in cluster A2 (p < 0.021, Odds Ratio 5.15, Fisher’s Exact Test).

Turning to the genes activated later, particularly, those associated
with pluripotency and germ-layer commitment in cluster A4, we find
comprehensive enrichment of FOXH1, SOX and POU motifs in their
promoters (Fig. 6d). These motifs correspond precisely with the early
pluripotency TFs whose transcripts are activated in cluster A1 (Fig. 6e).
Together, our high-resolution temporal profiling of the transcriptome
in control and high K+ conditions reveals a cascade of transcriptional
activation. We propose a model where a depolarized membrane opens
VGCCs and elevates intracellular calcium leading to the expression of
transcripts (including mTOR and pluripotency factors) whose pro-
motors are enriched with calcium responsive motifs. This is followed
by the activation of transcripts involved in pluripotency and germ-
layer commitment, driven by the pluripotency factors activated in
the early wave of gene expression.

Our transcriptome analysis not only revealed a potential gene
regulatory network but pointed towards a role for mTOR. mTOR is
critical for multiple cellular processes including autophagy, nutrient
sensing, and an emerging role in pluripotency26,36. Because the
expression of mTOR pathway members was increased in depolarizing
conditions and pathways associated with mTOR, we reasoned that
mTOR signaling was upregulated and maintained pluripotency in these depolarized embryos. To test this hypothesis, we applied the mTORC1 inhibitor, rapamycin, to depolarized gastrulating embryos to see if this could abolish the aberrant expression of pluripotency markers pou5f3.3 and ventx1.2 in the animal pole and activate germ-layer differentiation. Rapamycin dramatically lowered expression of pou5f3.3 and ventx1.2 in the animal pole in kcnh6 CR and high K+ treated embryos compared to those embryos treated with vehicle alone and appeared comparable to untreated control embryos (Fig. 7a, b, d, e). Conversely, the expression of the ectodermal marker, ectodermin, which was reduced in depolarizing conditions (kcnh6 depletion or exposure to high K+), was recovered with rapamycin treatment (Fig. 7c, f). Therefore, a polarized Vm at gastrulation onset is critical for limiting mTOR in order to suppress pluripotency genes and enter differentiation.

Finally, we tested whether our findings would also apply to human embryonic stem cells (hESCs). At stage 9, Xenopus animal cap cells are pluripotent in that they can, under appropriate conditions, form derivatives of any of the three germ layers (Fig. 4o). However, they contrast with hESCs in their limited capacity for self-renewal as the brisk pace of embryonic development proceeds. Therefore, we turned our attention to hESCs to test our findings in the context of a self-renewing pluripotent state and to determine their relevance to human development. hESCs are already highly pluripotent, and we wondered if depolarization would lead to elevations in the pluripotency markers OCT4 and SOX2. Indeed, hESCs grown for two days with Ergtoxin to specifically block KCNH channels showed a modest but significant elevation of these markers over their already high levels in the pluripotency state as indicated by immunostaining and qPCR against these markers (Fig. 8a, b, Supplementary Fig. 8a, b). While not as specific as Ergtoxin for KCNH channels, Barium showed similar trends but did not rise to statistical significance. qPCR for the markers OCT4, SOX2, and NANOG also revealed upregulation of these genes at the transcript level, with OCT4 and SOX2 upregulated by day 2 and all three genes upregulated on day 5 (Supplementary Fig. 8a, b).

We also tested whether blocking K+ channels with Ergtoxin affected the kinetics of differentiation. BMP4 induces differentiation to either mesodermal or extraembryonic fates in a dose-dependent manner57–59. Ergtoxin caused a significant delay in downregulation of pluripotency markers such as SOX2 at 12 h with a similar trend in NANOG (Supplementary Fig. 8c, d). As in Xenopus, the timing of differentiation of human embryonic stem cells appears significantly affected under depolarizing conditions.

To test whether the role of mTOR signaling downstream of membrane depolarization is conserved, we treated hESCs with rapamycin with or without Ergtoxin. Treatment with rapamycin led to...
regulation of membrane potential via KCNH channels promotes the exit from pluripotency and the activation of differentiated cell fates in hESCs and human cells. Although rapamycin did reduce the final cell number in the well (Supplementary Fig. 8f), taken together, our data support that the polarization of membrane potential via KCNH channels by Vm is critical for the exit from pluripotency factors, which is consistent with the notion that, because K+ is so abundant inside the cell, it is unlikely to act as a signaling moiety, unlike Ca2+.

Discussion
We propose a model in which membrane voltage regulates intracellular calcium during a critical stage of embryonic development, at which point cells need to extinguish pluripotency factors in order to activate a program of cellular differentiation (Fig. 8e). The K+ and Ca2+ channel network upstream of pluripotency factor expression potentially represents an extremely robust control mechanism over the first stages of organism development: ion channels are effective at low expression levels, modular and thus partially redundant with respect to each other (i.e., subunits of different channels can heterodimerize to form a channel if subunits of the same channel are unavailable), and their function depends on the existence of a simple ion concentration gradient across the plasma membrane. The basic elements of this regulation, K+ and Ca2+, are readily available extracellularly, conferring this system with some independence from protein-dependent cell signaling. Ultimately, ion channel networks may represent an additional mechanism to regulate cell fate during development, which is complementary to the established paradigm of gene expression regulation by secreted factors and ligand-receptor signaling (Fig. 8e).

Our work and the work by others supports the notion that membrane potential modulates development in a variety of contexts including LR patterning, chondrocyte differentiation in chick limbs as well as morphogenesis of the developing Drosophila wing. Additionally, our work adds to the mechanical mechanisms that have associated membrane potential with stem cells differentiation. Of note, previous work has suggested that K+ itself may have signaling properties that affect the directed differentiation of hESCs. However, our study indicates that it is Vm and not K+ itself that is critical. This is consistent with the notion that, because K+ is so abundant inside the cell, it is unlikely to act as a signaling moiety, unlike Ca2+.

While elevations in intracellular calcium do have physiological functions in the prospective mesoderm and ectoderm of the gastrula, both in patterning and morphogenesis, we show that regulation of voltage-gated calcium channels by Vm is critical for the exit from pluripotency. Based on this, we suggest that low intracellular calcium reduces the expression of mTOR and pluripotency factors, which is conductive to differentiation onset. While we have not eliminated other voltage responsive signaling, our results indicate that calcium is critical in this context.

Regulation by Vm and Ca2+ presents interesting implications for developmental timing, as they appear to be key in specifying the exact time frame of pluripotency exit. Indeed, previous work has also implicated mTOR in developmental timing of the pluripotent state.
and our work demonstrates the upstream regulation of mTOR in this context. The requirement of \( V_m \) for this developmental period is also underscored by measurements in developing Xenopus and Triturus showing a clear hyperpolarization of \( V_m \) towards the onset of germ-layer differentiation that is concurrent with a reduction in calcium levels. Therefore, it may be that membrane polarization may act as a timing mechanism to allow the embryonic cells to be synchronized before the onset of differentiation. To do so, the embryo employs ion channels, mTOR, and an array of pluripotency transcription factors. Then secreted factors can act on these pluripotent cells to activate different programs of differentiation across the embryo (Fig. 5e). Of note, tissues with abnormal ion homeostasis at gastrulation onset do show differentiation potential and may eventually differentiate at later stages, but the significant delay in exiting pluripotency dramatically affects gastrulation morphogenesis itself as well as LR patterning.

Importantly, genetic data from Htx/CHD patients identified the KCNH family and especially KCNH6. Recent work has identified ion channels as an interesting intersection between CHD and autism. Our work demonstrates the importance of \( V_m \) in vivo during early embryonic development as well as in vitro in human stem cells.

Inference of CRISPR edits (ICE) analysis

Genomic DNA from CRISPR and control embryos were obtained by lysing individual, stage 45 tadpoles in 50 mM NaOH as previously described and amplifying PCR fragments around the CRISPR target site that encompass ~200 bp upstream and 500 bp downstream of the site. The following primers were used for CRISPRs targeting exons 3 and 4 of the \( kcnh6 \) locus, respectively: CRex3-F: 5’-CATGGTATAAGAGCAAGCCG3’ and CRex4-R: 5’-GCTTATCCATTGTGACCAAGCCG3’. PCR fragments were Sanger sequenced using the same forward primers, and sequencing traces were uploaded for analysis with the Synthegene ICE analysis web tool to assess editing outcomes.

Organ situs

Stage 45 Xenopus embryos were paralysed with benzocaine or tricaine and scored with a light stereomicroscope. Cardiac looping was determined by position of the outflow tract; D-loop: rightward, L-loop: leftward; A-loop: midline. Normal intestinal looping was scored as counter-clockwise rotation of the gut, while abnormal intestinal looping was scored as completely inverse gut rotation (clockwise) or complete lack of looping (unlooped). While a completely inverted gut rotation is clearly an abnormality of LR patterning, an unlooped gut is less clear so we only considered an unlooped gut as abnormal situs when combined with abnormal placement (left-sided or midline) of the gall bladder. To quantify total abnormal organ situs, each tadpole was counted only once, regardless of whether multiple organs were affected.

Whole mount in situ hybridization

Digoxigenin-labeled antisense probes for pitx2 (TNeu083k20), dand5/coco (Teggo07d24), myoD (Tneu017h11), myf5 (Tgas127b01), tbx1 (Tneu024f07), foxJ1 (Tneu058m03), ectoderin (Tneu041j16), foxI1a (Tgas002h16), mixer (Tgas050b05), vegaT (Tgas066f22), gsc (Tneu077f20), xnr3 (Tgas011k89), vent2 (BG885317), oct25 (Tgas051h05), oct60 (IMAGE: 7526158), oct7 (IMAGE: 757764), vent1 (BG487195), sox2 (Tgas061h22), cytokeratin (IMAGE: 6991625), and sox17b (BG886038) were in vitro transcribed using T7 High Yield RNA Synthesis Kit (E2040S) from New England Biolabs. In order to generate a full-length antisense probe for X. tropicalis kcnh6, kcnh6 cDNA was cloned from stage 45 tadpole whole mRNA using primers xtkcnh6-F: 5’- ATGTTTATCCTAAACTTCGAGGACC-3’ and xtkcnh6-R: 5’-CTACTCTTCTGGAAGACCTGGG-3’ (XM_012952904.1). We note that kcnh6 had been misannotated as kcnh2 in the v7.1 model of the X. tropicalis genome (We used NCBI Annotation XP_012800383.2 to identify KCNH6). Embryos were collected at the desired stages, fixed in MEMFA for 1–2 h at room temperature (RT) and dehydrated in 100% ethanol. GRPs were dissected post fixation and prior to dehydration to detect dand5. To detect putative gene expression in the prospective endoderm (mixer, vegaT, kcnh6) gastrula stage embryos were bisected to facilitate better probe access. Briefly, whole mount in situ hybridization of digoxigenin-labeled antisense probes was performed overnight, the labeled embryos were then washed, incubated with anti-digoxigenin-AP Fab fragments (Roche 11093274910), and signal was detected using BM-purple (Roche 11442074001), as previously described in detail.
Medium conditions and treatments of embryos
Normal embryonic medium is 1/9x modified Ringer’s (MR) containing 11 mM NaCl, 0.2 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgCl₂ and 0.55 mM HEPES. To allow Ergtoxin to penetrate the embryos, we manually removed the vitelline envelope of stage 8 embryos and incubated embryos in 1/9x MR containing 50 nM Ergtoxin (Alomone STE-450) until stage 12. Embryos were then transferred back into 1/9x MR lacking Ergtoxin to develop until stage 45 in order to score organ situs. Barium chloride was applied into the medium at 20 mM and embryos were thoroughly rinsed in 1/9x MR after each incubation period for further development in Ba²⁺-free medium. For extracellular K⁺ manipulations, the KCi concentration in 1/9x MR was modified from 0.2 mM (normal) to 20 mM (high) or 0 mM (low). The ionophore valinomycin (ACROS) and L-type VGCC blocker Nifedipine (ACROS) were diluted in DMSo as stock solutions and applied to embryos in 1/9x MR at 2 and 10 μM, respectively. Treatments performed during gastrulation were applied from stage 8 through stage 12, and embryos were then rinsed thoroughly and returned into 1/9x MR. For rapamycin, we created a μM DMSO as stock solutions and applied to embryos in 1/9x MR at 2 and 10 μM. For calcium imaging, we treated embryos, stage 8

Intracellular recordings
For recordings, devitellinized, stage 10 kcnh6 or control MO-injected embryos were mounted into non-toxic clay with their animal pole exposed and covered with 1/9x MR. To investigate the resting potential, animal pole cells were impaled with a high-impedance (~70 MΩ). These recordings were made using a HEKA EPC10 ampliﬁer. We used HEKA PatchMaster v2x67 software for Windows. All electrodes were zeroed just before entry into the cells.

Calcium imaging
GCaMP6 and mCherry mRNAs were mixed and injected into embryos at the one-cell stage. Half of these embryos were then injected with kcnh6 MO, and the other half with control MO, still at the one-cell stage. Embryos were transferred at stage 10 into the round wells of a press-to-seal silicone isolator (Sigma S3685) mounted between two cover slips in 2% Methylcellulose-1/9x MR. GCaMP6 and mCherry fluorescence was then captured for 20 s (1 frame per second) via time lapse in the whole embryo of the same batch, then fixed at stage 17 in 4% paraformaldehyde-PBS for 2 h monitored in whole embryos of the same batch), then permeabilized for 30 min at RT using 0.5% Triton-PBS (PBST), then blocked in 1% BSA-PBST for 1 h at RT and incubated in primary antibodies diluted in 1% BSA-PBST overnight at 4 °C (anti-myoD 1:100; LsBio C143580-100 or anti-acetylated tubulin 1:2000 Sigma T-7939). Primary antibodies were then washed in PBST for 5 min and then incubated with secondary antibodies in 1% BSA-PBST for 1 h at RT. Phalloidin (1:50; Molecular Probes) and Hoechst 33342 (1:1000; Molecular Probes) were diluted into the secondary antibody solution. Images were acquired using a ZEISS 710 laser scanning confocal microscope.

For the series of intracellular recordings in high K⁺ and choline treated embryos, stage 8-9 embryos were impaled similarly with an electrode of ~40 MΩ. These recordings were made using an Axon 200B amplifier and digitized using a Digidata 1320 digitizer. Jclamp software for Windows was used in current clamp mode. All electrodes were zeroed just before entry into the cells.

Quantification RNA-seq
Stranded pair-end 100 bp RNA-seq reads were aligned to the Xt9.1 genome combined with ERCC spikes using STAR70 and quantified as transcripts per million (TPM) for each isoform with RSEM71 using the log₂(1 + tpm/1000000). We leveraged knowledge of spike-in concentrations to build a linear model to correct the TPM clicking peaks (in area), and GcAMP6 fluorescence, varying in intensity and spreading to multiple cells. The frames of each recording were sorted to identify the calcium transient peak (in area), and GCaMP6 fluorescence intensity was quantified at peak as a ratio to mCherry in mCherry− cells. The maximum Ca²⁺ transient area was calculated by demarcating in Fiji the GCaMP6(+)/ GCaMP6(−) area of the animal pole at transient peak. To avoid mosaic artifacts, only embryos with even, non-mosaic mCherry expression across the entire animal pole were considered. To avoid embryonic stage dependent fluctuations in Ca²⁺ transient size, we verified each embryo for stage by progression of blastopore closure and alternated recordings of control and kcnh6 MO embryos. Of note, there were no notable differences in mCherry expression between Control MO and kcnh6 MO embryos.

Animal cap pluripotency assays
After manually removing the vitelline envelope of stage 9 or 12 embryos, animal caps were excised and placed on agarose coated dishes in 1/9x MR solution. Caps were then directly placed into agarose coated wells of a 96-well plate in 1/3x MR containing 0.1% BSA and cultured without activin to allow for differentiation into epidermis, with low (20 ng/ml) activin to induce mesoderm, or high activin (200 ng/ml) to induce endoderm, as previously described72. Explants were raised at 25 °C until reaching the equivalent of stage 18 (monitoring in whole embryos of the same batch), then fixed in 4% paraformaldehyde, washed to eliminate pigmentation (0.5% SSC, 5% formamide, 1.2% H₂O₂), and then processed by in situ hybridization as described above.

RNA-seq
For RNA-Seq, embryos were kept at 25 °C either in 1/9x MR or in 10 mM KCl solution, and 10 embryos were harvested per time point and condition every 30 min starting at stage 8 and concluding at stage 13. Samples were immediately frozen and kept at ~80 °C until homogenized in 100 μl Trizol spiked with ERCC RNA Spike-In Mix. 10 μl ERCC RNA Spike-In Mix (Thermo Fisher Scientific) were first diluted into a final volume of 870 μl DEPC water and then further diluted 1:10 into Trizol, which was used to homogenize the samples. Total RNA was purified from the embryo Trizol homogenates according to the manufacturer’s recommendations. After isopropanol precipitation, RNAs were resuspended in DEPC water and any contaminating genomic DNA was removed by overnight precipitation in 5 M LiCl at 4 °C. RNA was subsequently pelleted and washed twice with 70% ethanol. All RNAs were resuspended in DEPC water (2 μl/embryo), and finally, RNA quality was verified by Bioanalyzer. All libraries were sequenced with 100 bp paired-ends on an Illumina NovaSeq6000.
We use the GLM.jl (https://github.com/JuliaStats/GLM.jl) in the Julia language to apply this model and add a pseudocount of 2 to all dinucleotide frequencies. As the GC effect varies between UC and high K+ samples (Supplementary Fig. 7b), we apply the correction independently to UC and High K+. The correction is able to explain a significant proportion of variance in spike TPM, increasing $R^2$ from 0.807 and 0.733 to 0.965 and 0.964 respectively for UC and high K+ samples. We apply this correction to each isoform of all genes quantified with the dinucleotide propensities of each isoform and RSEM isoform quantifications. We then sum all corrected quantifications at the isoform level to derive gene level quantifications. This allows us to account for differing isoforms of the same gene with differing dinucleotide propensities.

**Filtering of genes for differential expression analysis**

We first filtered 34,192 quantified genes to find those with sufficient temporal expression for further analysis, we selected genes that had runs of 6 consecutive samples with uncorrected TPM > 0.4. This resulted in 13,310 from which we excluded a further 162 genes which were excessively altered by the above described correction procedure, these had log2 fold changes between corrected or uncorrected quantifications outside of the interval (-2.5, 4.5). After dinucleotide correction and filtering we found excellent concordance between samples, with minimal evidence of outlying samples, by Spearman Correlation comparisons and principal components analysis (PCA) (Supplementary Fig. 7c, d). The two domains in visible in pairwise Spearman comparisons (Supplementary Fig. 7c) reflect the loss of maternal RNA and the commencement of widespread zygotenic transcription as we previously described14. Projection onto the first two principal components revealed that samples lie in appropriate order on a trajectory in 2D space, and the largest divergences between UC and high K+ occur midway through the time series in agreement with Gaussian process differential expression and clustering described below. Corrected dinucleotide gene expression abundances are used in all analyses.

**Temporal differentiation expression**

To determine genes temporally differentially expressed we used Gaussian process (GP) regression as we have previously applied15. All GP regression was performed with GaussianProcesses.jl (https://github.com/STOR-i/GaussianProcesses.jl; https://arxiv.org/abs/1812.09064). Due to the overdispersed nature of RNA-seq count data, we apply a variance stabilizing transform that puts all genes on the same scale: $y_{ni} = \frac{\sqrt{\alpha + \beta x_{ni}}}{m_n}$, with $x_{ni}$ the dinucleotide corrected abundance of gene $i$ in sample $s$, $m_n$ the maximum $x_{ni}$ over all samples, and $\alpha = 1$, $\beta = 1000$. We then perform exact GP regression (GP prior and a Gaussian likelihood) with Matern22 kernel, we optimize the three associated hyperparameters: $\sigma_\epsilon^2$ the signal variance, $\tau$ the timescale (using previous terminology16), this parameter is commonly referred to as the lengthscale $b$, and $\sigma_n^2$ the sample noise variance. Parameters are selected by optimizing marginal log-likelihood with parameters in log space: $\log_\beta \log_\sigma\log_\sigma\log_\tau \log_2$, and to ensure physiologically reasonable values for each we place Gaussian priors, $\mu(\sigma, \nu)$ over each of these variables respectively / (1,4,4,0), / (1,2,1,0), / (1,0,0,75). Finally, we reported GP median and 95% confidence intervals through our inverted data transformation $x_{ni} = m_n \left( y_{ni} - \alpha \right) / \beta$ and set $x_{ni} = 0$ for $y_{ni} < \alpha/\beta$.

To determine temporal differential expression, we calculate a marginal likelihood ratio for whether we prefer separate GP models for UC and high K+ or a single GP model for all data combined. If $L_{\text{UC}}$ and $L_{\text{HK}}$ are the marginal log-likelihoods for UC and high K+ respectively, and $L_{\text{UK}}$ is the marginal log-likelihood for a single regression through UC and high K+ together. Then we calculate log-likelihood ratio $LR = L_{\text{UC}} + L_{\text{HK}} - L_{\text{UK}}$ of evidence in favor of two models (essentially that the UC and High K+ have different expression trajectories for a given gene) and determine genes with $LR > 0$ as temporally differentially expressed. This resulted in 5144 differentially expressed genes, with 4043 activated and 1101 repressed (Supplementary Fig. 7d which shows that the max absolute divergence z-score between UC and high K+ trajectories increases with LR). We also considered a more stringent condition for differential expression using the Bayesian Information Criterion (BIC)17, that resulted in 2388 differentially genes. We found that this diminished differential expression gene set enrichments described below, indicating that the BIC was too conservative and we continued with our condition based on log-likelihood ratio.

**Clustering**

To determine sets of differentially expressed genes with similar trajectories, we applied K-means clustering to activated and repressed genes independently. We define a gene as activated if Gaussian process median for High K+ exceeds UC on average, and repressed if it does not, we found no genes for which the mean of High+ and UC differences was zero. We cluster UC and High K+ genes by taking Gaussian process medians and normalizing by the maximum value experience by UC or High K+. We then cluster both trajectories together employing the kmeans function offered by Clustering.jl (https://github.com/JuliaStats/Clustering.jl) with default settings and random seed 16. To select the cluster number, we calculated the silhouette score for activated and repressed clusters for $k = 2$–10. We found that the maximal mean silhouette score activated genes was $k = 3$ and for repressed genes was $k = 2$, but that scores were broadly similar for $k = 2$–4 and decreased significantly for $k > 4$, suggesting that $k = 4$ provides a reasonable partition of the data. In line with this we explored the clusters from $k = 2$–10, and found that key clusters were not well-resolved for $k < 4$ and that $k > 4$ clusters refined $k = 4$ behaviors. As $k > 4$ did not reveal new behaviors and did not improve gene set enrichments, we selected $k = 4$ to cluster activated and repressed genes.

**Gene set enrichments**

To assess the composition of each cluster we performed gene set enrichments using Enrichr45. We took genes from each cluster with a known Xenopus gene symbol and converted these to human symbols, by removing any “X” suffix for an integer N (for example, ventx1 becomes ventx1) and converting to uppercase. We then made the following substitutions to convert certain known Xenopus gene symbols to human where the name of the ortholog has diverged or only a paralog exists: pou5f3 → POU5F1, mix1 → mix1, dppa2 → DPPA4, lefty → lefty2, ventx1-3 → NANOg, mespb → MESP1, sox17a/b → SOX17. We remove any duplicate names that arose in this process. We calculated enrichments for the following gene sets: KEGG_2019_Human, BioPlanet2019, WikiPathways_2019_Human, GO_Biological_Process_2018, GO_Molecular_Function_2018, GO Cellular_Component_2018, CheA_2016. We calculate enrichments for each cluster individually and consecutive combinations of the 4 clusters: 1, 2, 3, 4, 12, 34, 123, 234. All enrichments can be found in Supplementary Data 1, selected enrichments are given in Fig. 6C and terms are given shortened labels for brevity (Table 2):

**Motif analysis**

To find motifs enriched in the promoters, we took the 500 bp upstream of the promoter of the maximally expressed isoform for each gene in the four activated and four repressed clusters, along with a background of the 500 bp upstream of all annotated TSS in the X.9.1 genome. We extracted fasta files for each of these sets of regions, and then used findMotifs.pl from Homer17 to search for known motifs with options: “findMotifs.pl clusterAB.fa fasta outAB –fasta background.fa --nomotif” where A ∈ {activated, repressed} and B ∈ {1, 2, 3, 4}. We filtered results to select best matching motifs from related families, namely we collapsed all ETS motifs to the canonical Homer ETS promoter motif; all SP and KLF motifs to SPI; SOX motifs to SOX2; all HOX

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motifs to HOXD13 (the highest scoring HOX); we represent all GFY and Ronin matches as ZNF143 (for which the motifs overlap); and we excluded motif annotated as PRDM10, due to low confidence in the motif. The motif annotated as ATF1 is an example of the CAMP response element (CRE) bound by CREB factors including ATF1, we label this as CRE/ATF1. The top 16 motif enrichments are given in Supplementary Fig. 7h, in Fig. 6d we give the top 6 motif matches, excluding ZNF143 due to divergence from the JASPAR database, to which we add CRE/ATF1 as a putative calcium responsive element motivated by CREM/CREB1 gene set enrichments Supplementary Fig. 7j).

To calculate CRE and ETS motif enrichment for mTOR and pluripotency genes, we took genes annotated with the terms mTOR signaling pathway and Signaling pathways regulating pluripotency of stem cells from KEGG_2019_Human as provided by Enrichr that are activated in high K+ (LR > 0) and are present in clusters A1 and A2. The resulting genes were subjected to the same promoter analysis, using ilastik software. This segmentation was cleaned (to remove debris and to separate merged cells) and mean nuclear protein intensity as well as Ca2+ transients were performed using student\'s t-test. Graphs were scored (gastrulation, left-right patterning, in situ hybridizations) we used Homer to calculate the occurrence of the maximal ATF/CRE family motif and the ETS motif in these promoters and the background set to report Fisher Exact test p-values and Odds Ratios.

Xenopus biological replicates, statistical methods, graphs, and models

In experiments where embryos were evaluated for phenotypes and scored (gastrulation, left-right patterning, in situ hybridizations) we carried out three to five biological replicates and Fisher\'s exact test to evaluate statistical significance. The animal cap experiment was performed twice with a total score of four to eight animal caps per experiment. For the calcium transient analyses, data was collected from three to five embryos in each experiment in three independent experiments, and statistical analyses on GCaMP/mCherry fluorescence intensity as well as Ca2+ transient area were performed using student\'s t-test. For whole cell electrophysiological recordings, three to five embryos (two cells each) were examined for their membrane potential and statistical significance was tested by student\'s t-test. Graphs were designed using GraphPad Prism software. Models were created with BioRender.com.

hESC culture

hESCs were grown in mTeSR1 (STEMCELL Technologies) in tissue culture dishes coated with Matrigel (Corning; 1:200 in DMEM/F12) and kept at 37 °C, 5% CO2. The cell lines used were ESIO17 (ESIBIO) and H9. Cells were routinely passaged using dispase (STEMCELL Technologies) and tested for mycoplasma contamination and found negative. For rapamycin experiments, cells were grown in MEF-conditioned HUESM media supplemented with 20 ng/ml bFGF as previously described. With or without 100 nM rapamycin, which we found to increase the survival of rapamycin treated cells compared to cells grown in mTeSR1.

hESC treatments and differentiation

Cells were dissociated with accutase and seeded onto eight-well imaging slides (ibidi 80826) at a density of 4–6 × 103/cm2. Cells were seeded and maintained in Rock-inhibitor Y27632 (MCE; 10 μM) to increase survival and the uniformity of response. Treatments with 1 mM BaCl2 or 10 or 25 nM Ergtoxin or 100 nM rapamycin were initiated 4 h after seeding. Differentiation was initiated 24 h after seeding where indicated. To initiate differentiation, the media was replenished with/without BaCl2 or Ergtoxin and treated with the indicated growth factors or small molecules. The media with any treatments was replenished daily.

Immunofluorescence of hESCs

Cells were fixed for 30 min in 4% paraformaldehyde, rinsed twice with DPBS (without Ca2+ and Mg2+, denoted DPBS−/−), and blocked for 30 min at room temperature. The blocking solution contained 3% donkey serum and 0.1% Triton X-100 in 1x DPBS−/−. After blocking, the cells were incubated with primary antibodies at room temperature for 2 hours. Antibodies and concentrations are listed in Supplementary Table 1. Cells were washed three times with DPBST (1X DPBS−/− with 0.1% Tween 20) and incubated with secondary antibodies (AlexaFluor 488 A21206, AlexaFluor 555 A31570 and A21432, and AlexaFluor 647 A31571, Thermo Fisher; 1:500) and DAPI for 30 min at room temperature. After secondary antibody incubation, samples were washed in DPBST and then DPBS at room temperature.

hESC Imaging and analysis

Images were acquired using a ×20, NA 0.75 objective on an Olympus IX83 inverted epifluorescence microscope or an Olympus/Andor spinning disk confocal microscope. Cell segmentation was performed using ilastik software. This segmentation was cleaned (to remove debris and to separate merged cells) and mean nuclear protein intensities as well as standard errors were quantified using a custom MATLAB code. Nuclear intensities were normalized by DAPI to correct for intensity variation due to optics. Code is available at https://github.com/warmflashlab/Sempou2022_Code.

qPCR

For qPCR, hESCs were grown with or without ErgToxin (25 mM) for the indicated times. RNA collection and DNase treatment were performed using the RNAqueous®-Micro Total RNA Isolation Kit (AM1931) and cDNA was synthesized with the SuperScript Vilo cDNA Synthesis Kit (Fisher Scientific 11754-050). qPCR measurements were collected using SYBR Green reagent (LifeTech-4367659) on a Step OnePlus instrument (Applied Biosciences). Data were normalized using the housekeeping gene GAPDH. Primers for qPCR were:

| Primer Forward | Primer Reverse |
|---------------|---------------|
| OCT4 5'-caagctctgcaagaccg3' | 5'-ccaaagcccacctgcctgt3' |
| SOX2 5'-ccattcagttgacagttgagtc3' | 5'-tcggcagactgatcaaatata3' |
| NANOG 5'-tgaaaaggattcagctg3' | 5'-aagacgacctcactcactc3' |
| GAPDH 5'-caagctctgcaagaccg3' | 5'-ggccctcagtgaattg3' |

Statistics and reproducibility

Embryo and cell sample sizes in this study were chosen according to the standards in the field. No data were excluded from the analyses. The investigators were blinded to allocation during experiments and outcome assessment whenever feasible.

For statistics, two-sided Fisher\'s exact test was used to assess significance of two variables with independent proportions, (treated/untreated) and their outcomes (normal/abnormal). This test was applied when scoring for morphological phenotypes of embryos and...
WMISH results, under control conditions or after microinjection (CR, MO, mRNA), medium manipulations or treatment with compounds. Two-tailed, unpaired t-tests or ANOVA were used to test whether the means of two populations differ largely from one another and were employed for V_m, calcium intensity and calcium transient area comparisons as well as all hESC experiments.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The RNA-seq time series of uninjected-control (UC) and high K+ embryos generated in this study have been deposited in the Gene Expression Omnibus under accession GSE186670. Source data are provided with this paper.

Code availability
All source data and code required to analyze RNA-seq time series and generate figures are available at https://github.com/owensnick/kCNHeGenomicsFigures.jl. Source code for quantitating protein intensities in human ES cells is available at https://github.com/warmashlab/Sempou2022_Code.

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Author contributions

E.S., M.C., D.Z., A.W., N.D.L.O., and M.K.K. conceived and designed the experiments and analyzed data from the experiments. E.S., V.K., W.H., and M.K.K. performed all Xenopus experiments. E.S., J.Z., L.T., and D.Z.
performed electrophysiological recordings in Xenopus. N.D.L.O. analyzed all genomic data. E.S., C.G., A.W., and M.K.K. conceived, designed, and analyzed experiments in human embryonic stem cells. C.G., E.C.-A., and A.W. performed human embryonic stem cell experiments. The manuscript was written by E.S., A.W., N.D.L.O., and M.K.K. and all authors critically evaluated the manuscript.

**Competing interests**
The authors declare the following competing interests: M.K.K. is a Founder and President of Victory Genomics, Inc. Yale University has filed a provisional patent application related to this work. The remaining authors declare no competing interests.

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