Timing is everything: Reiterative Wnt, BMP and RA signaling regulate developmental competence during endoderm organogenesis☆

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ARTICLE INFO

ABSTRACT

A small number of signaling pathways are used repeatedly during organogenesis, and they can have drastically different effects on the same population of cells depending on the embryonic stage. How cellular competence changes over developmental time is not well understood. Here we used *Xenopus*, mouse, and human pluripotent stem cells to investigate how the temporal sequence of Wnt, BMP, and retinoic acid (RA) signals regulates endoderm developmental competence and organ induction, focusing on respiratory fate. While Nkx2-1+ lung fate is not induced until late somitogenesis stages, here we show that lung competence is restricted by the gastrula stage as a result of Wnt and BMP-dependent anterior-posterior (A-P) patterning. These early Wnt and BMP signals make posterior endoderm refractory to subsequent RA/Wnt/BMP-dependent lung induction. We further mapped how RA modulates the response to Wnt and BMP in a temporal specific manner. In the gastrula RA promotes posterior identity, however in early somite stages of development RA regulates respiratory versus pharyngeal potential in anterior endoderm and midgut versus hindgut potential in posterior endoderm. Together our data suggest a dynamic and conserved response of vertebrate endoderm during organogenesis, wherein early Wnt/BMP/RA impacts how cells respond to later Wnt/BMP/RA signals, illustrating how reiterative combinatorial signaling can regulate both developmental competence and subsequent fate specification.

1. Introduction

The formation of organs from naïve progenitor cells is controlled by combinatorial signaling of a handful of pathways including Wnt, BMP, FGF, Notch, Hedgehog, and retinoic acid (RA) (Kraus and Grapin-Botton, 2012; Zorn and Wells, 2009). These signals are required in distinct temporal windows to progressively direct embryonic cells through a series of fate decisions into specific tissue lineages. In many cases these same signals can have dramatically different effects at different stages of development - promoting a given lineage at one time and then inhibiting at a subsequent time. This dynamic response is a commonly observed but poorly understood phenomenon in the development of many lineages, including cardiogenesis (Gessert and Kuhl, 2010; Loh et al., 2016), neurogenesis (Sasai et al., 2014) and endoderm organogenesis within the respiratory and digestive systems (Loh et al., 2014; Zorn and Wells, 2009). In most organ systems it is still poorly

understood how the ability or "competence" of a tissue to respond to signals changes over time, and how early signaling events impact subsequent responses to the same signals. A detailed understanding of these reiterative signaling dynamics is required for a comprehensive roadmap of organogenesis, which is likely necessary to facilitate the generation of multi-lineage organ tissues from human pluripotent stem cells (hPSCs) (McCauley and Wells, 2017).

Development of the respiratory and digestive systems by dynamic paracrine Wnt, BMP, and RA signaling between the embryonic endoderm and the adjacent mesoderm is a good example of reiterative, combinatorial signaling in organogenesis. In vertebrate embryos and hPSCs, the endoderm is patterned during gastrulation and early neural stages of embryogenesis wherein high levels of RA, Wnt, and BMP from the posterior mesoderm promote hindgut fate in posterior endoderm (Bayha et al., 2009; Deimling and Drysdale, 2009; Spence et al., 2011; Stevens, 2017), whereas low Wnt and BMP activity, due to antagonists

such as Dkk1, Sfrps, Noggin, and Chordin expressed in anterior mesendoderm, permit the development of foregut progenitors (McLin et al., 2007; Rankin et al., 2011). Within the next 24–48 h of development, RA, Wnt, and BMP produced by the anterior lateral plate mesoderm, have the opposite effect, and no longer suppress anterior identity but rather promote distinct foregut organ lineages including thyroid (Kurmann et al., 2015; Wang et al., 2011), lungs (Desai et al., 2004; Domyan et al., 2011; Goss et al., 2009; Harris-Johnson et al., 2009; Rankin et al., 2012), liver (Wandzioch and Zaret, 2009), stomach (McCracken et al., 2017, 2014) and pancreas (Kraus and Grapin-Botton, 2012). How these dynamic Wnt/BMP/RA signals and changing developmental competence are coordinated to control lineage specification in the primitive gut tube is still poorly understood.

We recently demonstrated that RA signaling is critical for foregut progenitors to activate respiratory fate in response to Wnt and BMP signals (Rankin et al., 2016). During early somitogenesis stages of development, RA produced by anterior lateral plate mesoderm is required to make the foregut endoderm competent to adopt respiratory fate a day later in response to lung-inducing Wnt2/2b and Bmp2/4/7 signals from the adjacent splanchnic mesoderm. The molecular basis for this RA-dependent respiratory competence is unknown. Moreover it remains untested if RA can instructively change the developmental competence of non-foregut endoderm or if RA acts in strictly a permissive fashion within the foregut.

In this study we used *Xenopus*, mouse, and hPSCs to interrogate if and how early Wnt/BMP/RA-mediated patterning impacts the competence and response of developing endoderm to subsequent Wnt/BMP/RA-mediated organ induction, with a focus on respiratory fate. We show that respiratory competence is restricted to anterior endoderm by the gastrula stage, much earlier that previously appreciated. Early Wnt and BMP signals make the posterior endoderm refractory to subsequent RA/Wnt/BMP-mediated lung induction and instead make the endoderm competent to adopt an intestinal fate in response to these same signals. We show that RA has a temporally dynamic, and permissive role in modulating how cells respond to Wnt and BMP. During gastrulation RA promotes posterior identity, but during early somite stages RA regulates respiratory versus pharyngeal fate in the anterior endoderm and midgut versus hindgut fate within posterior endoderm. These data illustrate that evolutionarily conserved, reiterative Wnt/BMP/RA signaling impacts dynamic competence and lineage decisions in the developing gut tube.

2. Results

2.1. Respiratory competence is restricted to anterior endoderm by the early gastrula stage in Xenopus

We previously demonstrated that induction of the respiratory lineage in *Xenopus*, mouse, and human foregut endoderm requires the sequential activity of RA followed by Wnt+BMP signaling (Rankin et al., 2016). However, in these *Xenopus* experiments where endoderm was isolated at the early somite-stages of development (stage NF14), only a subset of the explants expressed the respiratory markers *nkx2-1* and *sftpc* in response to RA/Wnt/BMP signals (Supplemental Fig. S1A), suggesting that a 'pre-pattern' or restricted domain of respiratory competence already existed in the foregut. To test if a restricted domain of lung-competent cells already existed by the gastrula stage, we micro-dissected gastrula stage *Xenopus* embryos (stage NF10.25) and pre-pared three types of explants (Fig. 1A): 1) the entire definitive endoderm (DE) region; 2) the anterior endoderm (*hhex*-expressing AE, which normally contributes to foregut endoderm including Nkx2-1+ respiratory endoderm (Supplemental Fig. S1C-J); and 3) the posterior endoderm (*ventx1/2*-expressing PE). We then subjected the DE, AE, and PE explants to a respiratory fate induction protocol (Fig. 1A), which consists of RA treatment during early somitogenesis (stages NF14-25) followed by culture in the GSK3β antagonist Bio

(which stabilizes βcatenin to mimic endogenous Wnt2/2b signals) + recombinant BMP4 protein from stages NF25-NF38. When sibling embryos reached stage NF38, the explants were analyzed by in-situ hybridization for *nkx2-1* and *sftpc*.

The DE explants treated with RA followed by Bio+BMP expressed respiratory markers only in a subset of the endoderm (Fig. 1C, I), indicating that a restricted domain of lung competent cells existed in the gastrula DE. This was confirmed in the AE and PE explants: AE explants robustly expressed *nkx2-1* and *sftpc* throughout the tissue (Fig. 1E, K) whereas PE explants failed to express the respiratory markers (Fig. 1G, M). RA prior to Bio+BMP was necessary for the gastrula DE and AE to express *nkx2-1* and *sftpc,* as omission of RA resulted in no induction of the lung markers by Bio+BMP (Fig. 1B, H, D, J) and RA alone was not sufficient to induce lung fate (data not shown and Supplemental Fig. S1A). These results indicate RA has a permissive role in enabling the endoderm to activate lung markers in response to Wnt/BMP, and we conclude that gastrula-stage DE already contains a restricted domain of respiratory competent cells, earlier than previously appreciated.

2.2. Stage-dependent effects of RA on Xenopus lung induction

One possible explanation for the restricted domain of lung competence in the gastrula DE was due to the timing of RA exposure. RA-synthesizing enzymes (*rdh10* and *raldh2*) are expressed in gastrula stage mesoderm, suggesting that RA acts early in development (Supplemental Fig. S2A). We tested if earlier RA treatment, starting immediately after dissection at the gastrula stage, might expand the domain of lung competence in DE explants (experiment diagrammed in Supplemental Fig. S2B). However we found that this early RA treatment of DE not only failed to expand the *nkx2-1* and *sftpc* domains in response to Bio+BMP, but rather completely suppressed the activation of lung markers (Supplemental Fig. S2B). In contrast, this early RA treatment resulted in the up-regulation of *pdx1 and cdx2* (Supplemental Fig. S2B), indicating that during this developmental time, RA promoted pancreatic and intestinal fate, consistent with previous reports (Bayha et al., 2009; Chen et al., 2004). We conclude that the timing of RA exposure elicits completely different responses in the developing endoderm: RA during gastrulation has a posteriorizing effect on the endoderm promoting pancreatic and intestinal fate, while RA starting at NF14 promotes respiratory fate in the AE.

2.3. RA regulates respiratory competence in mouse endoderm

We next sought to test whether RA also promotes respiratory competence in developing mouse endoderm. RA-dependent reporter activity in transgenic *Tg(RARE:LacZ)* mice (Rossant et al., 1991) was not detected in the mouse anterior endoderm at E8.0 (~3–5 somite stage, ss), however at E8.5 (8–10 ss) robust reporter activity was evident (Fig. 2A); this is prior to Wnt2/2b-dependent lung induction at ~E9.0 (16ss) when Nkx2-1 protein is first detectable in the ventral foregut (Havrilak et al., 2017). In *Raldh2-/-* embryos, which have a severe deficiency of RA production, Nkx2-1 protein is nearly absent from the respiratory domain but still present in the forebrain and thyroid domains (Fig. 2B), confirming that RA is required for normal lung but not thyroid induction (Desai et al., 2006; Wang et al., 2011).

To test if RA regulates lung competence of mouse endoderm, we performed a series of culture experiments using isolated endoderm tissue (Fig. 3). DE from the node to the headfold was isolated from E7.5 embryos (Fig. 3A; the posterior-most DE cannot be cleanly removed from the primitive streak at this stage) and cultured with or without RA for 24 h, followed by 5 days in the GSK3 antagonist CHIR99021 (which stabilizes β-catenin to mimic endogenous Wnt2/2b signals) + recombinant FGF7+ FGF10, which are required for survival of the mouse endoderm explants (we refer to this combination of factors hereafter as C/F7/10). Explants cultured without RA expressed little if any Nkx2-1
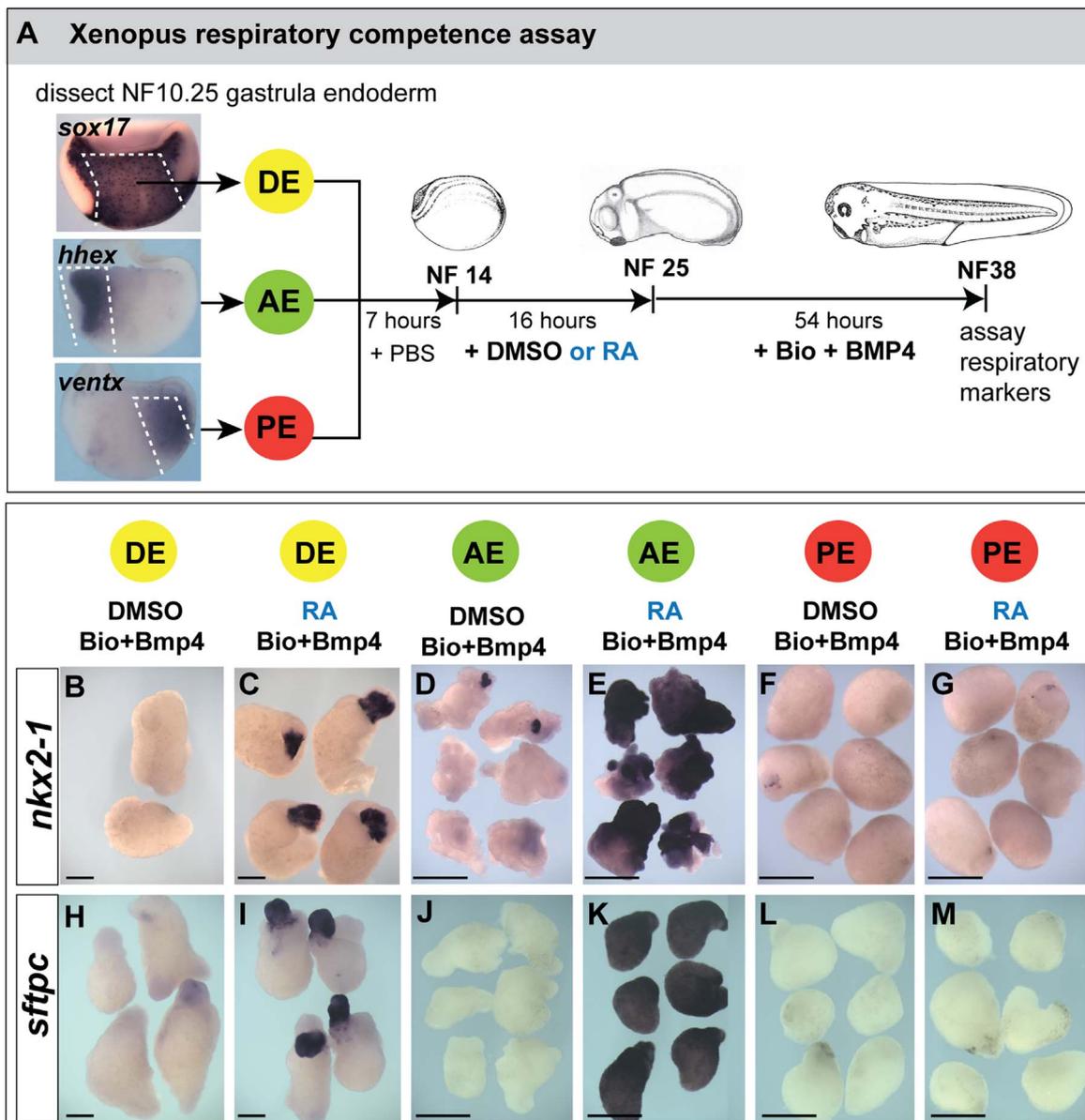
Fig. 1. Respiratory competence of *Xenopus* endoderm is restricted by the early gastrula stage. (A) Experimental diagram of the *Xenopus* respiratory competence assay. Bisected early gastrula stage (NF10.25) *Xenopus* embryos assayed by in-situ hybridization show the expression of *sox17α* throughout the definitive endoderm (DE), *hhex* in anterior endoderm (AE) and *ventx2* in posterior endoderm (PE). DE, AE, or PE explants were dissected at NF10.25, cultured in isolation until NF14, treated +/− 50 nM RA from NF14-25, and then in 3.5 μM Bio + 50 ng/mL BMP4 from NF25-38, at which time they were fixed and analyzed by in-situ hybridization for the respiratory endoderm markers *nkx2-1* **(B-G)** and *sftpc* **(H-M).**

(Fig. 3B), however 24 h of RA exposure made the endoderm broadly competent to express Nkx2-1 in response to C/F7/F10 (Fig. 3C,C'). Portions of each explant expressed E-cadherin and Sox2 but not Nkx2-1, indicating that not all endoderm cells had activated respiratory fate (Fig. 3C'). Exogenous BMP was dispensable in these cultures for Nkx2-1 expression; however we note that *Bmp2/4/7* transcripts are present in the E7.5 cardiac crescent and phospho-Smad1/5/8 is already detectable in the underlying anterior endoderm (Danesh et al., 2009; Wandzioch and Zaret, 2009), suggesting endogenous BMP signaling has occurred in E7.5 AE.

The observation that Nkx2-1 is only expressed in some but not all of the cells in each RA-treated explant suggested that, like in *Xenopus*, mouse DE contained restricted regions of lung-competent cells by the end of gastrulation. To test this we subdivided the E7.5 DE into rostral anterior endoderm (underlying the cardiac crescent) and more caudal endoderm (posterior to the cardiac crescent) (Fig. 3A). Moreover, since Nkx2-1 is expressed in both lung and thyroid, we performed these

experiments with *Tg(Sftpc:GFP)* transgenic mouse embryos (Lo et al., 2008), enabling unambiguous identification of respiratory fate. Explants were treated with RA for 24 h followed by 5 days in C/F7/10 followed by 1 day in a cocktail of factors (BFGM) optimized for growth of fetal lung tissue (Shannon et al., 1999). We found that the rostral anterior endoderm was much more competent than the more caudal endoderm to express Sftpc:GFP in response to the lung inducing signals (Fig. 3D,E); we note there was weak GFP expression in a small portion of the caudal explants, suggesting they contained a few anterior endoderm cells that can respond to RA.

Since it was not possible to cleanly isolate the PE from E7.5 mouse embryos we tested whether RA could impart respiratory competence to mouse hindgut endoderm (Hg endo) or foregut endoderm (Fg endo) explants from E8.5 (8−10 ss) embryos, which are roughly equivalent to *Xenopus* NF20 (Fig. 3F). *Tg(RARE:lacZ)* embryos indicated that both the foregut and hindgut endoderm had already experienced RA signaling by this time (Fig. 2A). Fg endo explants not exposed to RA
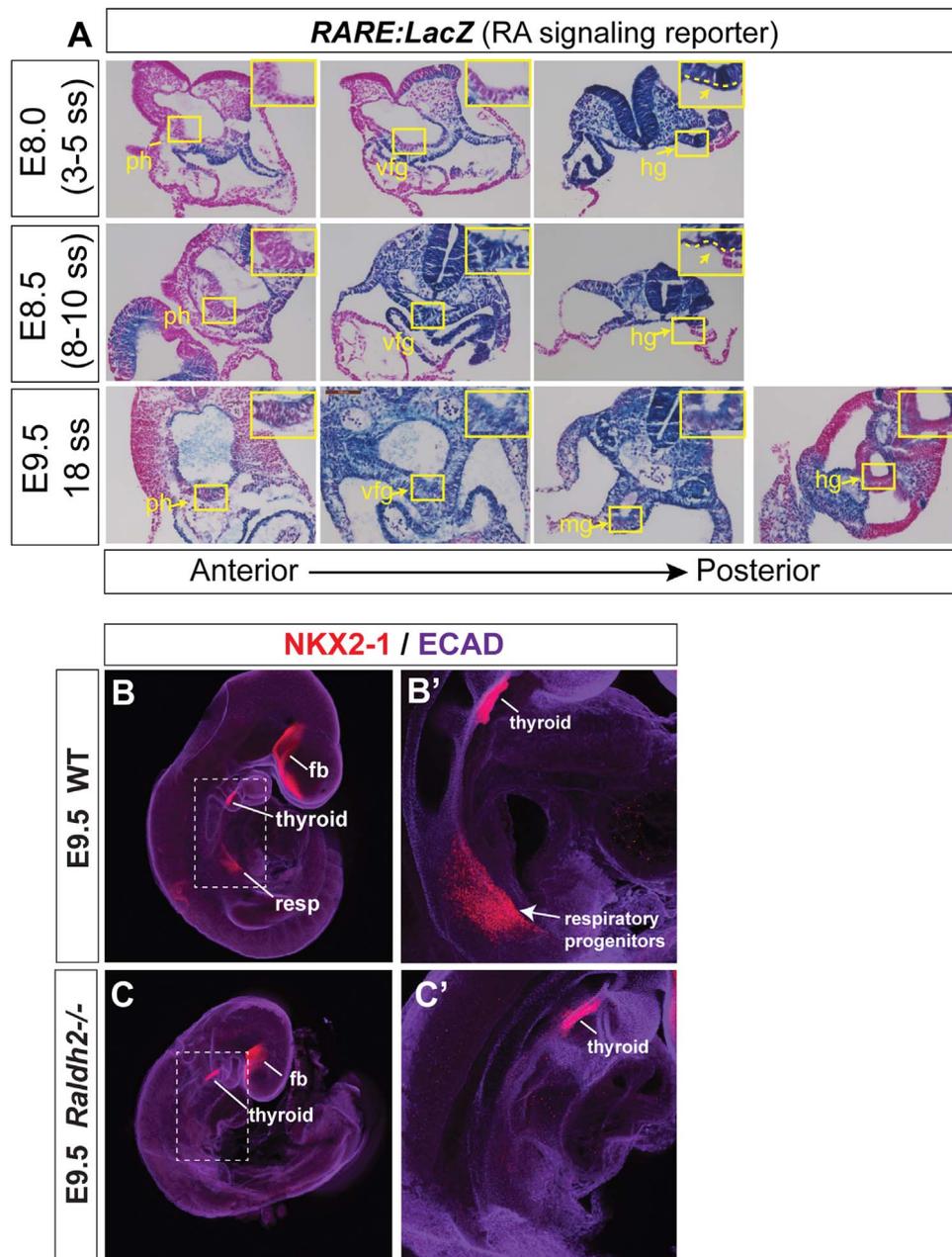
Fig. 2. Analysis of *RARE:lacZ* RA signaling reporter and *Raldh2-/-* mouse embryos. (A) Sections along the anterior-posterior axis of *RARE:LacZ* embryos stained for β-galactosidase (blue) and counterstained with fast red (pink) at the indicated developmental stages. Abbreviations: ph, pharynx; vfg, ventral foregut; hg, hindgut; mg, midgut. **(B-C)** Whole mount immunostaining of WT and *Raldh2-/-* embryos at E9.5 for Ecadherin (purple) and Nkx2-1 (red). Abbreviations: fb, forebrain; resp, respiratory domain. Thryoid and forebrain expression of Nkx2-1 is maintained in Raldh2-/- embryos, whereas there is a near complete loss of Nkx2-1 in the respiratory domain.

and then cultured in C/F7/10 expressed small discrete small patches of Nkx2-1+ cells (Fig. 3G,G'), indicating a limited amount of respiratory induction. RA treatment of E8.5 Fg endo resulted in a dramatic increase in Nkx2-1+ respiratory tissue in response to C/F7/10. In contrast, E8.5 Hg endo explants never had detectable Nkx2-1 in response to RA/C/F7/10 (Fig. 3I,I'). Thus at E8.5 mouse foregut but not hindgut endoderm is competent to activate respiratory fate.

An important hall mark of mammalian lung development is the stereotypical branching morphogenesis (Herriges and Morrisey, 2014; McCauley et al., 2017). We therefore set out to determine whether early RA treatment also imparted the ability to execute this lung morphogenesis program. To test this we performed tissue recombination experiments with isolated E12.5 distal lung mesenchyme (LgM), which is known to provide signaling cues sufficient to induce branching morphogenesis in competent respiratory epithelium (Shannon, 1994).

E7.5 DE from *Tg(Sftpc:GFP)* embryos was isolated, cultured +/- RA for 24 h followed by C/F7/10 and then recombined for 3 days with E12.5 LgM. To ensure that RA was only acting during the initial 24-h period, and that there was not any residual RA activity during induction of the respiratory lineage, we included the pan-RAR inhibitor BMS493 to the media containing C/F7/10. We found that DE cultured without RA had few GFP+ cells and the endoderm did not branch (Fig. 3J-J"); in contrast RA treated explants exhibited robust GFP+ respiratory epithelium that underwent extensive early branching morphogenesis at the distal tips of the growing lung buds (Fig. 3K-K"). Together these data suggest that, similar to *Xenopus*, RA acts permissively to impart respiratory competence to a restricted region of mouse AE and that this restricted domain of lung competence exists at E7.5, much earlier than previously appreciated.
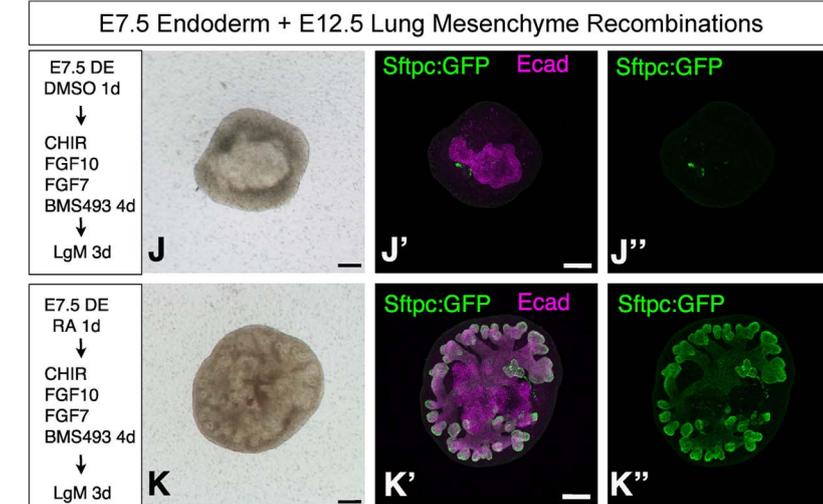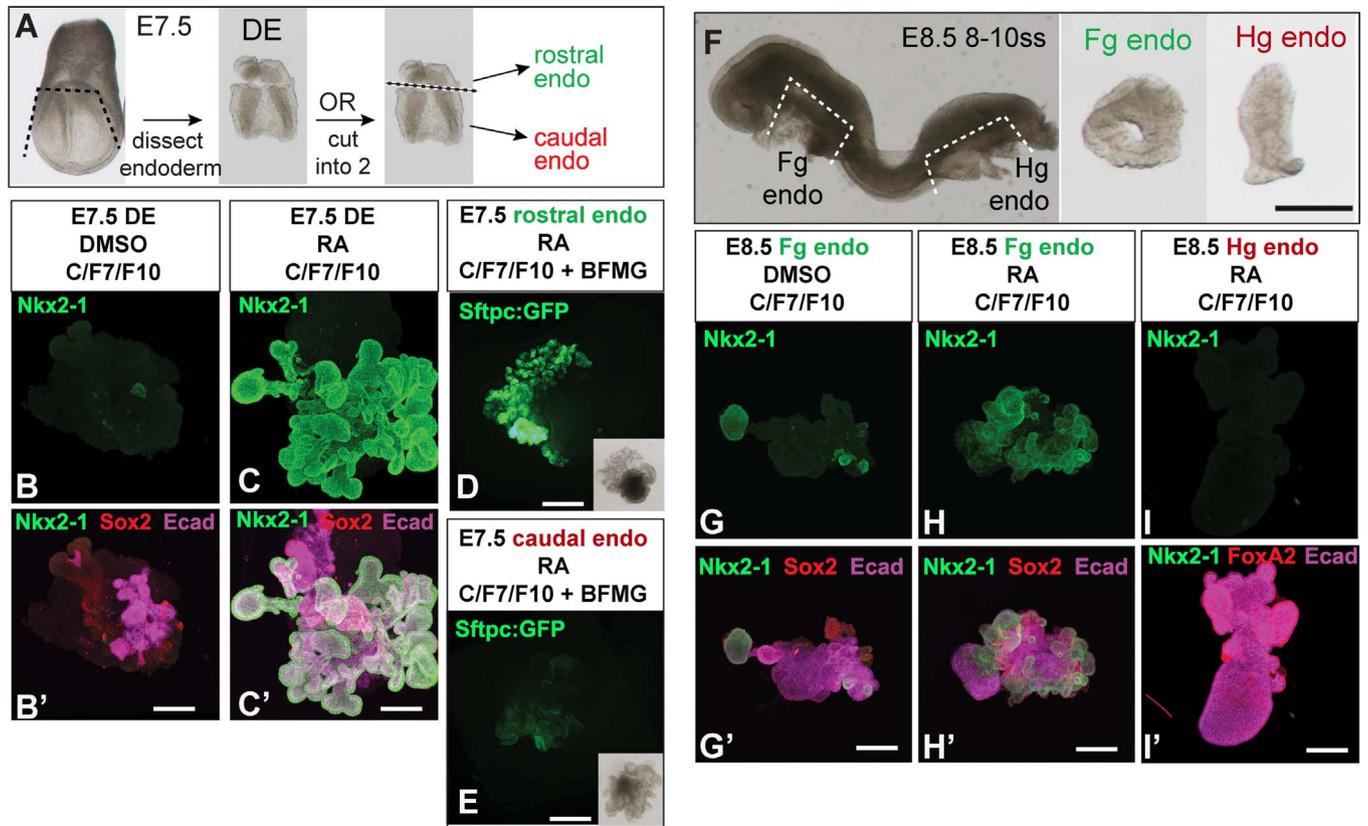
Fig. 3. RA imparts respiratory competence to mouse definitive endoderm. (A-E) Analysis of respiratory competence of E7.5 mouse endoderm. **(A)** Experimental dissection diagram. **(B-C)** E7.5 DE was isolated and cultured +/- RA for 24 h followed by CHIR99021+FGF7+FGF10 (C/F7/F10) and analyzed by immunofluorescence for Nkx2-1 (green), Sox2 (red), and E-cadherin (purple). **(D-E)** GFP fluorescence observed after E7.5 endoderm from *Tg(Sftpc:GFP)* transgenic embryos was isolated, separated into rostral AE underlying the cardiac crescent **(D)** or more caudal endoderm located posterior to the cardiac crescent **(E)**, cultured 24 h in RA, followed by C/F7/F10 + BFGM outgrowth media. **(F-L)** Analysis of respiratory competence of E8.5 endoderm. Foregut (Fg endo; **G,H**) or hindgut endoderm (Hg endo; **I**) was isolated from E8.5 embryos **(F)** and cultured +/- RA for 24 h followed by C/F7/F10 and analyzed by immunofluorescence for Nkx2-1 (green), Foxa2 (red) or Sox2 (red), and E-cadherin (purple). **(J-K)** RA-dependent, specified respiratory endoderm is competent to undergo early branching morphogenesis when recombined with distal lung mesenchyme. E7.5 DE from *Tg(Sftpc:GFP)* embryos was isolated, cultured without RA **(J)** or with RA **(K)** for 24 h followed by C/F7/10+ the pan-RAR antagonist BMS493 (in order to disrupt any RA signaling after the 24-h pulse), and then recombined and cultured for 3 days with E12.5 distal lung mesenchyme (LgM) and analyzed by immunofluorescence for GFP (green) and E-cadherin (purple). J and K are brightfield images of the recombinants.

2.4. Gastrula Wnt/BMP-dependent patterning impacts endoderm competence and the response to subsequent RA/Wnt/BMP inductive cues in Xenopus

Our data suggest that a restricted domain of lung competence exists in frog and mouse endoderm by the end of gastrulation. Given the well-established role of Wnt and BMP in gastrula stage A-P patterning, we hypothesized that these signals might predispose how different regions of the endoderm respond to later RA/Wnt/BMP-dependent organogenesis cues. To test this we disrupted early A-P pattern in *Xenopus* via Wnt and BMP loss-of-function and determined how this would impact the ability of the endoderm to respond to subsequent RA/Wnt/BMP signals and activate different endoderm lineage markers (Fig. 4). To inhibit endogenous Wnt and BMP signaling, we microinjected
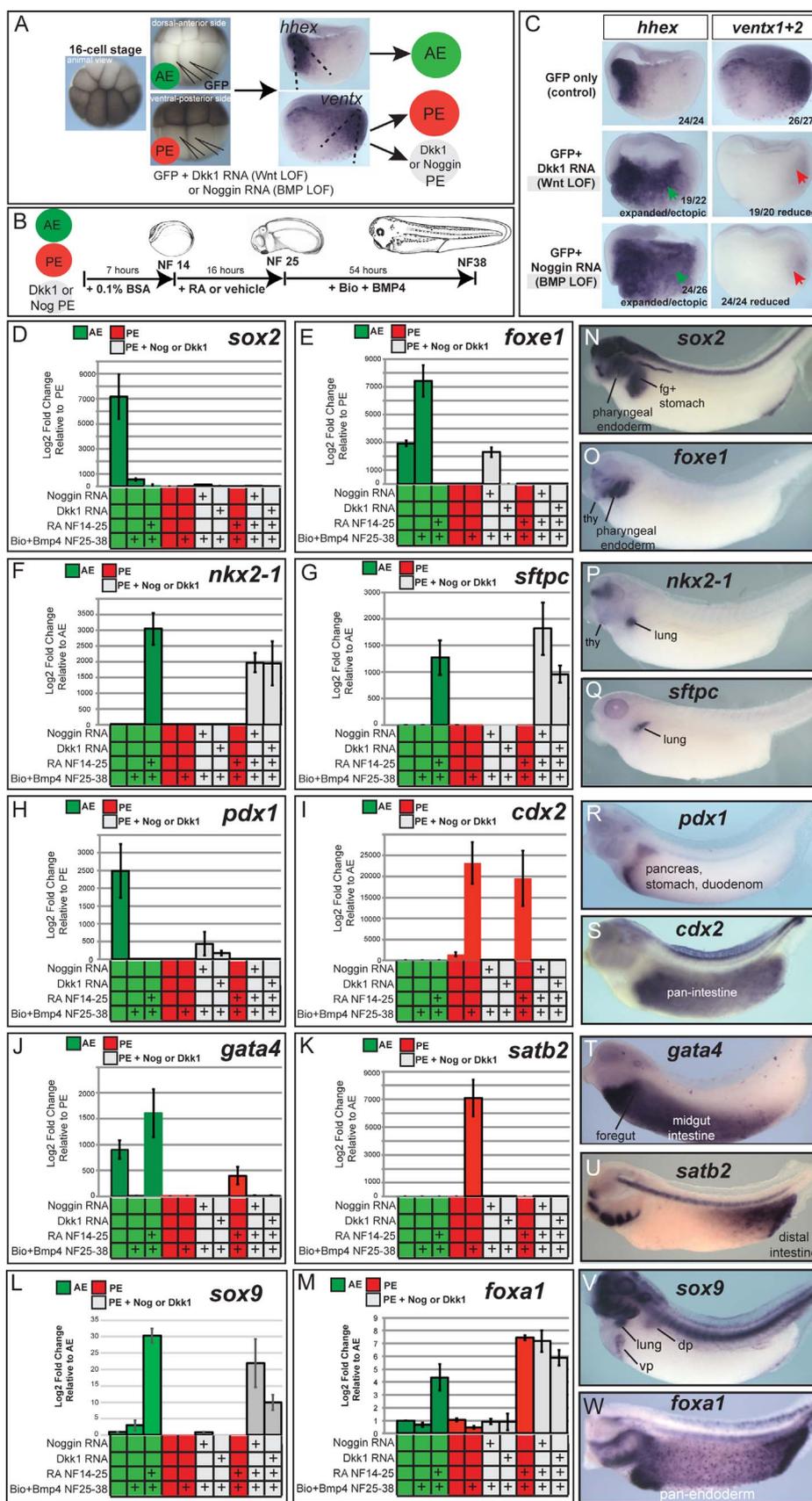
Fig. 4. Wnt/BMP-mediated A-P patterning impacts developmental competence. (A,B) Experimental diagram. *Xenopus* embryos at the 16-cell stage with clear pigment differences (animal pole, dorsal-anterior, and ventral-posterior views of such embryos are shown) were injected with 25 pg of Noggin or 100 pg of Dkk1 RNAs (to inhibit BMP and Wnt/βcatenin signaling, respectively) into each ventral-vegetal V2.1 blastomere along with 25 pg eGFP RNA as a lineage tracer, which targets the *ventx1/2*-expressing posterior mesendoderm. *Hhex+* AE tissue was targeted by injection of each dorsal-vegetal D1.1 blastomere with 25 pg eGFP RNA. At gastrula stage NF10.25, GFP fluorescence was monitored and used to dissect AE, PE, and BMP- or Wnt-inhibited PE explants, which were then treated as indicated in panel B (50 nM RA from NF14-25 followed by 3.5 μM Bio + 50 ng/mL BMP4 from NF25-38). **(C)** *In-situ* hybridization analysis of *hhex* and *ventx1+2* (both probes mixed together) in bisected gastrula NF10.25 embryos confirms effective inhibition of the posteriorizing Wnt and BMP pathways by dkk1 and noggin RNA injection. Numbers in the lower left corner indicate numbers of embryos assayed with the gene expression pattern shown. **(D-M)** Relative gene expression analysis (RT-qPCR) of different anterior-posterior endoderm lineages assayed in explants as prepared/treated in panels A,B. Graphs display the average $2^{-\Delta\Delta Ct}$ value +/- SEM of 3 biological replicates. **(N-W)** *In-situ* hybridization of control embryos showing the endogenous spatial domains of expression along the anterior-posterior axis.

synthetic RNAs encoding either the antagonists Dkk1 or Noggin (along with GFP RNA lineage tracer) into ventral-vegetal cells of 16-cell stage embryos (Moody, 1987), which targets the *ventx1/2*-expressing PE (Fig. 4A). In situ hybridization at NF10.5 confirmed that both Dkk1 and Noggin suppressed *ventx1/2* and expanded the *hhex* domain (Fig. 4C), indicating a loss of posterior and an expansion of anterior identity. AE and PE explants were micro-dissected and subjected to the respiratory fate induction protocol, consisting of RA from stages NF14-25 followed by Bio+BMP4 from stages NF25-38 (Fig. 4B). We then assayed explants for respiratory fate as well as a panel of markers reflecting different endoderm lineages along the A-P axis (Fig. 4N-W). In all conditions expression of the pan-endoderm transcription factor *foxa1* was robustly expressed, with a modest fold-change increase in response to RA (Fig. 4M).

As expected control PE explants failed to express lung markers or any of the other anterior lineage markers analyzed (*sox2, foxe1, pdx1*; Fig. 4D-H) in response to any of the treatments of +/-RA, +/- Bio +BMP4, confirming that gastrula stage PE is not competent to active foregut gene expression. PE explants did express *cdx2* as predicted; the addition of Bio+BMP4 dramatically increased *cdx2* and induced *satb2* (Fig. 4I,K), indicating distal intestine fate (Munera et al., 2017). Exposing PE to RA followed by Bio+BMP resulted in *satb2* suppression (Fig. 4K) and up-regulation of *cdx2* and *gata4* (Fig. 4J,) indicating a shift to midgut fate. Strikingly, both the Noggin and Dkk1-injected PE explants expressed *nkx2-1, sox9, and sftpc* in response to RA followed by Bio+BMP4 (Fig. 4F,G, L); these explants failed to express *cdx2, satb2* or *gata4* (Fig. 4I,J,K), confirming they had lost their posterior identity. RA was essential for the respiratory markers to be induced, since Bio+BMP treatment alone of Noggin or Dkk1-injected PE was not sufficient to induce *nkx2-1* or *sftpc*.

Although AE explants cultured without RA/Wnt/BMP did not express the lung markers (Fig. 1, Fig. 4F,G); they did express *foxe1, sox2, gata4, and pdx1* (Fig. 4D,E,H,J), suggesting that in the absence of any exogenous or mesodermal derived signals AE adopted a mixed foregut fate including pharyngeal, pancreatic, and stomach character. If RA was omitted, the Bio+BMP treated AE cultures expressed *foxe1* (Fig. 4E) but not *gata4, pdx1, nkx2-1 or sftpc*; this is indicative of pharyngeal/thyroid identity. However when AE explants were treated with the full lung-inducing regime of RA followed Bio+BMP4 to induced *nkx2-1, sox9, and sftpc*, we observed a complete suppression of *foxe1* (Fig. 4E,F,G,L), suggesting that RA promotes lung competence, at least in part, by suppression of pharyngeal/thyroid fate in agreement with previous studies (Wang et al., 2011).

Together these data indicate that early Wnt/BMP-mediated patterning, present by the gastrula stage, impacts the competence of endoderm to activate gene expression reflective of different organ lineages in response to subsequent RA/Wnt/BMP signals. Moreover our data suggest that somite-stage RA signaling acts along the A-P axis, promoting respiratory while suppressing pharynx potential in the AE, and midgut over hindgut potential in the PE.

2.5. Reiterative Wnt/BMP/RA impacts developmental competence of human definitive endoderm derived from pluripotent stem cells

We next set out to determine whether the reiterative Wnt and temporal RA signaling that we observed in *Xenopus* are conserved in hPSC cultures. To assess how early endoderm patterning impacts sub-

sequent Wnt/BMP/RA-mediated lineage induction, we adapted a step-wise protocol to induce respiratory fate in hPSCs (Green et al., 2011; Rankin et al., 2016). Following the differentiation of hPSCs into DE by treatment with Activin A for 3 days (d), we generated foregut endoderm by inhibiting BMP activity in the cultures via Noggin treatment from d3-6 (72 h), which mimics gastrula stage A-P patterning. Finally to induce *NKX2-1+* respiratory progenitors, cultures were treated with the GSK3 antagonist CHIR99021 and BMP4 from d6-9 (72 h).

We first set out to assess the temporal role of RA, and compared two different RA treatment periods: RA treatment for 72 h (d3-6) versus only a later 24-h RA treatment (d5-6), both in the presence or absence of Noggin (Supplemental Fig. S3). All conditions were followed by exposure to CHIR+BMP4 for 72 h (d6-9). RT-qPCR analysis of d9 cultures revealed that robust *NKX2-1* induction required d3-6 Noggin treatment and RA on d5-6 followed by CHIR+BMP4 (Supplemental Fig. S3C), consistent with previous reports (Huang et al., 2014; Rankin et al., 2016). Without RA the Noggin treated cultures exposed to CHIR +BMP4 expressed the pharyngeal marker *TBX1* but not *NKX2-1* (Supplemental Fig. S3B,C). Interestingly the 24-h RA treatment on d5-6 resulted in suppression of *TBX1* as early as d6 (data not shown), whereas *NKX2-1* was not robustly expressed until d9, supporting the idea that suppression of pharyngeal gene expression was a prerequisite for lung induction. In contrast to d5-6 RA treatment, earlier addition of RA from d3-6 was inhibitory to *NKX2-1* (Supplemental Fig. S3C) and resulted in the expression of mid-hindgut markers *GATA4* and *CDX2* (Supplemental Fig. S3E,F), indicating a posteriorizing effect of early RA. Overall these data are similar to our *Xenopus* results, as early RA treatment right after DE formation had a posteriorizing effect, suppressing anterior foregut lineages (pharynx and lung) and promoting mid-hindgut fates. RA treatment during a later time window makes the endoderm competent to activate respiratory gene expression in response to Wnt+BMP.

We next tested if early Wnt-dependent patterning also regulates the ability of human DE to respond to subsequent RA/Wnt/BMP signals (Fig. 5). In these experiments we kept the timing of RA exposure consistent on d5-6, and manipulated Wnt/β-catenin activity (via addition of CHIR99021) for 72 h during d3-6 when the DE is patterned into foregut by Noggin (experiment diagrammed in Fig. 5A). We then examined how the culture responded to subsequent CHIR+BMP4 on d6-9, assaying a range of endoderm lineage markers (Fig. 5B-J). Strikingly, we found that early CHIR treatment from d3-6 suppressed the induction of *NKX2-1* by RA+CHIR/BMP4 (Fig. 5F); instead the cultures expressed *GATA4* and *PDX1* consistent with a midgut/pancreatic fate (Fig. 5G,I). These results suggest that early Wnt treatment makes human endoderm refractory to respiratory induction. Interestingly we also found that when RA was omitted, the cultures treated with d3-6 Noggin (or CHIR+Noggin) followed by d6-9 CHIR +BMP expressed *FOXE1* and *PAX8*, reflecting pharyngeal / thyroid fate (Fig. 5C,D); RA treatment suppressed expression of these genes, which further suggests RA suppresses pharyngeal/thyroid potential in the foregut. Finally if both Noggin and RA were omitted, d3-6 CHIR followed by d6-9 CHIR+BMP treatment resulted in robust expression of *CDX2* and *SATB2* (Fig. 5H,J) but no other foregut or midgut markers, consistent with a distal hindgut identity (Munera et al., 2017). We conclude that, similar to *Xenopus*, early Wnt patterning impacts how hPSC-derived endoderm can respond to subsequent Wnt-mediated organ induction.
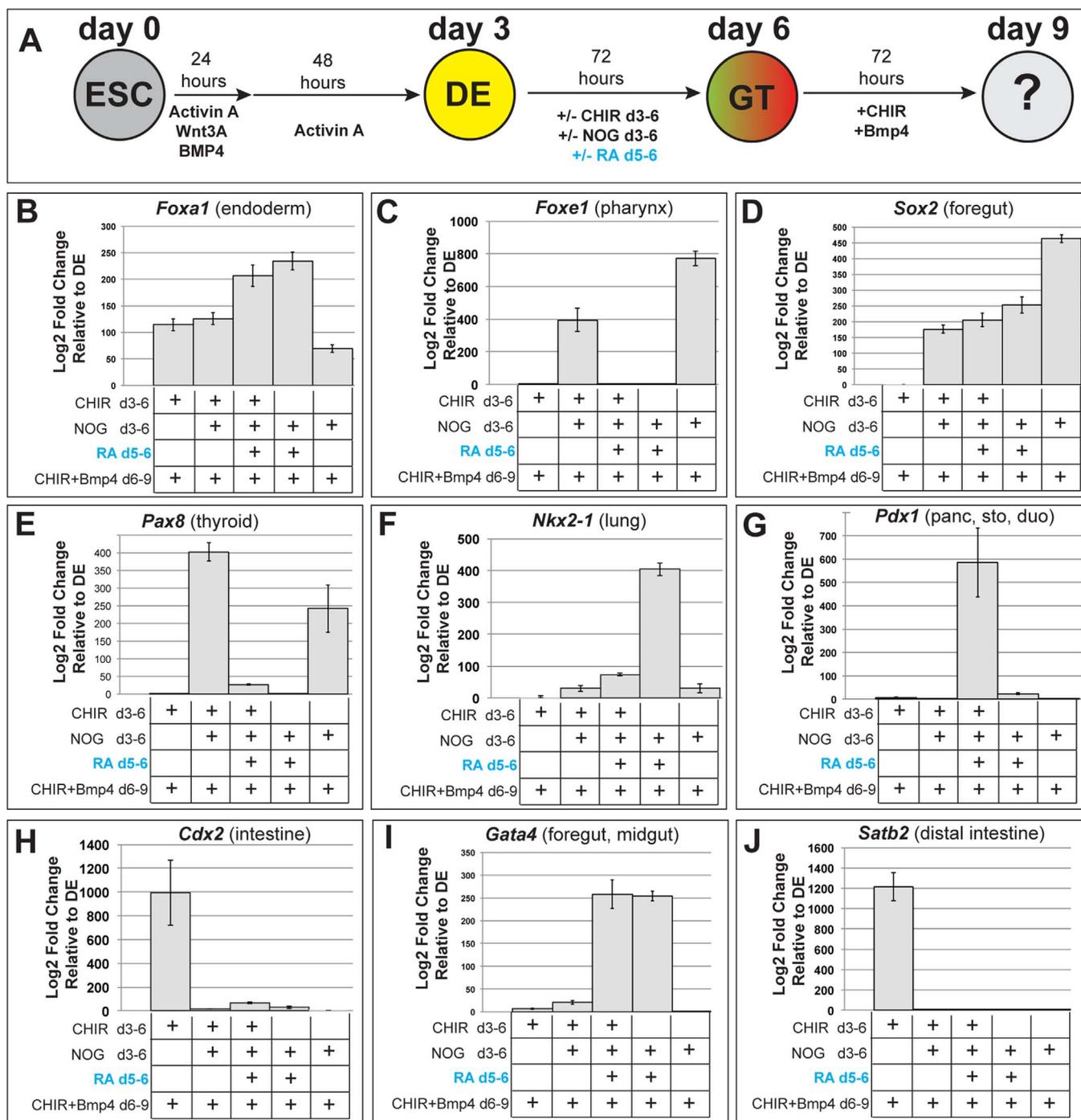
Fig. 5. Early CHIR treatment of human DE changes the ability of subsequent RA/CHIR/BMP4 to induce respiratory fate. (A) Experimental diagram. Definitive endoderm (DE) was induced from human embryonic stem cells (ESC) and then patterned into different types of gut tube (GT) endoderm by exposure to one or more of the following: 2 µM CHIR99021 (72 h, days 3–6 to posteriorize DE), 200 ng/mL Noggin (72 h, days 3–6, to anteriorize DE), and/or 2 µM RA (24 h, day5-6). Subsequently all types of GT endoderm was then exposed to 3 µM CHIR99021 + 50 ng/mL Bmp4 for 72 h (days 6–9). Cultures were then assayed by RT-qPCR for expression of the indicated genes (B-J). All culture conditions were performed in biological triplicate. Gene expression was normalized to the housekeeping gene *PBGD* and then log2 fold changes determined using the $2^{-\Delta\Delta Ct}$ method relative to expression in DE. Graphs display the average $2^{-\Delta\Delta Ct}$ value +/- SEM of the 3 biological replicates.

3. Discussion

In this study we investigated how reiterative signaling impacts developmental competence and lineage allocation in the developing endoderm. Although previous studies have examined individual roles of RA, Wnt, or BMP in the endoderm (Kraus and Grapin-Botton, 2012; Zorn and Wells, 2009), here provide an integrated view of how combinatorial Wnt/BMP/RA signaling orchestrates endoderm organogenesis, with remarkably similar temporal and spatial dynamics in

Xenopus, mouse, and hPSCs (summarized in Fig. 6). We draw three main conclusions: **1)** Wnt/BMP-mediated pattern present in the gastrula restricts domains of competence in the endoderm and impacts how cells respond to the same signals during lineage induction. Specifically early Wnt/BMP makes PE tissue refractory to subsequent RA/Wnt/BMP-dependent lung induction. **2)** RA has permissive role in AE respiratory competence and cannot instructively impart respiratory competence to the PE. **3)** RA has distinct spatial and temporal functions. During early gastrula stages RA inhibits foregut and promotes pancreatic and hindgut
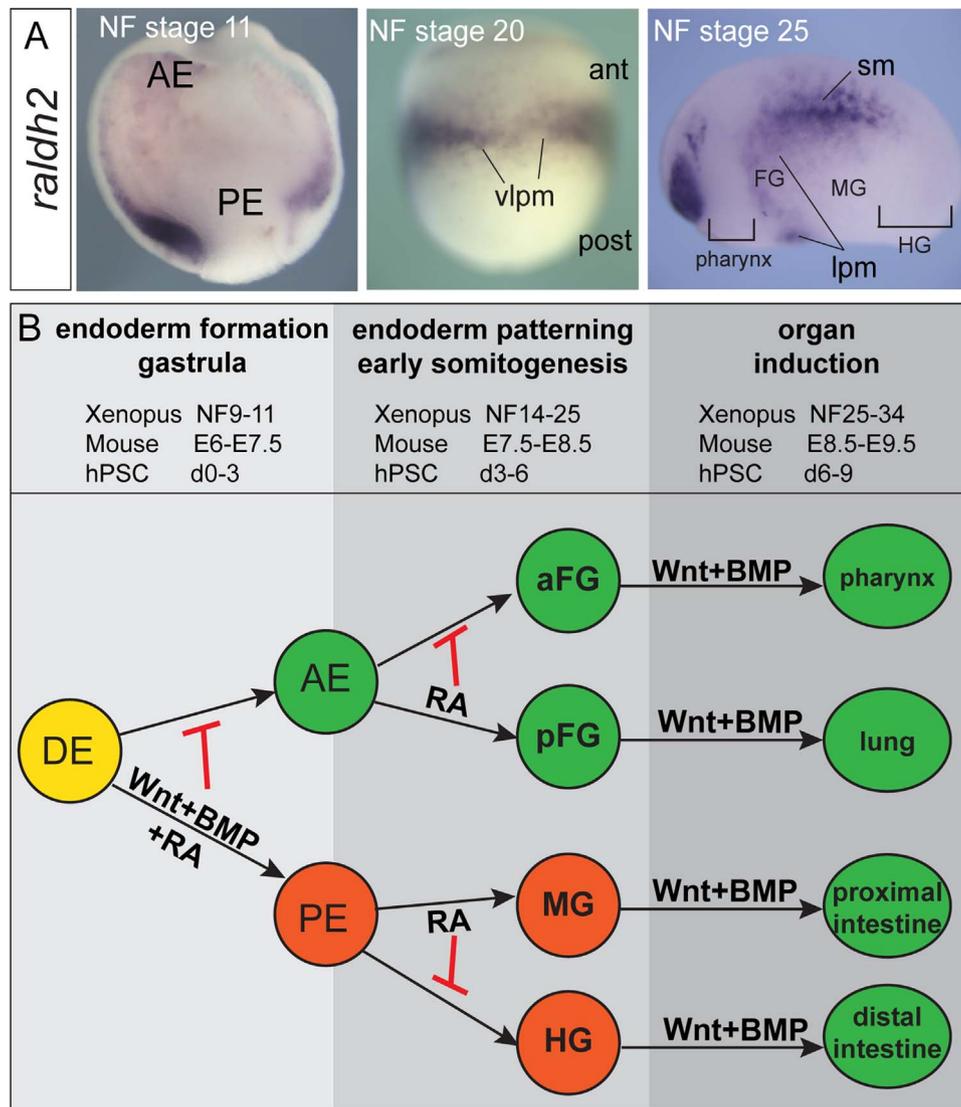
Fig. 6. Model depicting the reiterative use of Wnt, BMP, and RA signals during endoderm development. (A) In situ hybridization showing expression of the RA-synthesizing enzyme *raldh2* (*aldh1a2*) during early *Xenopus* development. Abbreviations: AE, anterior endoderm; PE, posterior endoderm; lpm, lateral plate mesoderm; FG, foregut; MG, midgut; HG, hindgut; sm, somitic mesoderm; ant, anterior; post, posterior. (B) Schematic depicting the reiterative roles for Wnt, BMP and RA signals during endoderm vertebrate development.

fate. Then during early somitogenesis stages RA promotes respiratory and suppresses pharyngeal fate within the AE, and promotes midgut over hindgut fate within posterior endoderm.

We demonstrated that cellular responses to RA/Wnt/BMP-mediated organ induction are dictated in part by earlier Wnt/BMP patterning in the gastrula. Although the underlying molecular mechanisms remain to be resolved, one likely possibility is that each signaling event not only activates a given lineage program but also suppresses alternative fates (Loh et al., 2014). For example, the hindgut transcription factor CDX2, which is activated by gastrula stage Wnt and BMP in the posterior, might not only promoting intestinal gene expression but also actively repress lung transcription, thus making PE refractory to lung induction. Our data also suggest that RA permissively promotes lung competence in the anterior foregut endoderm in part by suppressing pharyngeal fate, and in the posterior endoderm RA promotes midgut while suppressing distal *Satb2*+ hindgut fate. These functions of RA could occur directly or indirectly. RA-bound retinoic acid receptors (RARs) can either activate or directly repress transcription depending on the recruitment of different proteins to target genes. For example, RA signaling is required for recruitment of the PRC2 repressive complex and NCOR1/2 nuclear co-repressors to the *Fgf8*

locus to directly repress *Fgf8* transcription (Cunningham and Duester, 2015; Kumar et al., 2016). RARs may similarly repress key pharyngeal genes such as *Tbx1 and FoxE1*, thereby permitting lung genes to be activated by Wnt and BMP.

One caveat of our study is that we focused on the timing of RA rather than the concentration. In vivo bioavailability is tightly controlled by RA production and degradation (Cunningham and Duester, 2015), and this may play a key role in refining boundaries of competence. The concentrations of RA and other growth factors that we used in our ex vivo experiments were based on those from many previous publications (Bayha et al., 2009; Chen et al., 2004; Desai et al., 2006, 2004; Green et al., 2011; Loh et al., 2014; Wang et al., 2011, 2006) and on pilot experiments testing a range of concentrations (for RA; 5 µM to 50 nM) to identify the lowest dose in each system that would give a robust response in the 24-h period, which is the time frame that competence appears to be established in vivo (Rankin et al., 2016). In the future, it would be interesting to test whether a short pulse of high concentration RA has the same impact on as low RA concentrations over a longer period of time.

In addition to the role of RA, Wnt and BMP in endoderm patterning and lineage specification that we have examined here, RA, Wnt and

BMP signaling are also required in mammals to regulate later fetal lung growth, branching morphogenesis and proximal-distal pattern, (Desai et al., 2004; Frank et al., 2016; Herriges and Morrisey, 2014; McCauley et al., 2017; McCulley et al., 2015; Wang et al., 2006). While we did not formally test the impact of early RA on subsequent proximal-distal patterning, our mouse explants experiments cultured with lung mesenchyme underwent extensive growth and branching morphogenesis suggesting that both distal and proximal fates were probably present.

It is unclear how Wnt and BMP activate different transcriptional programs in different contexts. Wnt and BMP they are typically thought to activate transcription by simulating the recruitment of βcatenin and Smad1, along with co-activators, to transcription factor complexes bound to DNA cis-regulatory elements (Cadigan and Waterman, 2012; Gaarenstroom and Hill, 2014). However recent genome-wide studies in *Xenopus* have revealed a different picture where βcatenin and Smad1 chromatin binding is also associated with genes that are repressed by Wnt and BMP signaling (Kjolby and Harland, 2017; Nakamura et al., 2016; Stevens, 2017). Interestingly, approximately 25% of the foregut-specific transcriptome at NF20 appears to be directly repressed by βcatenin binding (Stevens, 2017); this supports the posteriorizing function of Wnt during endoderm patterning. These genomic analyses also found that 70–80% of the βcatenin and Smad1 binding in the genome were not associated with actively expressed genes. This raises the possibility that early Wnt and BMP signaling may result in βcatenin or Smad1 being recruited to cis-regulatory elements of lineage genes that will not be expressed until later in development and this may impact how these genes respond to later signaling. For example, gastrula stage Wnt-signaling may not only direct the activation of hindgut genes in PE but also actively repress lung genes in PE, and this inhibition may persists later in development, perhaps by modulating the epigenetic status of the chromatin.

Studies of endoderm differentiation in hPSCs indicate that 'epigenetic priming' of chromatin by histone modifications is a key step in the ability of developmental enhancers respond to inductive cues (Loh et al., 2014; Wang et al., 2015; Xie et al., 2013). But how cell signaling regulates epigenetic priming is still poorly understood. In one example, activated βcatenin has been shown to recruit the histone arginine methyltransferase Prmt2 to target promoters in the *Xenopus* blastula, and the resulting H3R8 dimethylation is essential for transcription several hours later (Blythe et al., 2010). In the context of A-P patterning of the nervous system, RA and Wnt interaction are reported to dynamically remodel the chromatin landscape of several HOX clusters by selectively removing H3K27me3 repressive marks from distinct chromatin domains to allow expression (Mazzoni et al., 2013). RA could similarly control whether respiratory versus pharyngeal loci available for activation by Wnt and BMP in the gut tube.

Resolving how RAR, βcatenin, and Smad effectors can bind cis-regulatory elements to activate or repress distinct genes in different contexts will require the genome wide identification of RA/Wnt/BMP-responsive cis-regulatory elements. For the most part such respiratory lineage enhancers are poorly characterized; even the cis-regulatory elements controlling *Nkx2-1* transcription in respiratory progenitors are still unknown in any vertebrate. Our work has defined a temporal framework for how reiterative signaling impacts endoderm lineage allocation, and we are poised to apply chromatin profiling technologies, such as ATAC-seq, at different developmental times in multiple model systems to help identify conserved lineage specific enhancers and define the molecular basis of developmental competence and transcriptional specificity during endoderm organogenesis.

4. Materials and methods

4.1. Xenopus methods

Xenopus laevis were maintained according to CCHMC IACUC protocols. Ovulation, in-vitro fertilization, and de-jellying of embryos

were performed as described (Sive et al., 2000). pBSRN3-Noggin, pBSRN3-eGFP (both linearized with Sfi1), and pCS107-Dkk1 (linearized with Not1) plasmids were used to synthesize mRNAs according to manufacturer's instructions. 25 pg of Noggin or 100 pg of Dkk1 RNA was injected into each ventral-vegetal V2.1 blastomere of 16-cell *Xenopus* embryos along with 25 pg eGFP RNA as a lineage tracer, which targets the *ventx1/2*-expressing posterior mesendoderm (Moody, 1987).

For *Xenopus* explant studies, DE, AE, or PE tissue was microdissected in 1X MBS (Modified Barth's Saline) + 50 µg/mL gentamycin sulfate (MP Biochemicals 1676045), and explants were then cultured in 0.5X MBS + 0.1% Fatty Acid Free BSA (Fisher BP9704) + 50 µg/mL gentamycin sulfate with the following concentrations of small molecules or recombinant proteins: 50 nM all-trans retinoic acid (Sigma R2625), 3.5uM Bio (TOCRIS 3194), 50 ng/mL recombinant human BMP4 (R & D systems 314-BP), 10 uM DEAB (Sigma D86256). For *Xenopus* whole embryo treatments, embryos were cultured in 0.1X MBS + 50 µg/mL gentamycin sulfate, with 10 uM DEAB from NF14-20 (Supplemental Fig. S1).

In-situ hybridization of *Xenopus* embryos and explants was performed mostly as described in Sive et al. (2000). Briefly, embryos and explants were fixed overnight at 4 °C in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), dehydrated directly into 100% ethanol, and stored at −20 °C. The following minor modifications to the in-situ protocol were used: proteinase K (ThermoFisher AM2548) on day 1 was used at 2 µg/mL for 10 min on explants and gastrula stage embryos, and at 5 µg/mL for 10 min on stage NF20-35 whole embryos; the RNAse A step was omitted on day 2; and finally the anti-DIG-alkaline phosphatase antibody (Sigma 11093274910) was used at a 1:5000 dilution in MAB buffer (maleic acid buffer, 100 mM Maleic acid, 150 mM NaCl, pH7.5) + 10% heat-inactivated lamb serum (Gibco 16070096) + 2% blocking reagent (Sigma 11096176001) on day2/3. Anti-sense DIG labeled in-situ probes were generated using linearized plasmid full-length cDNA templates with the10X DIG RNA labeling mix (Sigma 11277073910) according to manufacturer's instructions. Details on plasmids use to make in situ probes are available upon request.

For *Xenopus* immunofluorescence, embryos were fixed in 100 mM NaCl, 100 mM HEPES (pH7.5), 1.7% methanol-free paraformaldehyde for 1–1.5 h at room temperature and then dehydrated directly into Dent's post-fixative (80%Methanol / 20% DMSO) and stored at −20 °C. Primary antibodies were as follows: chicken anti-GFP (Aves GPF-1020; diluted 1:1000), rabbit anti-Nkx2-1 (Santa Cruz Biotechnologies sc-13040X; diluted 1:500), mouse anti-Sox2 (Abcam ab79351; diluted 1:1000), and rabbit anti-B-catenin (Santa Cruz Biotechnology sc-7199; red membrane staining of the gastrula embryo of Fig. S1C). Secondary antibodies were donkey anti-Chicken 488, donkey anti-rabbit Cy3, and donkey anti-mouse Cy5 (Jackson ImmunoResearch nos. 703-546-155, 711-166-152, and 715-175-151, respectively; all used at 1:1000 dilution). Briefly, embryos in Dent's fixative were serially rehydrated into PBS+0.1% TritonX-100 (PBSTr). Embryos, were bisect with a razor blade in PBSTr on an agarose-coated dishes and whole-mount staining was then performed on the gastrula halves or the tailbud foreguts. Both sample types were blocked for 2 h in PBSTr + 5% normal donkey serum (Jackson Immunoresearch 017-000-001) + 0.1% DMSO at room temperature and then incubated overnight at 4 °C in this blocking solution + primary antibodies (dilutions listed above). After extensive washing in PBSTr, samples were incubated overnight at 4 °C in PBSTr + secondary antibodies (dilutions listed above). Samples were again extensively washed in PBSTr, dehydrated directly into 100% methanol, cleared and imaged in Murray's Clear (2 parts benzyl benzoate, 1 part benzyl alcohol) using a metal slide with a glass coverslip bottom using a Nikon A1R confocal microscope.

RT-qPCR analysis of *Xenopus* explants was performed in biological triplicate with n=4 explants (DE, AE or PE) for each replicate. RNA was extracted using TRIzol (ThermoFisher 15596018) and purified using

the Direct-zol RNA miniprep plus kit (ZymoResearch R2070); 500 ng of RNA was used in cDNA synthesis reactions using Superscript Vilo Mastermix (ThermoFisher 11755050), all according to manufacturer's instructions. Real-time PCR reactions were carried out using the Powerup Sybrgreen Mastermix (ThermoFisher A25742) on ABI StepOnePlus or QuantStudio3 qPCR machines. Ornithine decarboxylase (*ocd*) was used as a reference gene for *Xenopus* samples. All Ct values for the experimental genes were normalized to the *ODC* Ct value, and then Log2 fold changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method, relative to the experimental gene's *ODC*-normalized expression in DE, AE, or PE cultured in isolation. Graphs display the average $2^{-\Delta\Delta Ct}$ value +/- SEM of the 3 biological triplicate samples. *Xenopus* RT-qPCR primer sequences are listed in Table S1.

4.2. Mouse methods

Wild-type CD1 mice were purchased from Charles River Laboratories (Wilmington, MA). *Tg(Sftpc:GFP)* mice were provided by Dr. Brigid Hogan (Duke University Medical Center, Durham, NC), and were originally generated by John Heath. *Raldh2+/-* heterozygous mice (Molotkov et al., 2005) were obtained from Dr. Gregg Duester (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA), and also carried the *RARE:lacZ* reporter allele. All mice were housed according to CCHMC IACUC protocols. Timed pregnant mice were sacrificed on gestational days E7.5, E8-8.5, or E9-9.5 and individual embryos were dissected out and staged according to somite number.

Mouse embryos were micro-dissected Hank's balanced salt solution (HBSS; ThermoFisher 14025092) using tungsten needles. To promote separation of germ layers, dissected embryo regions were incubated in HBSS + 1.25% pancreatin (Sigma P3292), 0.25% Difco trypsin 250 (ThermoFisher 27250018) for 20 min. The proteolytic enzymes were neutralized with HBSS containing 10% bovine serum albumin (Invitrogen 15561020) and 0.1 mg/mL DNase I (Sigma 11284932001) to reduce tissue stickiness. To isolate E8.5 foregut endoderm (FgE) from 8 to 10ss embryos, tungsten needles were used to cut at the anterior intestinal portal (AIP), and then as much of the neuro-epithelium as possible was removed without damaging the endoderm, and the foregut region was incubated for 20 min in the proteases listed above before endoderm isolation. To isolate E8.5 hindgut endoderm (HgE), the enzyme incubation was increased to 30 min. E7.5 and E8.5 endoderm explants were rinsed in complete serum-free differentiation medium (cSFDM) (2012), with B27 was used as a supplement instead of B27 + retinoic acid. Explants were enrobed in Cultrex Basement Membrane Matrix (Trevigen) and cultured in the matrix in cSFDM media with or without the following small molecules or recombinant proteins: 1 μM all-trans RA (Sigma R2625), 2uM CHIR99021 (C; TOCRIS 4423), 100 ng/mL human FGF10 (F10; R & D 345-FG), 25 ng/mL mouse FGF7 (F7; R & D 5028-KG). RA treatment was for 24 h, followed by 4–6 days of culture in C/F7/F10 as described in the figure legends.

Whole-mount immunostaining of mouse embryos and detection of B-gal activity in *Tg(RARE:lacZ)* experiments was performed as described in Rankin et al. (2016). The following primary antibodies were used: rabbit anti-Nkx2-1 (Seven Hills BioReagents WRAB-1231, 1:500) and rat anti-Ecadherin (R & D systems MAB7481, 1:1000). For immunostaining of mouse explants, they were carefully removed from the matrix, fixed overnight in PBS + 3.7% PFA at 4 °C, dehydrated into 100% methanol, and stored at −20 °C. Explants were rehydrated into PBSTr, blocked in PBSTr + 10% normal donkey serum (Jackson ImmunoResearch) for 1−2 h at room temperature, and then incubated overnight at 4 °C in blocking buffer + the following primary antibodies: rabbit anti-Nkx2-1 (Seven Hills BioReagents WRAB-1231, 1:500), rat anti-Ecadherin (R & D systems MAB7481, 1:1000), goat anti-FoxA2 (Santa Cruz Biotechnology sc-6554, 1:500), mouse anti-Sox2 (Abcam ab79351, 1:1000). After extensive washing, explants were incubated overnight in PBSTr + secondary antibodies (1:500, all raised in donkey;

Jackson Immunoresearch) at 4 °C. Explants were washed extensively in PBSTr, dehydrated in 100% methanol, and imaged in Murray's Clear using a Nikon A1R confocal microscope.

4.3. hPSC methods

Human ESC line WA01 (H1; WiCell) was maintained in feeder-free conditions on Matrigel (BD Biosciences) in mTesR1 media (Stem Cell Technologies). 3-day induction of DE was performed as briefly follows: hESCs were dissociated into single cell suspension using accutase and plated into a 24-well plates in mTesR1 with ROCK inhibitor Y-27632 (10 μM; Stemgent 04–0012). On the following day, cells were exposed to Day1 DE induction media: RPMI 1640 (ThermoFisher 61870036) + 1X non-essential amino acids (NEAA; ThermoFisher 11140050) + 25 ng/mL Wnt3a (R & D systems 5036-WN) + 10 ng/mL BMP4 (R & D 314-BP) + 100 ng/mL Activin A (R & D 338-AC). Day 2 DE induction media consisted of RPMI 1640 + 0.2% defined fetal bovine serum (dFBS; Gibco 16141079) + 1X NEAA + 100 ng/mL Activin A. Day 3 DE induction media consisted of RPMI 1640 + 2% dFBS +100 ng/mL Activin A. DE was then exposed to various patterning conditions over the next 72 h, all of which used RPMI 1640 media + 2% dFBS +1X NEAA containing the following small molecules or protein concentrations: 200 ng/mL human Noggin (R & D 6057-NG), 2uM CHIR99021 (TOCRIS 4423), or 2uM all trans retinoic acid (Sigma R2625). For the last 72 h of the culture, patterned endoderm was exposed to RPMI 1640 media + 2% dFBS +1X NEAA + 3uM CHIR99021 + 50 ng/mL BMP4.

All culture conditions were performed in biological triplicate. RNA was extracted from each condition and purified using the Nucleospin RNA II kit (Machery-Nagel 740955.250), and 500 ng of RNA was used in cDNA synthesis reactions using Superscript Vilo Mastermix (ThermoFisher 11755050) according to manufacturer's instructions. Real-time PCR reactions were carried out using the Powerup Sybrgreen Mastermix (ThermoFisher A25742) on ABI StepOnePlus or QuantStudio3 qPCR machines. Porphobilinogen deaminase (*PBGD*; Loh et al., 2014) was used as a reference gene for hPSC samples. All Ct values for the experimental genes were normalized to the *PBGD* Ct value, and then Log2 fold changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method, relative to the experimental gene's *PBGDC*-normalized expression in DE. Graphs display the average $2^{-\Delta\Delta Ct}$ value +/- SEM of the 3 biological triplicate samples. Human RT-qPCR primer sequences are listed in Table S1.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.11.018.

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