HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2017 February 17.

Published in final edited form as:

Cell Rep. 2016 June 28; 16(1): 66–78. doi:10.1016/j.celrep.2016.05.060.

A retinoic acid-hedgehog cascade coordinates mesoderm-inducing signals and endoderm competence during lung specification

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Abstract

Organogenesis of the trachea and lungs requires a complex series of mesoderm-endoderm interactions mediated by WNT, BMP, retinoic acid (RA) and hedgehog (Hh), but how these pathways interact in a gene regulatory network is less clear. Using *Xenopus* embryology, mouse genetics, and human ES cell cultures we identified a conserved signaling cascade that initiates respiratory lineage specification. We show that RA has multiple roles; first RA pre-patterns the lateral plate mesoderm and then it promotes *Hh* ligand expression in the foregut endoderm. Hh subsequently signals back to the pre-patterned mesoderm to promote expression of the lung-inducing ligands *Wnt2/2b* and *Bmp4*. Finally, RA regulates the competence of the endoderm to activate the Nkx2-1+ respiratory program in response to these mesodermal WNT and BMP signals. These data provide insights into early lung development and a paradigm for how mesenchymal signals are coordinated with epithelial competence during organogenesis.

In Brief

The regulatory network controlling respiratory epithelium specification is poorly understood. Rankin et al. find that an evolutionarily conserved RA-Hh signaling cascade is essential for

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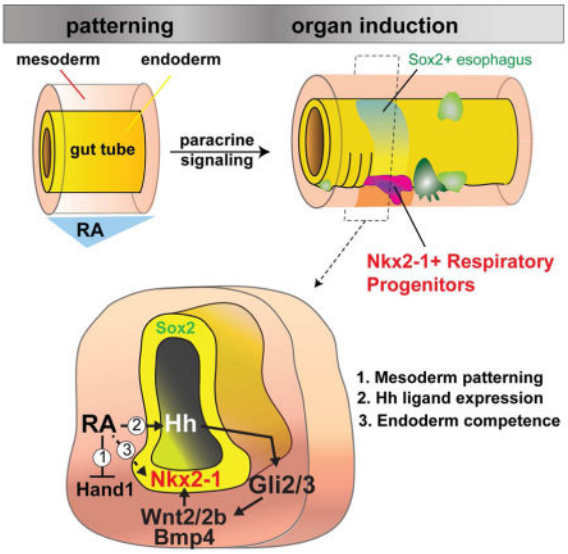
Author Contributions

Conceived and designed experiments: SAR, LH, KWM, JMS and AMZ. Performed Xenopus experiments: SAR, APK, CTA and AMZ. Performed mouse experiments: LH, EAG and JMS. Performed: hES cell experiments KWM, CMC. Analyzed the data and wrote the manuscript SAR, LH, KWM, JMW, JMS and AMZ. The manuscript has been seen and approved by all authors.

respiratory specification, and coordinates expression of lung-inducing Wnt2/2b ligands in the mesoderm with endodermal competence to respond.



Keywords

Retinoic acid; Hedgehog; Wnt; Bmp; Nkx2-1; Lung development; Organogenesis; *Xenopus*

Introduction

Embryonic development of the respiratory and digestive organs is controlled by a reiterative series of paracrine signals between the endodermal epithelium and the surrounding mesenchyme. Genetic analyses in mice have identified some of these key signals, but how different pathways interact to orchestrate organogenesis is still poorly understood. During gastrulation the endoderm is subdivided along the anterior-posterior (A-P) axis into broad foregut and hindgut domains by FGF, RA, Wnt, and BMP signals from the posterior mesoderm that promote hindgut progenitors, whilst secreted Wnt- and BMP-antagonists protect the anterior endoderm providing a low Wnt/BMP environment that is required to establish foregut progenitors (Zorn and Wells 2009; Kraus and Grapin-Botton 2012). Approximately one day later, spatially and temporally distinct Wnt, BMP FGF and RA signals no longer suppress anterior fate but instead direct the foregut progenitors into different foregut organ lineages (Zorn and Wells 2009; Kraus and Grapin-Botton 2012). How the different foregut lineages are specified is an active area of interest with implications for generating human tissue from pluripotent stem cells (PSCs) for disease modeling and regenerative medicine. The focus of this study is to resolve the complex interplay of mesoderm and endoderm signals that initiate lung organogenesis.

The respiratory lineage, marked by expression of the homeobox gene *Nkx2-1,* is induced during early somite stages of embryogenesis in a subset of the ventral foregut endoderm by canonical Wnt2 and Wnt2b (Wnt2/2b) ligands from the adjacent lateral plate mesoderm (lpm) (Goss et al. 2009; Harris-Johnson et al. 2009; Rankin et al. 2012). Mesodermal Bmp

signals cooperate with Wnt2/2b and also promote respiratory identity in the ventral endoderm by repressing expression of the transcription factor Sox2, which antagonizes *Nkx2-1* and promotes esophageal fate in the dorsal foregut (Domyan et al. 2011; Rankin et al. 2015). Despite the critical role of Wnt2/2b and Bmp4, how the localized expression of these ligands is coordinated with earlier A-P patterning of the gut tube is unclear.

After respiratory specification, reiterative Wnt, BMP and FGF signals continue to promote the expansion of the Nkx2-1+ progenitors and regulate branching morphogenesis. The RA and Hh signaling pathways also have important roles in lung development (Chen et al. 2010; Kugler et al. 2015; McCulley et al. 2015; Swarr and Morrisey 2015). Rat fetuses from vitamin A-deficient dams lacking RA and mice homozygous null for the RA-synthesizing enzyme *Raldh2* (Wilson et al. 1953; Wang et al. 2006) exhibit lung agenesis. Similarly, mouse embryos in which Hh signaling has been abrogated by dual deletion of the Hh transcriptional effectors Gli2 and Gli3 lack lung buds (Motoyama et al. 1998). However, it is currently unclear whether RA and Hh are simply required for expansion of lung progenitors or if they function earlier along with Wnt2/2b and Bmp4 to specify the respiratory lineage.

In general the gene regulatory network controlling respiratory lineage induction is poorly understood and the fact that epithelial competence to activate organ specific programs changes dynamically during development provides added complexity (Bossard and Zaret 2000; Horb and Slack 2001; Zaret and Carroll 2011). Indeed, the molecular mechanisms coordinating the regional expression of mesenchymal inductive signals with the acquisition of epithelial competence to respond to those signals are largely unknown for any visceral organ. This coordination ensures that the mesenchyme and epithelial lineages develop in synchrony for their integration into functioning organs. The importance of understanding these mechanisms is underscored by recent observations that the co-differentiation of epithelial and mesenchymal progenitors is critical for the generation of complex organoids from human PSCs (Spence et al. 2011; Takebe et al. 2013; McCracken et al. 2014).

Here we have used a combination of *Xenopus* embryology, mouse genetics, and human ES cell cultures to elucidate a conserved signaling network that orchestrates respiratory lineage specification. Our data indicate that a bidirectional RA-Hh signaling regulates lung specification. We show that RA has multiple roles; initially pre-patterning the lateral plate mesoderm and then promoting *Hh* ligand expression in the foregut endoderm. Hh subsequently signals back to the pre-patterned mesoderm to promote expression of the lung-inducing ligands *Wnt2/2b* and *Bmp4*. Finally, RA regulates the competence of the endoderm to activate the Nkx2-1+ respiratory program in response to mesodermal Wnt and Bmp. These studies link our understanding of early axial patterning to early lung organogenesis, and may inform strategies to generate complex multi-lineage respiratory tissue from PSCs.

Results

Bidirectional signaling between mesoderm and endoderm coordinates lung specification

To better understand the mesenchymal-epithelial signaling network regulating respiratory system induction (Figure 1A), we performed a series of explant culture experiments (Figure 1B) micro-dissecting foregut tissue from *Xenopus* embryos 20 hours post-fertilization (hpf)

at stage NF20; 4–7 somite pairs (4–7s). At this stage the endoderm is being patterned and cell identity is labile (Horb and Slack 2001; McLin et al. 2007). Three types of explants were prepared: 1) intact explants containing the endoderm with adjacent mesoderm, and non-neural ectoderm; 2) endoderm-only explants, in which the mesoderm and ectoderm were removed; and 3) mesoderm and ectoderm-only explants (referred to as mesoderm). Explants were cultured until 50 hpf (NF36; 36s), at which point they were assayed for the expression of genes involved in early lung development. Control embryos and intact explants confirmed that the orthologs of mammalian genes implicated in lung development were expressed in *Xenopus* with similar temporal-spatial dynamics including: *nkx2-1*, *sox2, shh, and foxa2* in the foregut endoderm as well as *wnt2*, *wnt2b*, *bmp2, bmp4, bmp7*, *gli1, gli2, gli3*, *foxf1*, *fgf7, and fgf10* in the splanchnic lpm (Figures 1C and S1) (Rankin et al. 2015).

As expected, respiratory epithelium markers *nkx2-1* and *sftpc* were not expressed in the endoderm-only explants, confirming the that mesodermal signals are necessary (Figures 1C). Surprisingly *wnt2* and *wnt2b* were not expressed in the mesoderm-only explants in contrast to *bmp2/4/7 and fgf7/10,* which were still robustly expressed (Figure 1C and S1). Mesoderm explants also expressed the RA-synthesizing enzymes *rdh10* and *raldh2* (*aldh1a2*), as well as the Hedgehog pathway transcriptional effectors *gli2 and gli3.* Interestingly, *gli1* and the Hh-receptor gene *patched 1* (*ptch1),* which are direct transcriptional targets of Gli2/3 and Hh signaling (Robbins et al. 2012), were almost undetectable in the mesoderm only explants, but were robustly expressed in the lpm of control embryos and intact explants (Figures 1C and S1). These data suggest that *wnt2/2b* expression in the *Xenopus* lpm requires signals from the endoderm, with Hh being a strong candidate. The Wnt2/2b-expressing mesenchyme then signals back to the endoderm to induce *nkx2-1+* respiratory fate.

Hh/Gli signaling is required for mesenchymal *wnt2/2b* and *bmp4* expression and respiratory induction in *Xenopus*

Although Hh signaling is known to have multiple functions in mammalian lung development (Kugler et al. 2015), its role in initial lineage specification is unclear. Analysis of Hh pathway components in *Xenopus* showed that *shh* and *dhh* are expressed in foregut endoderm, whereas *gli1/2/3, ptch1* and *smoothened* (*smo*) are expressed in the surrounding lpm prior to and during lung induction (Figure S2A). To test the hypothesis that Hh/Gli signaling is required for expression of the lung-inducing ligands *wnt2/2b* (Figure 2A), we used both pharmacological inhibition with cyclopamine (a Smo antagonist) and previously validated antisense morpholino oligos (MOs) to deplete Gli2 and Gli3 (Nguyen et al. 2005). Co-injection of Gli2-MO and Gli3-MO into the presumptive foregut region, or cyclopamine treatment from 20–48 hpf (NF20–35) resulted in a loss of *wnt2/2b*, reduced *bmp4,* and a failure to induce the *nkx2-1+* respiratory lineage (Figures 2B and S2B). Specification of the *nkx2-1+* thyroid (Figure S2B), liver, pancreas, and intestine were unaffected based on marker analysis (data not shown). Knockdown of Gli2 or Gli3 individually had no obvious effect (data not shown). Dramatically reduced *ptch1* and *gli1* expression confirmed the disruption of Hh signaling in Gli2/3-MOs and cyclopamine embryos (Figure S2B). Importantly injection of mRNA encoding human Gli2-activator (hGli2A) (Roessler et al. 2005) robustly rescued lung development in the Gli2/3-MO embryos (Figure 2B and S2B).

Examination of transgenic *Xenopus* embryos harboring a canonical Wnt-reporter *Tg(7xTcf:deGFP)* (Rankin et al. 2015) confirmed that cyclopamine treatment abolished β-catenin/Tcf transcriptional responses in the foregut epithelium (Figure S2C). Consistent with reduced *bmp4*, immunostaining revealed a loss of nuclear pSmad1 in the ventral foregut and a failure to down-regulate the dorsal marker Sox2 in cyclopamine treated explants (Figures 2B and S2B). We also observed a severe reduction of the mesenchymal transcription factor Foxf1, a known Hh-target (Kugler et al. 2015); however mesenchymal expression of Raldh2 was largely unaffected (Figure 2B). Consistent with a role for Hh signaling affecting cell proliferation and cell survival, we observed fewer phospho-histone H3+ mitotic cells in both the foregut epithelium and mesenchyme at 50 hpf (NF35; Figure S2D) and elevated activated caspase-3+ cells at a time point when the lung buds form (76 hpf, NF41; Figure S2E).

We conclude that Gli2 and Gli3 are redundantly required for respiratory specification in *Xenopus*. The data suggest that endodermal Hh signals, acting in a paracrine fashion via mesenchymal Gli2/3, are required for *wnt2/2b* and *bmp4* expression. Wnt2/2b and BMP then signal back to the endoderm to induce *nkx2-1* (Figure 2A). Consistent with this model, the Hh-agonist SAG was sufficient to induce *wnt2b* expression in mesoderm explants in the absence of the endoderm (Figure 2C). Moreover the GSK3 antagonist Bio (which stabilizes β-catenin) rescued and expanded the *nkx2-1*+ respiratory field in Gli2/3-MO embryos (Figure 2D), suggesting that Wnt/β-catenin activation is sufficient to specify respiratory progenitors downstream of Hh/Gli.

Gli2 and *Gli3* are necessary for *Wnt2/2b* expression and specification of respiratory progenitors in mice

We next sought to determine whether Hh/Gli signaling was required for *Wnt2/2b* expression and respiratory specification in mammals. Previous studies have shown that *Gli2−/−Gli3−/−* mutant mouse embryos arrest around E10.5 with lung bud agenesis (Motoyama et al. 1998). However the molecular basis of this phenotype is uncharacterized; it is unknown whether Nkx2-1+ progenitors are ever induced or alternatively that they simply fail to expand.

We confirmed that Hh pathway components are expressed in foregut prior to and during respiratory induction between E8.5 and E9.5, and analysis of *Gli1*^{LacZ} reporter embryos indicated that Hh signaling was robustly active in the foregut mesenchyme (Figure S3A). Examination of *Gli2−/−;Gli3−/−* embryos revealed a hypoplastic foregut that completely lacked Nkx2-1+ respiratory progenitors at E9.5 and E10.5, with the dorsal marker Sox2 being inappropriately expressed in the ventral epithelium (Figure 3A–F; data not shown). Consistent with our hypothesis, *Wnt2/2b* and *Bmp4* transcripts were lost or dramatically down-regulated, and pSmad1 was almost absent in E9.5 *Gli2−/−;Gli3−/−* embryos compared to controls (Figure 3G). We note that a thin layer of *Wnt2b+* lpm persists in the double mutants, but this was insufficient to support expression of the Wnt-target gene *Axin2* (Figure S3B). Analyses of various heterozygous combinations demonstrated that a single copy of either *Gli2* or *Gli3* was sufficient to support relatively normal Nkx2-1 expression. Thyroid, liver, pancreas, and hindgut appeared unaffected in double mutants at E9.5 (Figure S3B). Between E8.5–9.5, we observed decreased proliferation and elevated apoptosis in the

foreguts of *Gli2−/−;Gli3−/−* embryos relative to controls (Figure 3H–I). While some hypoplastic lpm was still clearly present in the *Gli2/3* mutants based on Vimentin staining, this remaining lpm displayed reduced expression of transcription factors Foxf1, Gata6, Islet1, Tbx5, *Osr1*, all of which are implicated in cardio-pulmonary mesoderm development (Figure S3B).

We next used an *ex vivo* culture assay to test whether loss of *Wnt2/2b* and *Bmp4* could account for the failed respiratory specification in Hh-deficient mouse embryos. Foregut explants from wild type E8.5 (8–12s) embryos were cultured for 2 days with or without the Hh-antagonist cyclopamine (Figures 3J–M and S3C). Control explants formed Nkx2-1+/Foxa2+ lung and Nkx2-1+/Foxa2- thyroid buds with relatively normal morphology (Figure 3J; n=4/5). In contrast, cyclopamine-treated explants lacked Nkx2-1+ respiratory cells and exhibited elevated cell death, but the thyroid was unaffected (Figure 3L; n=3/4), phenocopying the *Gli2/3* mutants. We then evaluated whether activation of the canonical Wnt and BMP pathways was sufficient to rescue respiratory fate in cyclopamine-treated explants. Treatment with CHIR99021 (a GSK3β inhibitor that stabilizes β-catenin) and recombinant BMP4 potently rescued (and expanded) Nkx2-1+/Foxa2+ respiratory progenitors in cyclopamine-treated (and control) explants (Figures 3J–M; n=3/3). CHIR and BMP4 did not rescue Foxf1 expression or mesenchymal cell death (Figure S3C). Thus when Hh signaling is disrupted, the endoderm is still competent to adopt a respiratory fate in response to Wnt and BMP activation. We conclude that in both *Xenopus* and mouse, Gli2/3 are redundantly required for robust expression of *Wnt2/2b* and *Bmp4* in the mesoderm, which in turn are necessary for the induction of Nkx2-1+ respiratory progenitors.

RA is required for *hh* ligand expression and lung induction in *Xenopus*

We next sought to identify the pathway upstream of Hh/Gli during lung induction. A good candidate was RA, because it regulates *Shh* expression in other developmental contexts (Rhinn and Dolle 2012; Cunningham and Duester 2015). In addition, RA acts as a morphogen defining A-P positional identity in the gut tube (Bayha et al. 2009) and is required for normal lung development in *Xenopus* and mouse (Desai et al. 2004; Desai et al. 2006; Wang et al. 2006; Wang et al. 2011). Although prior studies established a role for RA in pulmonary organogenesis, it was unclear if and how RA might regulate the Hh/Wnt-signaling cascade controlling specification. To investigate this possibility, we first confirmed that the RA signaling machinery is present in the *Xenopus* foregut mesendoderm from gastrulation and throughout lung induction stages. The RA-synthesizing enzymes Rdh10 and Raldh2 are expressed in the anterior lpm whereas the retinoic acid nuclear receptors, RAR alpha and RAR gamma, are present in both the lpm and endoderm (Figures S4A).

To test the hypothesis that RA promotes endodermal *hh* expression and Wnt2/2b-mediated lung induction, we disrupted endogenous RA signaling in *Xenopus* using three independent approaches: 1) depletion of Rdh10 and Raldh2 with previously validated Morpholinos (Strate et al. 2009); 2) pharmacologic inhibition of Raldh activity with the antagonist DEAB; or 3) treatment with the pan-RAR antagonist BMS493. Inhibitors were added from 17–27 hpf (NF15–25) prior to robust *shh* or *wnt2/2b* expression. All three types of RA-deficient embryos exhibited a severe reduction of *shh* and *dhh* in foregut endoderm at 50 hpf (NF36)

and lower *gli1* expression in the lpm, indicating reduced Hh activity (Figures 4A and S4C). RA-deficient embryos failed to express *wnt2/2b,* lacked Nkx2-1+ respiratory progenitors, and later exhibited complete agenesis of *sftpc+* lung buds (Figure 4A). Immunostaining confirmed effective MO knockdown of Raldh2 and Rdh10 (Figure S4B) and analysis of the direct RA target gene *hoxd1* validated the efficacy of the inhibition (Figure S4C). Importantly exogenous RA rescued *shh*, *wnt2/2b,* and lung development Rdh10+Raldh2-MOs embryos (Figure 4A). In addition, treatment of control embryos with exogenous RA expanded the *wnt2b* expression domain and caused ectopic *nkx2-1* and *sftpc* expression in the pharynx (Figure S4D) (Wang et al. 2011). We conclude that in *Xenopus* RA is required for Hh/Wnt-mediated lung specification.

Previous studies have established that RA regulates mouse lung bud outgrowth after respiratory lineage specification, by promoting Fgf10 expression (Desai et al. 2004; Chen et al. 2010), but this appeared to be later in pulmonary development than what we were examining in *Xenopus*. To test if there are temporally distinct roles for RA we treated intact *Xenopus* foregut explants (mesoderm + endoderm) with the RAR-antagonist BMS493 during two different time periods. Early RA-inhibition from stages NF15–25 resulted in a loss of *wnt2b, fgf10, nkx2-1* and *sftpc* (Figure S4E). In contrast, later RA-inhibition from stages NF25–38 caused a loss of *fgf10* and *sftpc*, but *wnt2b* and *nkx2-1* were still expressed, albeit at a reduced levels, indicating that respiratory fate was specified but that pulmonary progenitors failed to differentiate. Addition of recombinant FGF10 during the late RA-inhibition period (NF25–38) rescued *sftpc* and restored *wnt2b and nkx2-1* expression, however during the early BMS treatment period (NF15–25) FGF10 could not rescue *wnt2b, nkx2-1*, or *sftpc* (Figure S4E). Thus in *Xenopus* RA appears to have two temporally separable roles; an early requirement for *wnt2b* expression and respiratory specification, and a later Fgf10-dependent role in maintaining *wnt2b* and *nkx2.1* and promoting lung bud differentiation similar to what has been reported in mouse (Desai et al. 2004; Chen et al. 2010).

RA signaling is required for Wnt-mediated lung induction in mice

We next investigated whether early RA signaling was required for *Hh* and *Wnt2/2b* expression in mammalian lung induction. *Raldh2−/−* mutant embryos arrest right around the time that Nkx2-1 is first expressed (Desai et al. 2006), making it difficult to address the role of RA in respiratory specification. Maternal supplementation with exogenous RA between E7.5-E8.5 has been used to rescue this early arrest and demonstrate that RA promotes Fgf10-mediated lung bud outgrowth after specification (Wang et al. 2006; Chen et al. 2010). However, our *Xenopus* studies predicted that RA would regulate mouse lung induction around E7.5–8.5, precluding the use of this approach. We therefore turned to a whole embryo culture system where mouse embryos were isolated just after gastrulation at E7.5 and cultured in suspension for two days, by which time Nkx2-1+ respiratory progenitors can be detected. RA inhibition with DEAB or BMS493 resulted in axial truncations similar to *Raldh2−/−* mutants (Cunningham and Duester 2015), and a loss of Nkx2-1 expression in the respiratory domain relative to vehicle controls (Figure 4B). In contrast, Nkx2-1 expression in the brain and thyroid was present. Moreover BMS treated embryos exhibited a loss of *Shh* and *Ihh* in the foregut epithelium and a severe reduction of *Wnt2 and Wnt2b* (Figure 4C).

These data suggest that the role for RA upstream of Hh/Wnt-mediated respiratory specification is conserved in mammals.

RA regulates early lpm patterning independent of Hh/Gli

Our data suggest that early RA signaling acts upstream of Hh. To test this we activated the Hh pathway in RA-deficient *Xenopus* embryos by treating them with the Hh-agonist SAG. If the simple model was correct then SAG should rescue *wnt2/2b* and *nkx2-1* expression, but surprisingly it did not rescue even though an up regulation of *gli1* confirmed that the Hh pathway was activated (data not shown). This suggested that RA has roles in addition to promoting *shh* and *dhh.* One possibility was that early RA signaling, which patterns the nascent mesoderm and developing heart fields (Keegan et al. 2005; Ryckebusch et al. 2008; Deimling and Drysdale 2009), might be required for proper foregut lpm pattern and the ability to express *wnt2/2b* at NF34; we therefore examined lpm markers in more detail.

In control embryos at NF34 (50 hpf) *hand1* was broadly expressed in heart, pharyngeal mesenchyme, and posterior lpm, but was absent from the foregut lpm region that co-expresses *foxf1* and *wnt2/2b* (Figure 4A). However in Raldh2+Rdh10-depleted embryos, *hand1* was ectopically expressed in this foregut lpm whereas *foxf1* was severely reduced (Figure 4A). Addition of exogenous RA to the Raldh2+Rdh10-morphants had the opposite effect; it suppressed *hand1* and expanded *foxf1* (Figures 4A). Examination of RA-deficient embryos earlier at 27 hpf (NF25) revealed disrupted lpm patterning even before *shh* and *wnt2/2b* were expressed. Consistent with previous reports, RA-inhibition expanded expression of the both the pharyngeal / heart field marker *islet1* and the mid-hindgut mesoderm marker *wnt8* (Figure S4F). These expanded domains came at the expense of reduced *foxf1, osr1, and gata4/5/6,* which are normally expressed in a domain of anterior lpm overlying the presumptive lung field (boxed region in Figure S4F). Intriguingly, Hh-inhibition did not result in loss or reduction of *foxf1, osr1* and *gata4/5/6* nor in the expansion of *wnt8* at this stage (Figure S4F), although *islet1* was modestly expanded. Thus it appears that RA promotes lung-inducing mesenchyme through two mechanisms: 1) RA acts within the mesoderm, independent of Hh to establish a *foxf1+/hand1-* domain in the foregut lpm that is permissive to express *wnt2/2b*; and 2) RA also stimulates expression of endodermal Hh ligands, which then are necessary for *wnt2/2b* expression within the *foxf1+/hand1-* domain.

RA is necessary in both the mesoderm and endoderm for *Xenopus* lung specification

To directly test the idea that RA has distinct tissue specific functions, we microinjected a dominant-negative RA receptor (Sharpe and Goldstone 1997) into either the presumptive endoderm or mesoderm (endo-dnRAR and meso-dnRAR, respectively) of *Xenopus* embryos. Co-injection of GFP mRNA tracer confirmed accurate lineage targeting (Figure S5). Meso-dnRAR resulted in loss of *wnt2/2b* and *nkx2-1* as well as ectopic *hand1*, but endodermal *shh* was unaltered (Figure 5A). On the other hand, endo-dnRAR resulted in loss of *shh, wnt2b*, and *nkx2-1,* but no change in *hand1* (Figure 5A). This supports the hypothesis that RA has distinct endoderm and mesoderm functions; the loss of *wnt2/2b* in meso-dnRAR is due to the early mesoderm patterning function of RA, whereas the loss of *wnt2/2b* in endo-dnRAR is due to RA's role in promoting Hh expression.

We further evaluated these distinct RA functions by determining if Hh or Wnt activation could rescue lung development in meso-dnRAR and endo-dnRAR embryos. Hh activation by SAG was unable to rescue *wnt2/2b* or *nkx2-1* in meso-dnRAR embryos (Figure 5B), supporting the model that early lpm patterning by RA is a prerequisite for Hh/Gli-dependent *wnt2/2b* expression. However, Wnt/β-catenin activation by Bio robustly rescued and expanded *nkx2-1* in meso-dnRAR embryos (Figure 5B). Hh and Wnt activation had a very different effect on endo-dnRAR embryos, where SAG rescued *wnt2/2b* expression. Thus the loss of *wnt2/2b* in endo-dnRAR embryos is due to the lack of paracrine Hh signals. Surprisingly, neither SAG, Bio nor SAG + Bio, rescued *nkx2-1* in endo-dnRAR embryos (Figure 5B and data not shown), suggesting that RA-deficient endoderm is unable to activate the lung program in response to Wnt/β-catenin. We conclude that RA acts in both mesoderm and endoderm to regulate both the expression of lung inducing signals and the competence to respond.

RA regulates the competence of the endoderm to adopt a respiratory fate in response to Wnt and BMP

We further tested the possibility that RA regulates competence of the endoderm to respond to lung-inducing signals using *Xenopus* endoderm explants. When foregut endoderm was isolated at NF22 (24 hpf; 7–10s), after the time when endogenous RA was likely to have acted (based on Figure S4E), and treated with a combination of Bio + BMP4 from NF25–38 they robustly expressed *nkx2-1* and *sftpc*, demonstrating that the endoderm was competent to adopt a respiratory fate (Figure 6A). BMP4 was required to down-regulate *sox2* expression, but neither BMP nor Bio alone induced *nkx2-1* (Figure 6A). If embryos were pretreated with the RAR-antagonist BMS493 from NF14–22 and then endoderm explants were dissected, they did not express *nkx2-1* and *sftpc* in response to Bio+ BMP4 (Figure 6A). The fact that the Wnt- and BMP-target genes *cyclind1* and *id4* were still up regulated indicated that the general ability of the explants to activate Wnt/BMP-target genes was not compromised by RA inhibition.

These experiments suggest that between NF14–22 (16–24 hpf) endogenous RA from the mesoderm is required to impart respiratory competence to the endoderm. Consistent with this hypothesis, if endoderm was isolated earlier at NF14, it did not express *nkx2-1* and *sftpc* in response to subsequent Bio and BMP4 treatment (Figure 6B). However, if this early endoderm was pretreated with exogenous RA from NF15–25, and then with Bio + BMP4, *nkx2-1* and *sftpc* expression was induced (Figure 6B) indicating that RA imparted competence. Consistent with our previous experiments, RA regulated *shh* expression in the explants, raising the possibility that competence might involve Hh signaling. However co-treatment with RA + cyclopamine to block Hh activity did not prevent respiratory induction by Bio+Bmp4 (data not shown). These data indicate that during early somite stages of development RA/RAR activity regulates the competence of the endoderm to execute the *nkx2-1+* respiratory program in response to Wnt and BMP, and that this function of RA is Hh-independent.

RA promotes respiratory competence in differentiated human ESCs

The embryonic lethality and limited material from RA-deficient mouse embryos make it very difficult to test the role of RA in epithelial competence with this system. We therefore turned to human ESCs as a model. Recent protocols to generate airway epithelium from hESCs include RA in a cocktail of factors to induce foregut fate (Huang et al. 2014), however it is not clear how RA acts in this context. To test whether RA promotes respiratory competence of human foregut (Figure 7A) tissue, we first induced definitive endoderm (DE) from hESCs by 3 days (d0–3) of Activin treatment. The DE was then patterned into SOX2+ foregut tissue by addition of Noggin for 3 days (d3–6), which inhibits BMP and mimics endogenous A-P patterning (Green et al. 2011; McCracken et al. 2014). We then evaluated whether addition of RA on d5–6 influenced the ability of the foregut cultures adopt a respiratory fate to respond BMP and WNT activation. In the absence of RA, the human foregut cultures failed to activate *NKX2-1* after 3 days (d6–9) of BMP4 and CHIR99021 (a GSK3 antagonists that stabilizes β-catenin) however *SOX2* was down-regulated as expected after BMP4 treatment (Figure 7B). In contrast, RA-treated foregut cultures robustly expressed *NKX2-1* mRNA at d9 in response to CHIR99021 and BMP4 (Figure 7B), and NKX2-1 protein was detected throughout the culture (Figure7C). Importantly the thyroid marker *PAX8* was undetectable in these cultures, indicating that *NKX2-1* reflected respiratory and not thyroid fate (data not shown). RT-PCR analysis at d6 revealed that the RA treatment alone was not sufficient to induce *NKX2-1*, however DE marker *FOXA1,* a critical endoderm “pioneering” transcription factor (Zaret and Carroll 2011), was significantly up-regulated in response to RA (Figure 7B). These results support the conclusion that, like in *Xenopus*, RA signaling is required at a distinct patterning stage for the competence of human foregut progenitors to adopt a respiratory fate in response to Wnt and BMP.

Discussion

Twentieth century embryology established that a series of paracrine interactions between the mesenchyme and epithelium directs gut tube organogenesis (Zorn and Wells 2009; Kraus and Grapin-Botton 2012), however the molecular mechanisms that coordinate dynamic inducing signals with progressive competence have remained elusive. We have defined a bi-directional signaling cascade between the mesoderm and the endoderm that links post-gastrula patterning to the earliest steps in lung organogenesis, and provides a mechanistic basis for how the expression of mesenchymal-inducing signals are coordinated with epithelial competence during respiratory lineage specification. Our results, integrated with previous studies, support the RA-Hh-Wnt signaling model in Figure 5C, which appears largely conserved between frog, mouse, and human. We show that RA initiates this signaling cascade governing respiratory development during early somite stages, and that in *Xenopus* RA has at least three distinct roles.

First, RA produced in the mesoderm patterns the early *Xenopus* lpm into distinct regions, including a *foxf1+/ hand1-* domain overlying the presumptive lung field. RA simultaneously restricts the cardiac field and the more posterior lpm, thereby allocating pulmonary, cardiac and intestinal mesoderm progenitors. This lpm patterning occurs about 12–15 hours prior to

lung induction in *Xenopus* and appears to be independent of Hh. RA regulation of the early cardiac field has been well established (Keegan et al. 2005; Ryckebusch et al. 2008; Deimling and Drysdale 2009), but the impact on lung-inducing mesenchyme was previously unexplored. We postulate that the overlap of *foxf1, gata4/5/6,* and *osr1* expression, combined with the lack of *islet1*/*hand1,* demarcates an anterior foregut lpm territory that is able to express *wnt2/2b* in response to subsequent HH/Gli signals.

Second, RA promotes *Hh* ligand expression in the foregut endoderm. Hh signals back to the mesenchyme to stimulate Gli2/3, which are redundantly required for robust *wnt2/2b* and *bmp4* expression. The fact that *bmp4* is reduced in Hh-deficient but not in RA-deficient embryos suggests that the regulation of *bmp4* expression is complex. Indeed *bmp4* was maintained in *Xenopus* mesoderm explants cultured without the Hh-expressing endoderm (unlike *wnt2/2b*). Precisely how *bmp* expression is regulated is unclear, but we postulate that RA and Hh might have both positive and negative inputs. Overall the reduced *Wnt2/2b* and *Bmp4* in Hh/Gli-deficient *Xenopus* and mouse embryos results in failure to induce Nkx2-1+ respiratory progenitors and complete lung agenesis. It is unclear whether *wnt2/2b* or *bmp4* transcription is directly regulated by Gli transcription factors or whether the primary role of Hh/Gli is to maintain survival and proliferation of the mesenchyme. In any case, the rescue of respiratory fate in Hh-deficient embryos by β-catenin activation indicates that Hh/Gli act upstream of canonical Wnt2/2b.

The third critical role of RA in this model is to impart respiratory competence to a subset of the foregut endoderm. In both *Xenopus* and hESCs RA was necessary and sufficient for the foregut progenitors to activate the NKX2-1+ respiratory program in response to Wnt and BMP. This role of RA may help explain why Wnt/β-catenin suppresses lung fate in the foregut endoderm during gastrulation (McLin et al. 2007; Zhang et al. 2013), whereas a day later, after the RA signal, Wnt/β-catenin induces lung fate. Thus by simultaneously acting in the mesoderm and the endoderm RA initiates a mutually dependent signaling cascade that keeps inducing signals in register with competence. Our data indicate that the RA functions described here are temporally distinct from RA's previously known role in promoting Fgf10-mediated lung bud growth in mice (Desai et al. 2004; Chen et al. 2010).

It remains to be determined whether RA acts directly on the *Nkx2-1* locus or if RA regulates competence indirectly via intermediate factors. The enhancers that control *Nkx2-1* expression in the respiratory progenitors have not been identified in any species, thus it is unknown whether RAR-RXR or even β-catenin/Tcf complexes directly regulate its transcription. A recent study in hESCs found that epigenetic priming of pancreatic and hepatic enhancers was functionally correlated with developmental competence (Wang et al. 2015). The binding of RA-activated RAR-RXR complexes to retinoic acid response elements (RARE) can cause the dissociation of histone deacetylases and co-repressors and the recruitment of histone acetylases, which mediate chromatin relaxation and enable transcription (Cunningham and Duester 2015). Determining if and how RA regulates the epigenetic landscape of prospective lung progenitors is a key question to address in the future.

It is also possible the RA regulates competence indirectly via Foxa, Gata, or Hox transcription factors. Hox factors provide positional information during embryogenesis (Wellik 2009) and can mediate competence to respond to FGF in the neural tube (Shimizu et al. 2006), although the mechanisms are unclear. Interestingly Hox5 promotes *Wnt2/2b* and *Bmp4* expression in the mouse fetal lung mesenchyme (Hrycaj et al. 2015). Zaret and colleagues have shown that in hepatic development Foxa and Gata4–6 are pioneering factors that bind and open chromatin making it permissive for transcription (Zaret and Carroll 2011). They could play a similar role in the respiratory lineage as our preliminary *Xenopus* experiments indicate that *foxa1, foxa2,* and *gata6* expression is RA-dependent (data not shown) and we found that RA treatment of human foregut cultures significantly up-regulated *FOXA1* expression. Interestingly, in breast cancer cell lines genome wide DNA-binding patterns of RA-RAR complexes are highly coincident with Foxa occupancy (Ross-Innes et al. 2010). RARs have also been reported to physically interact with β-catenin (Yasuhara et al. 2010), and thus it is possible that RAR, β-catenin, and Foxa factors cooperate to stimulate respiratory program transcription.

In sum, RA-Hh-Wnt regulatory modules have multiple temporal- and cell-context dependent roles during respiratory development and in the other organ systems (Cunningham and Duester 2015; Kugler et al. 2015; McCulley et al. 2015; Swarr and Morrisey 2015), illustrating the importance of determining the regulatory interaction in each tissue. Disruptions in these pathways have also been implicated in various congenital malformations. In the 1950s, in work performed at Cincinnati Children's Hospital, Wilson and Warkany suggested that maternal deficiency of Vitamin A, the precursor of RA, could result in offspring with foregut malformations including right lung agenesis (Wilson et al. 1953). Our data may help provide a mechanistic basis for understanding of such birth defects and may inform strategies to direct the differentiation of complex multi-lineage lung tissue from human iPSCs.

Methods and Materials

Xenopus and mouse methods

Animal experiments were performed according to CCHMC IACUC approved protocols. *Xenopus* embryo culture, microinjection, and small molecule treatments were performed as previously described (Rankin et al. 2012). Full details of the Morpholino, mRNA, and inhibitor experiments are provided in the supplemental experimental procedures. All experiments were repeated at least three independent times with similar results. Xenopus embryo in-situ hybridization and immunofluorescence were performed as previously described (Rankin et al 2012) using antibodies listed in supplemental table S1.

Gli2+/− and *Gli3+/−* mutant mice (Motoyama et al., 1998) and the $Gli1^{LacZ}$ reporter mice were provided by Drs. Sam Brugmann, and Rolf Stottmann. Control and mutant mouse embryos were aged matched by somite number. Details of mouse explant culture conditions, in situ hybridization and immunostaining are described in the supplemental experimental procedures, using antibodies listed in supplemental table S2.

Human ESC Methods

Human ESC line WA01 (H1; WiCell) was maintained in feeder-free conditions on Matrigel (BD Biosciences) in mTesR1 media (Stem Cell Technologies). DE differentiation was performed as previously described (McCracken et al., 2014). Briefly, hESCs were dissociated into single cell suspension using accutase, and plated into a 24-well plate in mTesR1 with ROCK inhibitor Y-27632 (10 μM; Stemgent). On the following day, cells were exposed to Activin A (100 ng/mL; Cell Guidance Systems) for three days in RPMI 1640 media (Invitrogen) containing increasing concentrations of 0%, 0.2%, and 2.0% defined fetal bovine serum (dFBS; Invitrogen). DE was patterned into Sox2+ foregut endoderm by three-day culture in RPMI 1640 media with 2.0% dFBS with recombinant Noggin (200 ng/mL; R&D Systems). All-trans RA (2 μM; Sigma R2625) was added to the media on final day of Noggin treatment (d5–6). Foregut cells were then cultured for three more days (d6–9) in RPMI 1640 with 2.0% dFBS, CHIR99021 (2 μM; Stemgent), and BMP4 (10 ng/mL; R&D Systems). qPCR and immunostaining methods including primer sequences and antibodies are described in the supplemental materials and methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by:

NIH grant R01 HL114898 to A.M.Z.

We are grateful to Drs. Brugmann and Stottmann for kindly providing reagents. We thank the Zorn and Wells labs as well as the Endoderm club for helpful discussions, and Brian Gebelein comments on the manuscript. This work was supported by grants NIH grants DK092456 and AI116491 to JWM, and HL114898 to AMZ.

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Highlights

- An RA and Hh cascade is essential to specify the respiratory epithelium lineage
- RA and Hh regulate expression of lung-inducing Wnt2/2b and Bmp4
- RA coordinates expression of mesoderm inducing signals with endoderm competence
- Respiratory specification pathways are conserved in Xenopus, mouse and hES cells

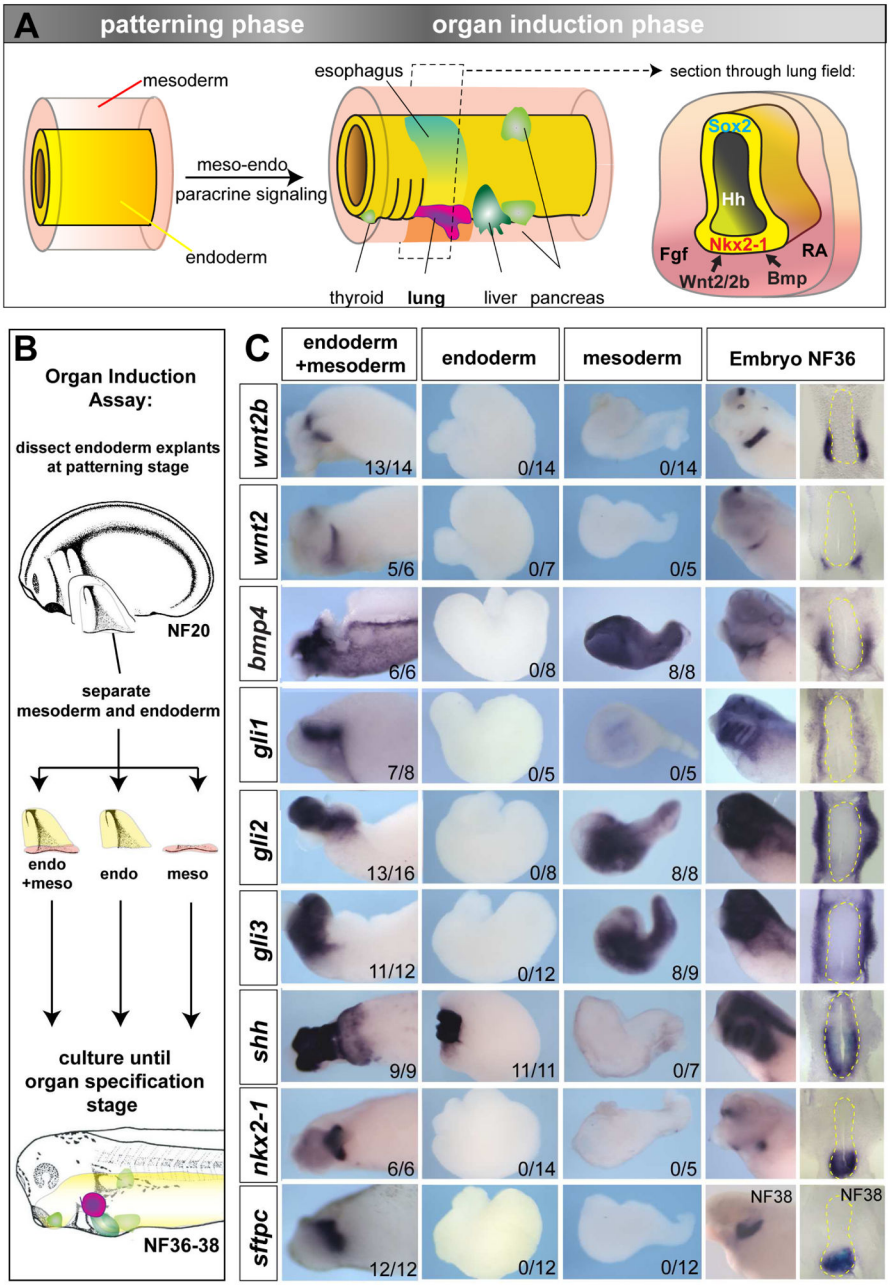

Figure 1. Bi-directional endoderm-mesoderm signaling is required for respiratory specification in the *Xenopus* foregut

(A) Model of mesoderm-endoderm paracrine signaling during foregut organogenesis.

(B) Schematic of the *Xenopus* organ induction assay. Foregut endoderm (yellow) and mesoderm (orange) were isolated at NF20 and cultured together or separately until NF36–38.

(C) In-situ hybridization using the indicated probes reveals that induction of *nkx2-1/sftpc+* respiratory progenitors requires the mesoderm, whereas *wnt2*, *wnt2b*, and *gli1* expression in the mesoderm requires the endoderm (outlines in yellow dashed line in embryo sections).

The number of explants expressing the marker is indicated in the lower right of each panel. See also Figures S1.

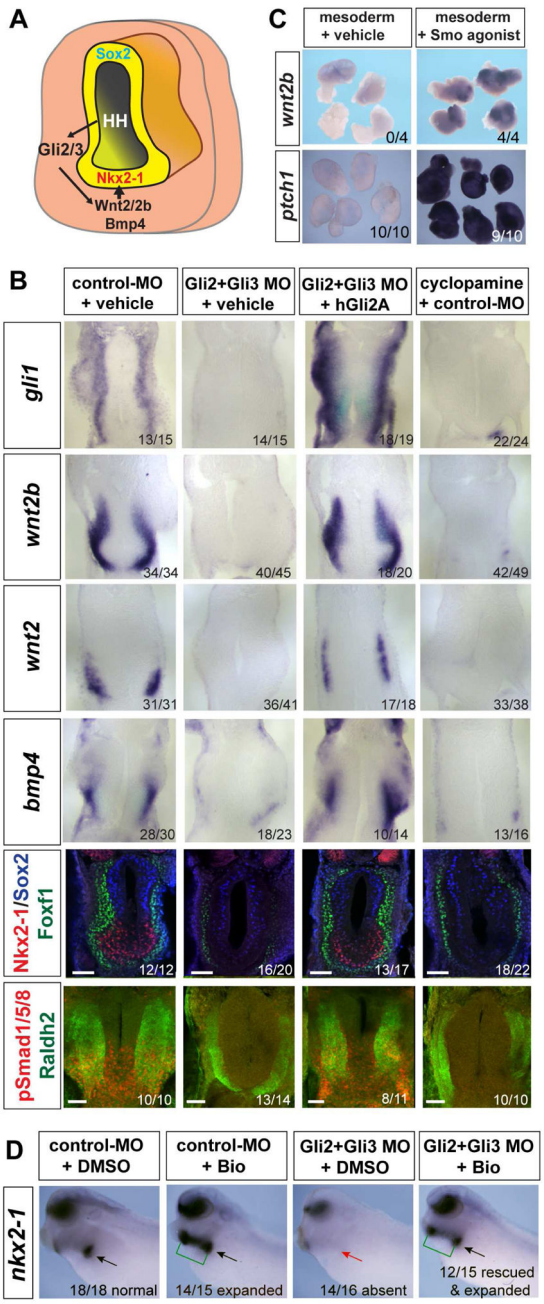

Figure 2. Hh/Gli signaling is required for respiratory Wnt-mediated specification in *Xenopus*

(A) Schematic of a section through the lung field showing a model of Hh/Gli regulation of Wnt/BMP-mediated respiratory induction.

(B) In-situ hybridization and immunostaining of foregut sections from NF34 stage control and Hh/Gli-deficient *Xenopus* embryos generated by either Gli2+Gli3-MO injection or cyclopamine treatment from NF20–34. Expression of the lung-inducing *wnt2/2b* and *bmp4* ligands and Nkx2-1+ respiratory specification requires Hh/Gli signaling, which can be rescued by injection of a human Gli2 mRNA. Foxf1+ lpm and nuclear pSmad1/5/8 are also reduced in Hh-deficient embryos. Scale bar 50 μM.

(C) Expression of *ptch1* and *wnt2b* are activated in mesoderm explants treated with the Hh-pathway agonist SAG from NF20–34.
(D) Activation of β-catenin by Bio treatment from NF28–34 rescued and expanded (bracket) the *nkx2-1* respiratory domain in Gli2+3 MO-injected embryos, indicating that Hh signaling acts upstream of Wnt. Red arrow indicated absent *nkx2-1* expression. See also Figures S2.

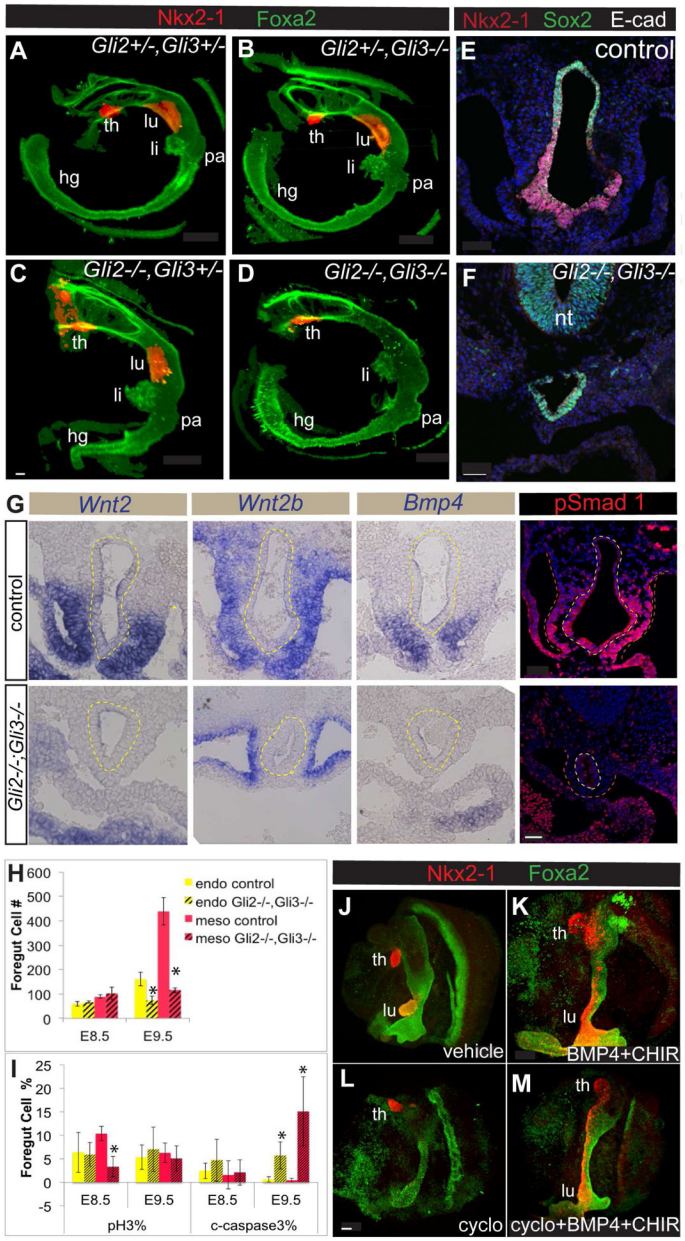

Figure 3. Hh/Gli signaling is required for mouse respiratory specification

(A–F) Immunofluorescence of control and *Gli2;Gli3* mutant mouse embryos at E9.5 (23–26s) in whole mount (A-D) and sections (E–F) reveal that Gli2 and Gli3 are required for Nkx2-1+ respiratory progenitors. th, thyroid; lu, lung; li, liver; pa, pancreas; hg, hindgut; nt, neural tube.

(G) In-situ hybridization and immunostaining of E9.5 (23–27s) sections show that *Wnt2, Wnt2b* and *Bmp4* expression as well as pSmad1 require Gli2 and Gli3. Yellow dotted line outlines the foregut epithelium and the red dotted line indicated the splanchnic lpm.

(H–I) The average number of foregut cells (H) as well as the average % of mitotic (phospho-Histone H3+) and apoptotic (cleaved caspase-3+) foregut cells (I) was quantified from immunostained sections (n=3 sections /embryo) of control and *Gli2;Gli3* mutant embryos at

E8.5 and E9.5 (n= 3 embryos each)..Total cell number was based on DAPI staining. +/− standard deviation, *P<0.05 in pairwise Student's t-test of mutant and age matched controls. (J–M) Treatment of E8.5 mouse foregut explants with cyclopamine (cyclo) for 2 days results in loss of Nkx2-1+ respiratory progenitors phenocopying the *Gli2−/−;Gli3−/−* mutants. Nkx2-1+/Foxa2+ respiratory fate in Hh-signaling deficient explants was rescued addition of by addition of CHIR (to stabilize β-catenin) and BMP4. th, thyroid; lu, lung. See also Figures S3.

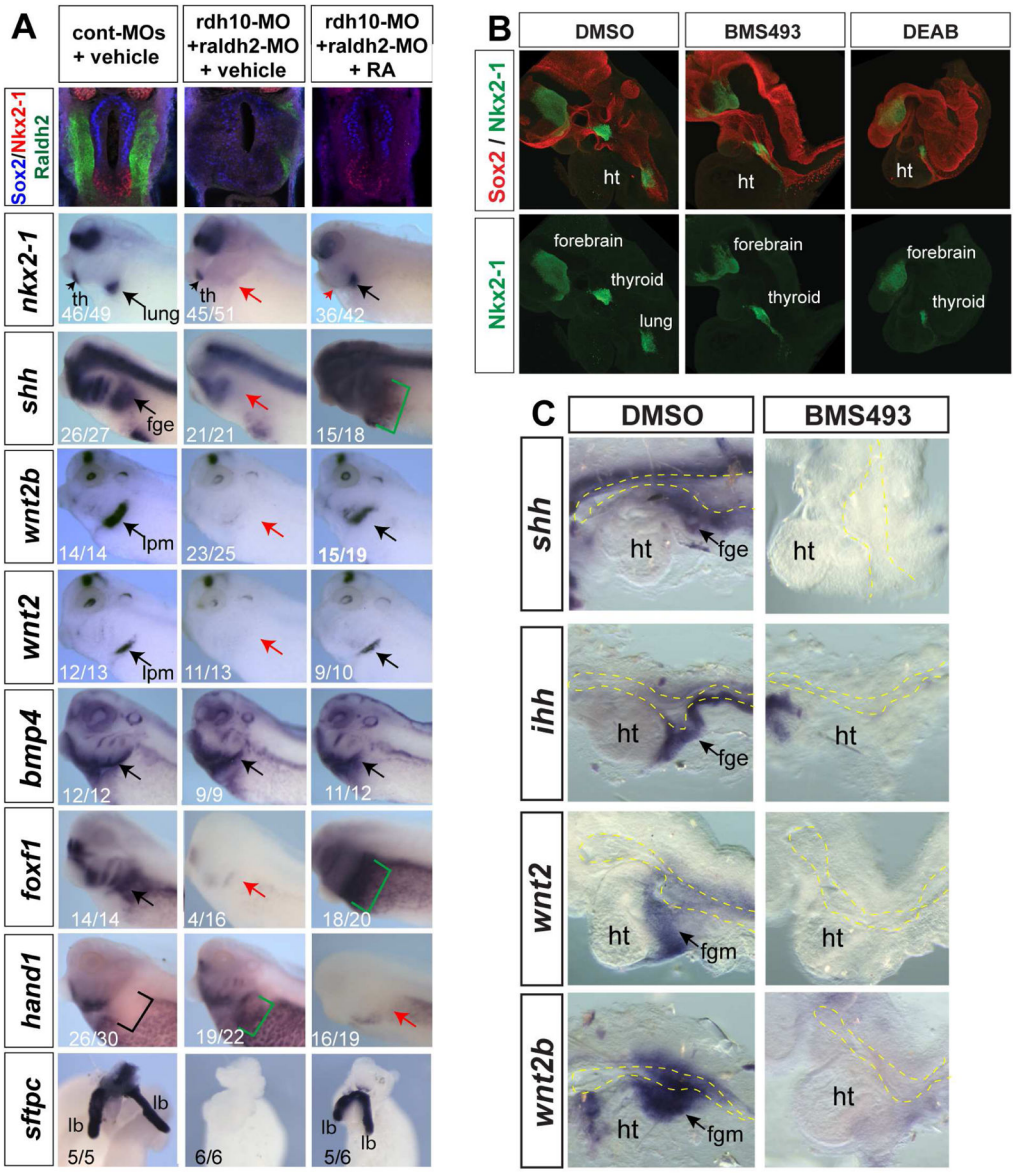

Figure 4. RA signaling is required for respiratory specification in *Xenopus* and mouse

(A) Depletion of RA-synthesizing enzymes in *Xenopus* embryos by rdh10+raldh2 MO injection results in loss of *shh, wnt2/2b*, *foxf1,* but expanded *hand1 in the lpm.* rdh10+raldh2 morphants fail to specify *nkx2-1+* respiratory progenitors at NF34, and exhibit a complete agenesis of *sftpc+* lung buds at NF43. RA treatment of rdh10+raldh2 MO embryos from NF15–34 rescues lung development, inhibits *hand1* and expands *foxf1*. Black arrows indicate normal expression, red arrows loss of expression and brackets expanded expression. The number of embryos with robust marker expression is indicated.

(B) Mouse embryos isolated at E7.5 and cultured for 2 days in either DMSO, BMS493, or DEAB to inhibit RA signaling results a failure of Nkx2-1+ respiratory progenitor induction, but does not inhibit Nkx2-1+ thyroid or forebrain.

(C) Sibling embryos from panel (B) analyzed by in-situ hybridization indicate that RA signaling is necessary for normal expression of *Shh, Ihh, Wnt2*, and *Wnt2b* in the mouse foregut. Dashed lines indicate the lumen of the foregut with arrows showing the lung field. ht; heart, fge; foregut endoderm, fgm; foregut mesoderm. See also Figures S4A–F.

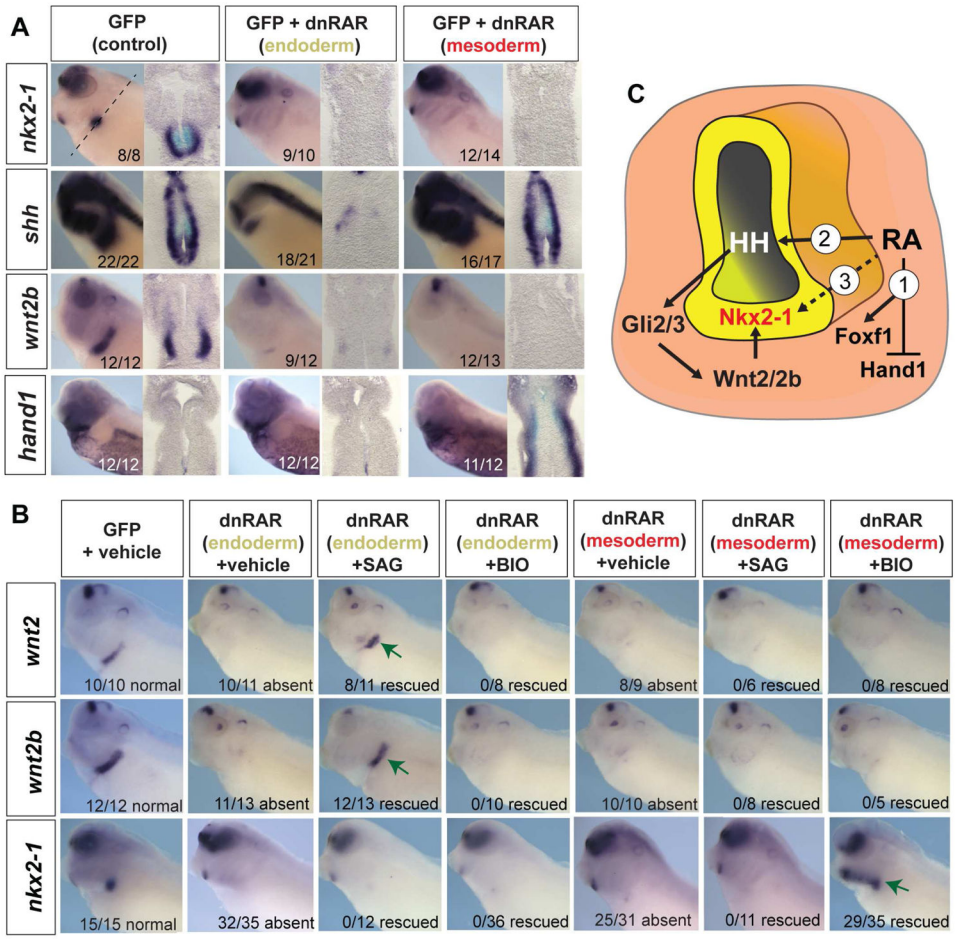

Figure 5. RA is necessary in both the mesoderm and endoderm for *Xenopus* lung specification

(A) Tissue-specific inhibition of RA response by targeted injection of mRNA encoding a dnRAR (+GFP) into either the endoderm or mesoderm. Disruption of RAR activity in the endoderm results loss of *shh, wnt2b*, and *nkx2-1,* but *hand1* was unaffected. In contrast, RAR-disruption in the mesoderm results in loss of *wnt2b* and *nkx2-1* and ectopic *hand1*, but *shh* is unaffected. Whole mount in-situ and sections through the lung region are shown. Also see Figure S5.

(B) Rescue experiments in dnRAR embryos using Hh or Wnt/β-catenin agonists SAG and BIO. SAG rescues *wnt2 and wnt2b* in endoderm-targeted dnRAR, whereas BIO rescues *nkx2-1* in mesoderm-targeted dnRAR. Green arrows indicate rescued expression.

(C) A model showing three roles of RA. 1) RA regulates early lpm patterning independent of Hh. 2) RA regulates *Hh* ligand expression and Hh/Gli then promote *Wnt2/2b* expression. 3) RA is required for the competence of the epithelium to activate *Nkx2-1* in response to Wnt/Bmp signals.

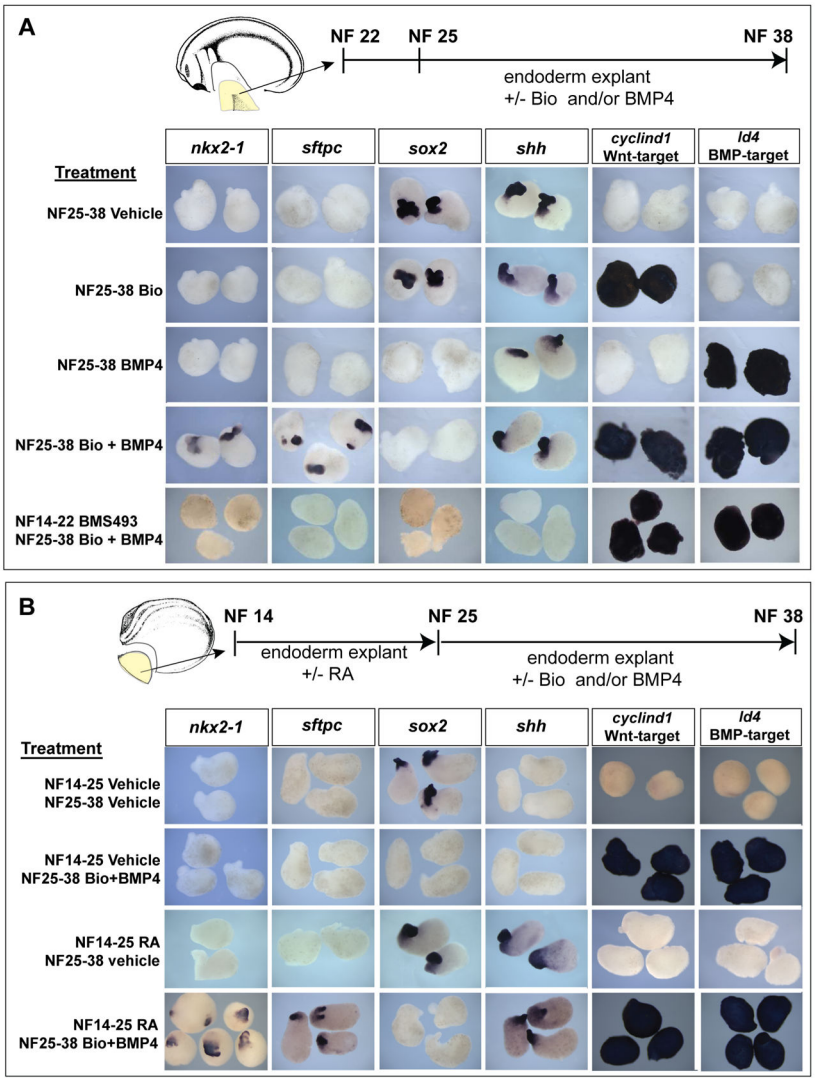

Figure 6. RA is required for respiratory competence of the *Xenopus* foregut endoderm

(A) Endoderm explants dissected at NF22 were treated with Bio, BMP4, or Bio+BMP4 from NF25–38 and assayed by in situ hybridization with the indicated probes. Bio + Bmp4 induced *nkx2-1/sftpc+* respiratory fate, but pre-treatment of embryos from NF14–22 with the pan-RAR antagonist BMS493 prevents respiratory induction and *shh* expression. BMP4 represses *sox2* and induces the BMP-target gene *id4*, whereas Bio induces the Wnt-target gene *cyclind1*.

(B) Endoderm isolated at NF14 is not competent to express *nkx2-1* or *sftpc* in response to subsequent Bio+BMP treatment from NF25-38. However after RA treatment of endoderm from NF14–25, *nkx2-1/sftpc+* respiratory progenitors are induced in response to Wnt and BMP4 activation.

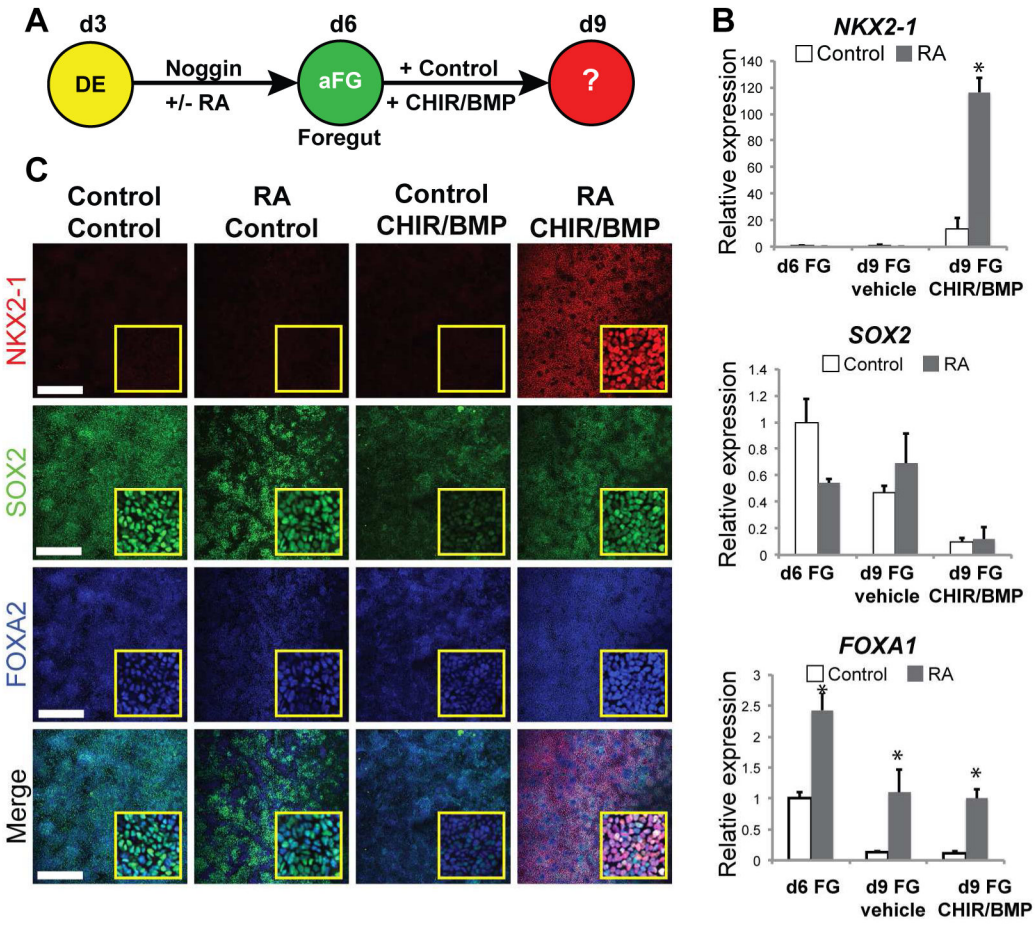

Figure 7. RA is required for the respiratory competence of human foregut endoderm cultures

(A) Schematic depicting the differentiation protocol of H1 hESCs to test the role of RA signaling in respiratory competence of human foregut endoderm culture.

(B) RT-qPCR analysis show that *NKX2-1* is robustly induced by CHIR + BMP4 (d6–9) when cultures were treated with RA on d5–6. *SOX2* and *FOXA1* confirm foregut identity. *P<0.05 in Student T-test of control versus RA treated (n=4).

(C) Immunofluorescence of d9 cultures reveals widespread expression of NKX2-1 in response to the RA/CHIR/Bmp4 treatment. Boxed insets show a 40X magnification.