Disruption of a Hedgehog-Foxf1-Rspo2 Signaling Axis Leads to Tracheomalacia and a Loss of Sox9+ Tracheal Chondrocytes

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Key words: Trachea, tracheomalacia, cartilage, Hedgehog

SUMMARY STATEMENT
This work provides a mechanistic basis for tracheomalacia in patients with Hedgehog pathway mutations.
ABSTRACT

Congenital tracheomalacia, resulting from incomplete tracheal cartilage development, is a relatively common birth defect that severely impairs breathing in neonates. Mutations in the Hedgehog (HH) pathway and downstream Gli transcription factors are associated with tracheomalacia in patients and mouse models; however, the underlying molecular mechanisms are unclear. Using multiple HH/Gli mouse mutants including one that mimics Pallister-Hall Syndrome, we show that excessive Gli repressor activity prevents specification of tracheal chondrocytes. Lineage tracing experiments show that Sox9+ chondrocytes arise from HH-responsive splanchnic mesoderm in the fetal foregut that expresses the transcription factor Foxf1. Disrupted HH/Gli signaling results in 1) loss of Foxf1 which in turn is required to support Sox9+ chondrocyte progenitors and 2) a dramatic reduction in Rspo2, a secreted ligand that potentiates Wnt signaling known to be required for chondrogenesis. These results reveal a HH-Foxf1-Rspo2 signaling axis that governs tracheal cartilage development and informs the etiology of tracheomalacia.
INTRODUCTION

Impaired formation of the tracheal cartilage, or tracheomalacia, occurs in 1 in 2100 live births and can result in life-threatening airway collapse and impaired breathing (Boogaard et al., 2005; Kamran and Jennings, 2019). Current surgical treatment includes insertion of stents to keep the airway open, but these frequently lead to localized inflammation and multiple subsequent surgeries as the patients age (Fraga et al., 2016; Wallis et al., 2019). Generating biologically accurate replacement tissue from pluripotent stem cells is an aspirational strategy to improving patient care, but this requires a detailed understanding of both normal fetal tracheal development and the etiology of tracheomalacia (Fraga et al., 2016; Wallis et al., 2019).

Tracheal cartilage development in the mouse begins by embryonic day (E) 11.5 with expression of the transcription factor Sox9, a master regulator of chondrogenesis, in the ventral and lateral splanchnic mesenchyme surrounding the fetal trachea (Hines et al., 2013). Sox9+ cells do not condense around the dorsal side of the trachea, which forms the trachealis smooth muscle. Between E11.5 and E14.5, as the trachea continues to lengthen and grow, the Sox9+ presumptive chondrocytes organize into distinct, C-shaped rings separated by fibroelastic tissue along the anterior-posterior axis of the trachea (Kishimoto et al., 2018; Park et al., 2010). By E15.5, the chondrocytes differentiate into cartilage rings (Park et al., 2010). Hedgehog (HH) and Wnt signaling are critical for tracheal cartilage development in mice and mutations in these pathways have been associated with tracheomalacia in patients, but how these pathways interact to regulate tracheal chondrogenesis is unclear (Sinner et al., 2019).
The transcription factor Sox9 is required for the development of chondrocyte progenitors throughout the body (Lefebvre et al., 2019). Genetic deletion of Wls, encoding the cargo protein essential for Wnt ligand secretion from the tracheal epithelium leads to a loss of Sox9 expression in the tracheal mesenchyme and a failure in chondrocyte development, causing eventual tracheomalacia (Snowball et al., 2015). Mutations in a number of other Wnt ligands or receptors expressed in the fetal foregut including Wnt4, Wnt5a, Wnt7b, Ror2, and Rspo2 also display deficits in cartilage development with varying extent of tracheomalacia (Bell et al., 2008; Caprioli et al., 2015; Kishimoto et al., 2018; Li et al., 2002).

Disruption in HH signaling can similarly result in tracheomalacia and loss of Sox9+ tracheal chondrocytes in mice (Litingtung et al., 1998; Miller et al., 2004; Motoyama et al., 1998; Park et al., 2010). The HH pathway regulates gene expression via zinc finger Gli transcription factors. In the absence of HH ligands, the HH receptor Smoothened is inhibited, leading to the proteolytical processing of Gli2 and Gli3 into isoforms that act as transcriptional repressors (GliR) (Briscoe and Therond, 2013). In the presence of HH, Smoothened is active, leading to the production of full-length Gli2 and Gli3 isoforms that activate target gene transcription (GliA). In general, Gli3 predominantly acts in the transcriptional repressor form, while Gli2 largely acts as a transcriptional activator (Litingtung et al., 2002; te Welscher et al., 2002; Vokes et al., 2008). Shh ligand is expressed in the developing foregut epithelium where it signals to the surrounding mesenchyme to regulate Gli activity (Ioannides et al., 2003). In Shh^-/- mutants, the primitive foregut tube fails to separate into distinct trachea and esophagus (Litingtung et al., 1998; Miller et al., 2004; Park et al., 2010). Cartilage never forms
around the mutant foregut and there is a dramatic reduction in Sox9 expression and proliferation of the ventral foregut mesenchyme (Litingtung et al., 1998; Miller et al., 2004; Park et al., 2010). Gli2\(^{-/-}\);Gli3\(^{+/+}\) mouse embryos that have only one copy of Gli3 also exhibit tracheomalacia, whereas Gli2\(^{+/+}\);Gli3\(^{-/-}\) embryos, which lack Gli3 but have a single copy of Gli2, do not (Motoyama et al., 1998; Nasr et al., 2019). These data suggest that the balance of GliA to GliR is critical for normal tracheal development.

Indeed, Pallister-Hall Syndrome (PHS) [Online Mendelian Inheritance of Man (OMIM): 146510] patients have a heterozygous mutation in GLI3 that leads to a truncated protein lacking the transcriptional activation domain. As a result, the mutant protein only has GLI3R transcriptional repression even in the presence of active HH signaling. PHS patients can exhibit multiple syndromic phenotypes and often present with laryngeal clefts and tracheomalacia (Bose et al., 2002; Johnston et al., 2005).

Thus, while both HH and Wnt are critical for tracheal development, how they functionally interact is unclear. Here, we use conditional Smo\(^{+/-}\) mouse mutants, which lack GliA, and Gli3\(^{TFlag/+}\) transgenic mice, which overexpress Gli3R, to show that imbalance of Gli activator and repressor activity disrupts specification of Sox9+ tracheal chondrocytes resulting in a tracheomalacia phenotype. We find that HH/Gli promotes the expression of Foxf1 in the ventral foregut mesenchyme, which in turn is required for Sox9 expression. Transcriptional profiling of Foxg1Cre;Gli3\(^{TFlag/+}\) foregut tissue reveals that in addition to loss of Foxf1 and Sox9, there is a dramatic reduction in expression of Rspo2, a secreted ligand known to potentiate Wnt signaling which is required for cartilage development (Bell et al., 2008). In situ hybridization confirmed reduced expression of Rspo2 as well as the Wnt response gene Notum in the ventral tracheal
mesenchyme (Gerhardt et al., 2018). Re-analysis of published ChIP-seq data suggests that Rspo2 is a direct transcriptional target of Foxf1. These data reveal a HH-Foxf1-Rspo2 axis where epithelial HH regulates Wnt signaling in the mesenchyme promoting the specification of Sox9+ tracheal chondrocytes.

RESULTS

Tracheal Chondrocytes Arise from the Splanchnic Foregut Mesoderm

In order to investigate the mechanisms of early tracheal chondrogenesis, we first performed lineage tracing experiments to confirm that the Sox9+ tracheal chondrocytes are derived from the lateral plate mesoderm and not the neural crest, which give rise to laryngeal cartilage (Tabler et al., 2017). For these experiments we crossed floxed mT/mG reporter mice to three different Cre lines: Foxg1Cre which recombines in the foregut mesendoderm beginning at E8.5; Dermo1Cre which recombines in the lateral plate mesoderm beginning at E9.5 (Supplemental Figure S1); or Wnt1Cre which recombines in the early neural crest cells (Boucherat et al., 2014; Hebert and McConnell, 2000; Lewis et al., 2013; Li et al., 2008; Muzumdar et al., 2007; Ustiyan et al., 2018). At E13.5, the Foxg1Cre and Dermo1Cre expressing splanchnic mesoderm lineage traced Sox9+ tracheal chondrocytes surrounding the trachea, as well as the Foxf1+ mesenchyme and smooth muscle of the esophagus and dorsal trachealis muscle, but they did not trace the Sox9+ cells between the smooth muscle layers of the esophagus (Supplemental Figure 1C and 2A-C). In contrast, the Wnt1+ cells did not trace the Sox9+ tracheal chondrocytes or Foxf1+ smooth muscle (Supplemental Figure 2B-C), but did lineage trace the Sox9+ enteric neurons between the esophageal smooth
muscle layers (Supplemental Figure 2D) as well as Sox9+ chondrocytes in more anterior sections through the larynx (data not shown), consistent with previous reports (Adachi et al., 2020; Tabler et al., 2017). This demonstrates that the laryngeal and tracheal cartilages have distinct origins with the latter arising from the lateral plate mesoderm.

**HH/Gli Imbalance Leads to Tracheomalacia**

Pallister-Hall Syndrome patients, with a mutated copy of GLI3 that leads to excessive GLI3R, frequently present with tracheomalacia (Bose et al., 2002; Johnston et al., 2005). To better understand how disrupted HH/Gli signaling results in tracheomalacia, we analyzed a series of conditional mouse mutants where we either deleted the HH receptor Smo, which effectively removes GliA, or we ectopically expressed Gli3Tflag/+, which, like PHS patients, has elevated Gli3R activity but preserved GliA function (Vokes et al., 2008). We also took advantage of the different times of Foxg1Cre and Dermo1Cre recombination to examine the temporal roles of HH/Gli activity.

At E15.5 all the Gli3Tflag/+ and Smo^flo^ mutants showed varying degrees of tracheomalacia, with reduced cartilage development as indicated by Alcian Blue staining (Figure 1A). The early Foxg1Cre mutants were more severe than the later Dermo1Cre mutants. Foxg1Cre;Smo^flo^ mutants had the most severe tracheomalacia, as well as tracheal stenosis and a hypoplastic foregut, while the later-acting Dermo1Cre;Smo^flo^ mutant tracheas had relatively more cartilage than the other mutants. All mutants also showed varying losses of the dorsal trachealis muscle (Figure 1B) as
well as some degrees of esophageal stenosis, supporting that HH/Gli signaling is also required for esophageal development (Jia et al., 2018; Litingtung et al., 1998).

We next asked if Sox9+ tracheal chondrocytes were present at E15.5 but undifferentiated as a result of disrupting the HH/Gli pathway. However, all mutants showed reduced Sox9 levels that correlated with the level of Alcian Blue staining (Figure 1B), suggesting the loss of cartilage was not due primarily to a failure in differentiation, but rather due to a loss of Sox9+ chondrocytes. We also observed a reduction in Foxf1, a direct Gli target that is required for foregut smooth muscle development (Hoffmann et al., 2014; Hoggatt et al., 2013; Ustiyan et al., 2018). Co-staining with Acta2 confirmed reduced smooth muscle differentiation, particularly in the Gli3T mutants (Figure 1B). The more dramatic loss of Sox9+ tracheal chondrocytes and Foxf1+ muscle in Foxg1Cre mutants compared to Dermo1Cre mutants correlates with the more efficient early recombination by Foxg1Cre at E9.5. Dermo1Cre is less efficient and does not recombine robustly until E10.5 (Supplementary Figure 1A-B) (Boucherat et al., 2014; Ustiyan et al., 2018). This suggests that HH/Gli signaling begins acting in the foregut lateral plate mesoderm between E8.5-E9.5, consistent with previous reports (Rankin et al., 2016).

**Dynamic Foxf1 and Sox9 Localization during Tracheal Development**

Since the phenotypes suggested an early disruption in chondrocyte development, we set out to better characterize the earliest expression of Sox9. Immunostaining showed that at E10.0, prior to separation of the foregut into distinct trachea and esophagus, the splanchnic mesoderm uniformly expressed Foxf1 with only
rare interspersed Sox9+ cells (Figure 2A). Robust Sox9 was first detected in the ventral-lateral mesoderm surrounding the trachea at E10.5 just after foregut separation, with the staining intensity and number of Sox9+ cells increasing by E11.5 (Figure 2A) (Hines et al., 2013). Foxg1Cre;mTmG lineage tracing experiments indicate that the Sox9+ cells surrounding the ventral-lateral trachea are mesoderm-derived chondrocytes, whereas the dispersed Sox9+ cells around the presumptive esophagus are neural crest cells that give rise to Tubb3+ enteric neurons (Supplemental Figure S1C). Initially Sox9 and Foxf1 were co-expressed in the ventral mesoderm, but as development proceeds, the upregulation of Sox9 in chondrocytes was coincident with a downregulation of Foxf1. By E11.5, the Sox9 and Foxf1 expression domains were largely distinct, with Foxf1 being restricted to the presumptive trachealis muscle, indicating a segregation of chondrocyte and smooth muscle lineages (Hines et al., 2013). Interestingly, Sox9/Foxf1 double-positive cells persisted at the cartilage-smooth muscle boundary (Figure 2A; E11.0 inset).

Since upregulation of Sox9 and downregulation of Foxf1 in the ventral tracheal mesenchyme follows tracheoesophageal separation, we asked if these expression dynamics were dependent on tracheoesophageal separation and/or epithelial identity. Nkx2-1 and Sox2 are transcription factors required for development of the tracheal and esophageal endoderm epithelial, respectively (Minoo et al., 1999; Que et al., 2007). Nkx2-1−/+ mutants have a single undivided foregut tube of esophageal character, whereas deletion of Sox2 from the foregut results in an undivided foregut tube of tracheal character (Kuwahara et al., 2020; Que et al., 2009; Que et al., 2007; Teramoto et al., 2020; Trisno et al., 2018). A reanalysis of these mutants showed that the single
undivided foregut in both the Sox2 and Nkx2-1 mutant embryos was correctly patterned. However in Nkx2-1 mutants there appeared to be fewer Sox9+ chondrocytes compared to controls or Sox2 mutants, whereas the Sox2 mutants seemed to have far fewer Foxf1+ cells compared to controls or Nkx2-1 mutants (Supplemental Figure 3). Thus, the emergence of tracheal chondrocytes with an upregulation of Sox9 and a downregulation of Foxf1 is influenced by the epithelial identity, but not dependent on tracheoesophageal separation.

**Hedgehog/Gli Activity is Required for Specification of the Tracheal Mesenchyme**

We next considered whether dynamic HH signaling might account for the reciprocal Sox9-Foxf1 expression pattern. Analysis of *ShhGFP/+* embryos with GFP knocked into the *Shh* locus as well as RNAScope® *in situ* hybridization showed that at E10.5 *Shh* was enriched in the tracheal epithelium, but by E11.5 *Shh* was more strongly expressed in the esophageal epithelium (Supplemental Figure S4), consistent with previous reports (Ioannides et al., 2003). In contrast *Ihh* was weakly expressed in the E10.5 trachea epithelium and mesenchyme but undetectable by E11.5 (Supplemental Figure 4B). We postulated that this expression pattern might result in an overall reduction of HH response in the ventral tracheal mesoderm correlating with reduced Foxf1 and increased Sox9. We took advantage of *Gli1LacZ* reporter mice since Gli1 is a direct transcriptional target of HH-Gli2/3 signaling, enabling us to examine the overall impact of both Shh and Ihh activity (Briscoe and Therond, 2013). Contrary to our hypothesis, Gli1LacZ was uniformly expressed in the foregut mesoderm surrounding the gut tube at both at E10.0 and E11.0 with no obvious difference in trachea versus
esophageal mesenchyme (Figure 3B). RNAscope in situ hybridization confirmed this Gli1 expression pattern and also showed uniform Smo expression in the foregut, supporting the conclusion that HH/Gli signaling is still active in the E11.5 ventral tracheal mesoderm (Supplemental Figure 4C).

Next, we performed Foxf1 and Sox9 immunostaining on Gli3TFlag/+ and Smo−/− mutants at E11.5 to examine the initial defects in tracheal chondrogenesis (Figure 3). The Foxg1Cre;Smof/f and Foxg1Cre;Gli3TFlag/+ mutants had reduced number of Sox9+ chondrocytes and also exhibited reduced ventral Foxf1 expression compared to controls (Figures 3A-D). Dermo1Cre mutants appeared to be mostly unchanged in both overall tracheal mesoderm cell number and lineage-specific populations, although they did exhibit a trend of reduced Foxf1 expression levels (Figure 3A-C). Since we observe loss of Sox9 and Foxf1 at E15.5 in both Dermo1Cre mutants, this suggests a continuing role for HH/Gli signaling in maintaining Foxf1 and Sox9 expression and promoting tracheal chondrogenesis.

Since HH signaling is known to maintain cell proliferation and survival in many contexts (Bohnenpoll et al., 2017; Li et al., 2004), we assessed whether this might contribute to the tracheomalacia phenotype in Smo−/− and Gli3TFlag/+ mutants. At E11.5 there were no statistically significant changes in either tracheal mesodermal cell proliferation or apoptosis in any of the mutants as determined by quantification of phospho-Histone H3 or cleaved caspase-3 immunostaining, respectively (Figures 3E-F and Supplemental Figure 5A). However, previous studies have demonstrated that HH/Gli does indeed promote splanchnic mesoderm proliferation and survival from E8.5 to E9.5, which likely explains the reduced cell number in Foxg1Cre mutants, which
recombines starting at E8.5 (Figure 3B) (Li et al., 2008; Rankin et al., 2016). Overall, however, the reduced cell numbers in Foxg1Cre mutant is not sufficient to explain the loss of Foxf1 and Sox9. Together the results indicate that HH/Gli is required between E8.5-10.5 to maintain Foxf1 and specify Sox9+ chondrocytes with prolonged signaling between E10.5 and E15.5 maintaining cartilage and smooth muscle development.

**Foxf1 Is Required for Development of Sox9+ Chondrocytes**

Previous work has shown that loss of one Foxf1 allele leads to impaired tracheal and esophageal development (Mahlapuu et al., 2001; Ustiyan et al., 2018). The reciprocal expression pattern of Foxf1 and Sox9 and the fact that both are reduced in the Smo<sup>fl/fl</sup> and Gli3<sup>Flag/+</sup> mutants suggest that Foxf1 may initially be required for the development of Sox9+ progenitors, but that Sox9 and Foxf1 might then antagonize each other’s expression. To test this, we conditionally deleted Foxf1 using both Foxg1Cre and Dermo1Cre. Foxg1Cre;Foxf1<sup>fl/fl</sup> mutants exhibited a large reduction in Sox9+ ventral foregut mesoderm cells (Figure 4A), consistent with the small cartilaginous nodules previously observed in Foxf1<sup>+/−</sup> tracheas (Mahlapuu et al., 2001). Dermo1Cre;Foxf1<sup>fl/fl</sup> mutants also exhibited fewer Sox9+ cells compared to controls (Figure 4A). Quantification of cell numbers revealed that at E11.5, Foxf1 mutants had a general trend of around 200 fewer tracheal mesoderm cells compared to controls, while only the Foxg1Cre;Foxf1<sup>fl/fl</sup> mutants had significantly fewer Sox9+ cells in the tracheal mesoderm (Figures 4C-E). Interestingly, some of the remaining Sox9+ cells also expressed Foxf1, suggesting that they escaped Cre recombination (Figure 4A). The fact that Sox9 was not upregulated in the Dermo1Cre;Foxf1<sup>fl/fl</sup> mutants indicates that Foxf1 does not repress
Sox9, which was one possibility suggested by their reciprocal expression patterns. We also found that the Foxg1Cre;Foxf1ff mutant foregut failed to separate into a distinct trachea and esophagus (Figure 4A), consistent with our recent work suggesting that the early lateral plate mesoderm is required for morphogenesis (Nasr et al., 2019).

We next asked if Sox9 might suppress the ventral expression of Foxf1 as chondrocytes emerge. Examination of E13.5 Foxg1Cre;Sox9ff mutants revealed that loss of Sox9 had no impact on the ventral downregulation of Foxf1 (Figure 4B). This suggests that Sox9 and its downstream targets are not responsible for the ventral reduction in Foxf1 expression. Thus, Foxf1 and Sox9 do not repress each other’s expression during tracheal chondrogenesis.

Since previous work showed that few Dermo1Cre;Foxf1ff mutants survive beyond E16.5 due to impaired growth and survival of the cardiovascular and pulmonary mesenchyme (Ustiyan et al., 2018), we examined cell proliferation and apoptosis. However, at E11.5 we did not detect any significant differences in cell proliferation or cell apoptosis in either Foxg1Cre;Foxf1ff or Dermo1Cre;Foxf1ff compared to controls (Figures 4F-G and Supplemental Figure 5B). As neither changes in cell proliferation nor cell death can explain the relative reduction in Sox9+ cells in Foxg1Cre;Foxf1ff mutants, we conclude that Foxf1 is required for initial specification of Sox9+ tracheal chondrocyte.
Gli3 and Foxf1 Regulate Expression of Rspp2, a Known Wnt Modulator of Tracheal Chondrogenesis

Previous studies have shown in different cellular contexts that both Foxf1 and Sox9 are direct transcriptional targets of HH/Gli (Bien-Willner et al., 2007; Hoffmann et al., 2014; Madison et al., 2009; Tan et al., 2018). In order to discover additional Gli-regulated genes that might mediate tracheal chondrogenesis, we performed RNA sequencing on E10.5 foreguts and E11.5 tracheas dissected from control and Foxg1Cre;Gli3TFlag/+ embryos. Differential expression analysis (Log2 Fold Change $>|1|$, p<0.05) identified 708 transcripts (70 reduced and 638 increased) with altered expression in mutants at E10.5 and 738 Gli-regulated transcripts (352 reduced and 386 increased) at E11.5 (Figures 5A and B, Supplemental Table 1). Of these, 144 genes were differentially expressed at both E10.5 and E11.5. The reduced expression of Hhip, a direct HH target gene, was consistent with Gli3T repressive activity (Beachy et al., 2010). Gene ontology (GO) enrichment analysis of the downregulated genes identified epithelial tube morphogenesis and respiratory system development, consistent with HH signaling being required for foregut organogenesis, whereas upregulated genes were associated with cell signaling and muscle development, indicative of the relative increase in muscle progenitors in the absence of Sox9+ chondrocytes (Supplemental Figure S6A-B).

We next intersected the Gli3R-regulated transcripts with a manually curated list of 61 genes implicated in tracheal chondrogenesis and/or tracheomalacia in mice or humans (Supplemental Table 2). These were identified from a review of the literature (Sinner et al., 2019) and by searching the Monarchinitiative.org, an online
knowledgebase that aggregates human disease and animal model genotype-phenotype associations (Shefchek et al., 2020). This intersection revealed five genes, all of which were downregulated in Gli3T transgenic embryos (Figure 5C). In addition to Sox9 and Foxf1, this identified Rspo2, Wnt4, and Notum, all key regulators of the canonical Wnt pathway (Figure 5A-C) and all of which exhibit impaired tracheal chondrogenesis when mutated in mice (Bell et al., 2008; Caprioli et al., 2015; Gerhardt et al., 2018). Focusing on the Wnt pathway, we additionally observed reduced expression of Wnt11 (Supplemental Figure 6C), whose role in tracheal development has not yet been identified but is known to support Sox9+ chondrocyte maturation in other tissues (Liu et al., 2014; Tada and Smith, 2000). Rspo2, a secreted protein that interacts with Lgr4/5/6 and Lrp6 receptor complexes to potentiate Wnt/β-catenin signaling, was one of the most downregulated transcripts at both E10.5 and 11.5 (-1.86 and -2.73 Log2FC, respectively) (Bell et al., 2008; Carmon et al., 2011; de Lau et al., 2011; Gong et al., 2012; Kazanskaya et al., 2004; Kim et al., 2008; Lebensohn and Rohatgi, 2018; Ruffner et al., 2012). Wnt4 was modestly downregulated in the E10.5 foregut (-1.54 Log2FC), whereas Notum, a known Wnt target gene and feedback inhibitor was reduced about two-fold in the E11.5 Gli3T trachea (Figure 5B-C, Supplemental Figure 6C) (Gerhardt et al., 2018). These data demonstrate that HH/Gli transcriptionally regulates components of the canonical Wnt pathway, which are known to activate Sox9 expression in the tracheal mesenchyme (Snowball et al., 2015).

We next examined published ChIP-seq data to examine whether Rspo2, Notum, Wnt4, and Wnt11 were likely to be direct target genes of Gli and Foxf1 transcription factors. We used previously published Gli3-3xFlag ChIP from E10.5 limb buds and
Foxf1 ChIP from E18.5 lungs (Dharmadhikari et al., 2016; Lex et al., 2020); these datasets are the most similar to tracheal chondrocytes currently available. We also examined previously published ATAC-seq and H3K4me3 ChIP-seq performed in the E9.5 cardiopulmonary foregut progenitors to help identify active promoter and enhancer regions (Steimle et al., 2018). Examination of genome browsers showed that Gli3 can bind to both the Foxf1 and Sox9 promoters overlapping with H3K4me3 peaks (Figure 5D), consistent with previous reports that they are direct HH/Gli targets (Hoffmann et al., 2014; Tan et al., 2018; Vokes et al., 2008). Gli3 binding regions were also detected on putative regulatory elements of Notum and Wnt11 but not on the Rspo2 or Wnt4 loci (Figure 5D and Supplemental Figure 6F), suggesting that Rspo2 and Wnt4 might be indirectly regulated by Gli. Indeed, the Foxf1 ChIP-seq data showed that Foxf1 binding was associated with putative intronic enhancers of Rspo2; with the Sox9, Notum, Wnt4 and Wnt11 loci; as well as Foxf1’s own promoter (Figure 5D and Supplemental Figure 6F) (Ustiyan et al., 2018). Although the ChIP data are not from the developing trachea, together with the RNA-seq, this analysis is consistent with Gli3-Foxf1 acting in a regulatory network to promote expression of Sox9, Foxf1, Rspo2, Notum, Wnt4, and Wnt11 in the presumptive chondrocytes.

**Wnt Signaling Is Disrupted in Gli3R and Foxf1 Mutants**

We performed RNAscope *in situ* hybridization on E11.5 embryos to validate the RNA-seq analysis and examine which cell populations exhibited a change in Rspo2, Notum and Wnt4 expression. In controls, Rspo2 and Notum were strongly expressed in the ventral tracheal mesoderm, whereas Wnt4 was weakly expressed in the mesoderm
surrounding the esophagus and trachea as well as the epithelium (Figures 6A and 6B, Supplemental Figure 6E), all consistent with previous publications (Bell et al., 2008; Caprioli et al., 2015; Gerhardt et al., 2018). In both Foxg1Cre;Gli3TFlag/+ and Foxg1Cre;Foxf1f/f mutants, Rspo2 and Notum were largely undetectable relative to controls, while there was a modest reduction of Wnt4 levels (Figures 6A and 6B, Supplemental Figure 4E). Since Notum is a direct Wnt target gene required for tracheal chondrogenesis (Gerhardt et al., 2018), this suggests that the cumulative reduction in Rspo2, Wnt4 and Wnt11 in the ventral mesenchyme of Foxg1Cre;Gli3TFlag/+ and Foxg1Cre;Foxf1f/f mutants results in an overall reduction in Wnt response that is unable to sustain Sox9 induction. Together, these data demonstrate that HH/Gli regulate a Foxf1-Wnt pathway required for tracheal chondrogenesis.

DISCUSSION

In this study we show that conditional mouse mutants with relatively high levels of GliR, mimicking PHS, exhibit tracheomalacia and fail to properly specify Sox9+ tracheal chondrocytes. Our data suggest a model of the epithelial-mesenchymal interactions that orchestrate tracheal chondrocyte differentiation (Figure 7) where: 1) HH ligands expressed in the ventral foregut epithelium from E8.5-11.5 signal to the surrounding splanchnic mesoderm to activate Gli transcription factors which promote Foxf1 transcription. 2) Foxf1 in turn maintains the lateral plate mesoderm and directly promotes Sox9 transcription at the initiation of tracheal chondrogenesis. 3) Downstream of HH, Gli3 and Foxf1 cooperate in a regulatory network to promote the transcription of Sox9, Rspo2, Wnt4, Wnt11, and Notum. 5) This Gli-Foxf1-Rspo2 axis promotes Wnt
signaling in the mesenchyme, which is known to be required for activation of Sox9 expression and tracheal cartilage development (Snowball et al., 2015). Disruptions in this HH-Wnt regulatory network result in the failure to induce and/or maintain Sox9 which is essential for chondrogenesis. Together, these data provide a mechanistic basis for the tracheomalacia in patients with mutations in HH/Gli pathway genes.

**Temporal Role of HH/Gli in Tracheal Chondrogenesis.**

Previous work indicates that conditional deletion of Shh from the respiratory epithelium between E8.5-12.5 resulted in minor disruptions to in tracheal cartilage ring patterning, whereas deletion after E13.5 had no effect (Miller et al., 2004). We postulate that the differences in this report compared to our current study may be due to a low level of Ihh acting redundantly with Shh; Ihh promotes chondrocyte proliferation in the endochondral skeleton (Long et al., 2001). We manipulated the Smo receptor or Gli3 downstream of any ligand redundancy. An alternative explanation is the efficiency of early Cre-mediated deletion. The fact that the early-acting Foxg1Cre had a more severe loss of Foxf1 and Sox9 than Dermo1Cre mutants suggests that HH starts acting on the lateral plate mesoderm between E8.5-10.5. However, by E15.5 the Dermo1Cre mutants did exhibit a dramatic loss of cartilage and reduced Sox9, suggesting that continued HH/Gli activity between E10.5-15.5 is necessary to maintain Sox9 and to promote chondrogenesis.

Our study also suggests that the balance of GliA to GliR activity is critical for specification of Sox9+ chondrocytes. Both the Smo<sup>ff</sup> mutants, which mimic an absence of GliA function, and Gli3<sup>Tflag+</sup> mutants which have excess Gli3R, exhibit
tracheomalacia and a reduction of Sox9. Similarly, Gli2+/Gli3+/ germline mouse mutants, with one copy of Gli3R but no Gli2A, displayed tracheomalacia, whereas Gli2++;
Gli3+/ mice, which lack Gli3R, do not (Litingtung et al., 1998; Miller et al., 2004; Nasr et al., 2019; Park et al., 2010). These phenotypes along with the genomic analysis suggests that too much Gli3R relative to Gli2A directly represses Sox9 transcription. Indeed, previous work indicates that HH target gene expression can be reduced by either the loss of GliA or by increased GliR relative to GliA, whereas in some cases loss of GliR is sufficient to activate some target genes (Falkenstein and Vokes, 2014).

It is also possible that spatiotemporal dynamics in HH signaling levels impact dorsal-ventral patterning of the peritracheal mesenchyme. Although we observed uniform expression of the HH target gene Gli1 in the mesenchyme around the trachea and esophagus, it is possible that by E11.5 there is insufficient HH activity in the ventral trachea to support Foxf1 expression. This could be a product of the shift in Shh expression from the ventral foregut to esophageal epithelium. Ultimately, this might result in a HH activity gradient that patterns the peritracheal mesenchyme and could explain in part why Foxf1 persists in the dorsal trachealis muscle next to the Shh-rich esophagus.

**Foxf1 Is Required for Specification of Sox9+ Tracheal Chondrocytes.**

Our analysis indicate that Foxf1 is required for specification of Sox9+ tracheal chondrocytes. We postulate that Foxf1 promotes Sox9 expression in several ways. First, Foxf1 is known to be essential for mesenchymal proliferation and survival of the early foregut mesenchyme (Rankin et al., 2016), and while this alone cannot account for
the phenotypes we observe, we expect it contributes to the ultimate expansion of chondrogenic mesenchyme. Second, the ChIP-seq analysis suggests that Foxf1 directly regulates Sox9 transcription. Finally, Foxf1 also promotes Sox9 expression indirectly by stimulating expression of Wnt pathway components.

The downregulation of Foxf1 in the ventral mesoderm as Sox9+ chondrocytes are induced initially suggested that Foxf1 and Sox9 might mutually repress one another, but the genetic analysis ruled out this possibility. Rather, our data together with previous studies suggests that downstream of HH/Gli and Foxf1, pathways including Wnt and possibly BMP likely contribute to regulatory feedback loops controlling dorsal-ventral patterning of the peritracheal mesoderm. This patterning likely leads to the restriction of Foxf1 to the dorsal trachealis and Sox9 to a ventral-lateral domain (Domyan et al., 2011; Rajagopal et al., 2008; Rankin et al., 2016; Snowball et al., 2015).

**HH/Gli Regulates a Foxf1-Rspo2-Wnt Axis**

While both HH and Wnt signaling were known to regulate tracheal chondrogenesis, how these pathways interact was previously unclear. Our analysis indicates that epithelial HH signals stimulate Gli activity in the adjacent ventral mesenchyme to activate a Foxf1-Rspo2-Wnt signaling axis which promotes Sox9 expression. During tracheal development, Wnt ligands secreted from the ventral respiratory epithelium (primarily Wnt7b) are required to signal to the adjacent mesenchyme to activate Sox9 expression (Rajagopal; et al., 2008). Conditional epithelial deletion of the Wnt cargo protein Wls, which is required for Wnt ligand secretion, results in a failure to specify Sox9+ chondrocytes (Snowball et al., 2015).
addition, a number of other Wnt pathway components including Rspo2, Wnt4, Wnt5a, Wnt2 and Notum are expressed in the peritracheal mesenchyme and contribute to development of Sox9+ chondrocytes (Bell et al., 2008; Caprioli et al., 2015; Gerhardt et al., 2018; Goss et al., 2009; Kishimoto et al., 2018; Li et al., 2002; Snowball et al., 2015). For example, Rspo2 and Wnt4 mutant tracheas have a fewer and dysmorphic tracheal cartilage rings, while Notum mutant tracheas exhibit impaired cartilage differentiation (Bell et al., 2008; Caprioli et al., 2015; Gerhardt et al., 2018).

Our analysis suggests that Rspo2, Wnt4, Wnt11, Notum and Sox9 are all direct Foxf1 targets, and that Gli3 might bind to the same Sox9, Notum and Wnt4 enhancers as Foxf1. This implies a positive feedback loop where Gli transcription factors first activate Foxf1 in the early lateral plate mesoderm. Foxf1 then cooperates with Gli to directly promote expression of Sox9 and the Wnt pathway, which in turn reinforces Sox9 transcription. Indeed, there is genomic evidence from the developing heart and long bones supporting such a Gli-Fox combinatorial activity (Hoffmann et al., 2014; Tan et al., 2018). One limitation of our study was that the ChIP-seq data was from other tissue. Future ChIP experiments of Gli3 and Foxf1 from the fetal trachea will be important to elucidate the genomic details of this Gli-Foxf1-Wnt regulatory network.

Our data further suggest that the combined, loss of Rspo2, Wnt4 and Notum in Foxg1Cre;Gli3TFlag/+ , Foxg1Cre;Smo/+/ and Foxg1Cre;Foxf1+/ mutants results in a reduction of Wnt activity insufficient to activate and/or maintain Sox9 transcription, similar to Wls mutants. Indeed, the tracheomalacia observed in Rspo2 mutants is made worse with additional reduction in Lrp6. This suggests that a dose-dependent disruption in Wnt activity may severely impact tracheal chondrogenesis (Bell et al., 2008).
The regulation of Sox9 expression by Wnt signaling appears to be context dependent. While Wnt-ß-catenin promotes Sox9 expression in the trachea and the gut, it appears to suppress Sox9 in the context of limb chondrogenesis (Blache et al., 2004; Kozhemyakina et al., 2015; Snowball et al., 2015). The prevailing view is that canonical Wnt signaling directly regulates Sox9 transcription, but to our knowledge, direct binding of the Wnt transcriptional effectors Tcf and ß-catenin to Sox9 enhancers remains to be demonstrated. It will be interesting to determine whether Tcf-ß-catenin complexes bind to the same enhancers as Foxf1 and Gli3. Finally, it is possible that Wnt also contributes indirectly as ß-catenin can promote FGF-dependent tracheal chondrogenesis (Hou et al., 2019). Altogether, our data provide a mechanistic understanding of how disruptions in HH/Gli signaling may impair specification of Sox9+ tracheal chondrocytes and ultimately lead to tracheomalacia.

MATERIALS AND METHODS

Animal models

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center (CCHMC) under protocol 2019-0006. Animals were housed within the CCHMC Veterinary Services Core in temperature-controlled rooms with regular access to food and water. Mice were maintained on outbred background. Most embryos were harvested prior to overt sexual differentiation and the entire litter was analyzed suggesting a equal distribution of male and females. Dr. Debora Sinner provided Foxg1Cre (Hebert and McConnell, 2000), Dermo1Cre (Sosic et al., 2003), and mTmG (Muzumdar et al., 2007) animals, as well as
Foxg1Cre;Sox9f/f (Kist et al., 2002) mutant and control samples. Dr. Vladimir Kalinichenko provided Foxf1fl/fl animals (Ren et al., 2014). Dr. Samantha Brugmann provided Wnt1Cre (Lewis et al., 2013), mTmG (Muzumdar et al., 2007), and Gli3TFlag/Flag (Vokes et al., 2008) animals. Dr. Joo-Seop Park provided Gli3TFlag/Flag animals (Vokes et al., 2008).

Immunostaining, in situ hybridization, and Alcian Blue staining

At least three embryos of each genotype were used for all experiments. For section immunostaining, embryos were collected and incubated in 4% paraformaldehyde solution overnight at 4°C. After two rinses in 1XPBS, embryos were incubated in 30% sucrose overnight at 4°C before embedding in OCT for cryosectioning. Sections were collected at 8 µm. Foxg1Cre;Sox9f/f embryos were embedded in paraffin before sectioning, and were deparaffinized before beginning immunostaining. On the first day of immunostaining, sections were washed in 1XPBS before incubation in 1XPBS with 0.05% Triton X-100. Sections were then blocked with 5% normal donkey serum in 1X PBS for one hour before overnight incubation in primary antibodies (Foxf1, goat, R&D, 1:300; Sox9, mouse, Invitrogen, 1:200; Sox9, rabbit, Millipore, 1:200; Acta2, mouse, Sigma, 1:800; Acta2, rabbit, Genetex, 1:800; pHH3, mouse, Millipore, 1:1000; CC3, rabbit, Cell Signaling, 1:200; GFP, chicken, Aviva Biosystems, 1:1000; DsRed, mouse, Living Color, 1:1000, ß-galactosidase/LacZ, chicken, Abcam, 1:1000) at 4°C. On the second day, sections were washed three times in 1XPBS before incubation in secondary antibodies (donkey anti-mouse 647, Jackson; donkey anti-goat 647, Jackson; donkey anti-rabbit 647, AlexaFluor; donkey anti-rat 647,
Jackson; donkey anti-rabbit Cy3, Jackson; donkey anti-mouse Cy3, Jackson; donkey anti-goat 488, Jackson; donkey anti-chicken 488, Jackson; donkey anti-chicken 647, Jackson; donkey anti-rabbit 405, Abcam; DAPI, ThermoScientific, all at 1:500) at room temperature, and were washed in 1XPBS three more times before coverslip placement. All antibodies have been validated by numerous previous publications and where practical loss of signal in genetic null mutants.

*In situ* hybridization was performed using an RNAScope Multiplex Fluorescent v2 kit according to manufacturer instructions (ACD Biosystems). For wholemount immunostaining, embryos were stored in methanol at -20°C before beginning staining. Foreguts were dissected out, incubated in Dent’s Bleach for two hours, and were serially rehydrated into 1XPBS before blocking in 5% normal donkey serum and 1% DMSO for two hours. Foreguts were then incubated in primary antibody diluted in blocking solution overnight at 4°C. After five washes in 1XPBS, foreguts were incubated in secondary antibody overnight at 4°C. The next day, after three washes in 1XPBS, foreguts were serially dehydrated into methanol and stored at 4°C overnight before clearing in Murray’s Clear solution for imaging. All images were taken on a Nikon LUNA upright confocal microscope in the CCHMC Confocal Imaging Core. All scale bars are 100 µm.

Alcian Blue staining was performed on dissected foreguts as previously described (Que et al., 2007). Foreguts were then serially rehydrated into 1XPBS before incubation in 30% sucrose overnight at 4°C. After embedding in OCT, foreguts were cryosectioned at 60 µm thickness and photographed using a Nikon LUN-A inverted widefield microscope.
Quantitative analysis

Confocal images were analyzed using Nikon Elements Analysis and Imaris programs. All statistical analyses were performed in Microsoft Excel on data obtained from single transverse sections from each embryo. Sections were selected based on their median location between the anterior separation of the larynx into the trachea and the posterior formation of the mainstem bronchi from the trachea. Calculations were performed in Microsoft Excel using a two-sided Student’s t-test with unequal variance and with significance defined as p<0.05. No specific power calculation was performed. The sample size is indicated in each figure and no data was excluded. The genotype of immunostaining results was blinded to co-investigators for interpretation. Graphs were generated using Prism. For relative expression of Foxf1 and Sox9 in the tracheal mesoderm (referred to as TMes in figures), the number of Foxf1+ or Sox9+ tracheal mesoderm cells was divided by the total number of tracheal mesoderm cells. For pHH3+ (mitotic indices) or CC3+ rates of mesenchyme cells, the number of tracheal mesenchymal cells positive for either pHH3 or CC3 was divided by the total number of tracheal mesenchymal cells. For quantification of immunostaining values are reported as mean ± standard error, with p<0.05 as calculated by a two-sided Student’s t-test with unequal variance.
RNA-seq and ChIP-seq analysis

RNA-Seq analysis was performed on control and Foxg1Cre;Gli3Flag/+ samples sequenced at stages E10.5 (foreguts) and E11.5 (tracheas) with three independent biological replicates (embryo dissections) for each condition. After storing in RNALater (Ambion) at -80°C, RNA was isolated using a Qiagen MicroEasy Kit and was amplified by the CCHMC Gene Expression Core before sequencing in the CCHMC DNA Sequencing Core using an Illumina 3000 high-throughput platform. Single end sequencing with read-depth of ~22-27 million and read length of 75bp was performed. Raw reads from the experiments were analyzed using Computational Suite for Bioinformaticians and Biologists (CSBB -v3.0, https://github.com/praneet1988/Computational-Suite-For-Bioinformaticians-and-Biologists). The following steps were carried out in analysis using CSBB. Quality check and trimming was performed using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Bbduck (https://jgi.doe.gov/data-and-tools/bbttools/bb-tools-user-guide/bbdock-guide/) respectively. Quality trimmed reads were then mapped to the mouse genome (mm10) using Bowtie2 and quantified using RSEM (https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-12-323).

Differential expression analysis was carried out using CSBB-Shiny (https://github.com/praneet1988/CSBB-Shiny) and volcano plots were generated using EnhancedVolcano (https://www.bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/d
oc/EnhancedVolcano.html) package in R. Differentially expressed genes were obtained at following thresholds: LogFC $> |1|$ and $0.05 >$ False Discovery Rate (FDR).

ChIP-Seq analysis was performed on following published datasets: 1) Foxf1 ChIP on dissected E18.5 lung, GSE77159 (Dharmadhikari et al., 2016); 2) Gli3-3xFlag ChIP on dissected E10.5 limb buds, GSE133710 (Lex et al., 2020); 3) ATAC-seq and H3K4me3 ChIP performed on E9.5 cardiopulmonary progenitors, GSE119885 (Steimle et al., 2018). These datasets were reprocessed using CSBB and for visualization purpose bigwig files were generated using deeptools (BamCoverage function) (Ramirez et al., 2016) from bam files. Peaks were called using Macs2 (default parameters) (Zhang et al., 2008). Genome browser views were generated using IGV.
ACKNOWLEDGEMENTS

We would like to thank members of the CLEARconsortium.org, the Zorn and Wells labs as well as Samantha Brugmann, Sheila Bell, and Steve Vokes for critical feedback. We also thank Leslie Brown, Kaulini Burra, and Natalia Bottasso Arias for technical assistance. We are grateful to the staff of the CCHMC Confocal Imaging Core, Veterinary Services Core, Gene Expression Core, and DNA Sequencing Core for their assistance.

COMPETING INTERESTS

The authors have no competing interests to declare.

FUNDING

This work was supported by P01HD093363 to A.M.Z., F30HL142201 to T.N., R01HL144774 to D.S., R01HL141174 and R01HL149631 to V.V.K., and T32 to the University of Cincinnati Medical Scientist Training Program. Confocal imaging and genomic analysis were supported in part by NIH P30 DL0778392 awarded to the CCHMC Digestive Health Center.

DATA AVAILABILITY

The RNA-seq data has been deposited into GEO: GSE Accession # pending.

CONTRIBUTIONS

T.N. and A.M.Z. designed experiments and wrote the manuscript. T.N., P.C., and K.A. performed bioinformatics analysis. T.N., A.M.H., J.L.K., K.D., V.U., J.M.S., S.L.T., and D.S. performed experiments. V.V.K., J.M.W., D.S. and A.M.Z. provided support.
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Figure 1: Imbalance in Gli Activity Leads to Tracheomalacia

(A) Alcian Blue wholemounts of dissected E15.5 foreguts from control, *Foxg1;Gli3*Flag/+,*Dermo1Cre;Gli3*Flag/+, *Foxg1Cre;Smof/f,* and *Dermo1Cre;Smo*ff embryos. Earlier mutations generated using *Foxg1Cre* produced more severe tracheomalacia compared
to *Dermo1Cre*-mediated deletions. N=3-5 embryos / genotype. (B) Sox9, Foxf1, and Acta2 wholemount immunostaining of dissected E15.5 foreguts from control, *Foxg1Gli3TFlag/+*, *Dermo1Cre;Gli3TFlag/+*, *Foxg1Cre;Smoff* and *Dermo1Cre;Smooff* embryos. *Foxg1Cre* mutants display more significant reductions in Sox9 and Foxf1 compared to *Dermo1Cre* mutants, suggesting that impaired tracheal mesenchymal specification may contribute to tracheomalacia. N=3-5 embryos / genotype. All scale bars, 100 µm.
Figure 2: Dynamic Sox9 and Foxf1 Expression during Tracheal Chondrocyte Specification

(A) Foxf1 and Sox9 immunostaining of control embryos between E10.0 – E11.5. Foxf1 is initially expressed throughout the lateral plate mesoderm, but then downregulated in the ventral tracheal mesoderm relative to the dorsal tracheal mesoderm by E11.5. Sox9 is only found in neural crest cells at E10.0, but progressively localized to the ventral
tracheal mesoderm between E10.5 and E11.5. The inset shows Sox9+/Foxf1+ co-expressing cells at the boundary between the presumptive smooth muscle and chondrocytes. N=3-5 embryos/stage. (B) Immunostaining of LacZ (β-galactosidase), Foxf1, and Sox9 in E10.0 and E11.5 Gli1\textsuperscript{LacZ/+} embryos, showing the direct HH-target Gli1 expressed throughout the mesoderm surrounding the esophageal and tracheal endoderm. All scale bars, 100 µm. e=esophagus, t=trachea.
Figure 3: Hedgehog/Gli Signaling Supports Tracheal Mesenchymal Specification

(A) Foxf1 and Sox9 immunostaining of E11.5 foregut transverse section from control, Foxg1Cre;Gli3TFlag/+, Dermo1Cre;Gli3TFlag/+, Foxg1Cre;Smo/−, and Dermo1Cre;Smo/− embryos. Foxg1Cre mutants have fewer Foxf1+ and Sox9+ mesoderm cells compared to Dermo1Cre mutants and control embryos. N=3-5 embryos / genotype. All scale bars,
100 µm. e=esophagus, t=trachea. (B-E) Quantification of E11.5 immunostaining for each genotype showing: B) the total number of cells in the tracheal mesoderm (TMes), C) the ratio of Foxf1+ / total TMes cells, D) the ratio of Sox9+ / total TMes cells, D) the mitotic index of phospho-histone H3 (pHH3+) / total TMes cells to indicate proliferation, and E) the ration of cleaved caspase-3 (CC3+) / total TMes cells to indicate apoptosis. All quantification shown as mean ± standard error of the mean, with p<0.05 as calculated by a two-sided Student’s t-test with unequal variance. N=3-5 embryos / genotype.
Figure 4: Foxf1 Is Required for Tracheal Sox9 Expression

(A) Foxf1 and Sox9 immunostaining of E11.5 control, *F*ox*g*1*Cre*;Foxf1+/−, and *Dermo1Cre;Foxf1+/−* mutants. *F*ox*g*1*Cre* mutants have fewer Foxf1+ and Sox9+ cells compared to controls. *Dermo1Cre* mutants have some Sox9+ cells in the ventral trachea that co-localize with Foxf1 suggesting that these cells escaped Cre recombination. (B) Foxf1 and Sox9 immunostaining of E13.5 control and *F*ox*g*1*Cre*;Sox9+/− embryos. (C-G) Quantification of E11.5 immunostaining for each
genotype showing: C) the total number of cells in the tracheal mesoderm (TMes), D) the ratio of Foxf1+ / total TMes cells, E) the ratio of Sox9+ / total TMes cells, F) the mitotic index of phospho-histone H3 (pHH3+) / total TMes cells to indicate proliferation, and G) the ratio of cleaved caspase-3 (CC3+) / total TMes cells to indicate apoptosis. N=3-5 embryos / genotype. All scale bars, 100 µm. e=esophagus, t=trachea. All quantification shown as mean ± standard error of the mean, with p<0.05 as calculated by a two-sided Student’s t-test with unequal variance.
Figure 5: Gli3 Regulates Expression of Wnt Pathway Components

(A-B) Volcano plot of differentially expressed transcripts in control versus Foxg1Cre;Gli3T foreguts as determined by Log2FC >1, p<0.05 at (A) E10.5 and (B) E11.5. (C) Venn diagram intersecting genes differentially expressed in Foxg1Cre;Gli3T mutants with genes known to be involved in human or mouse tracheal chondrogenesis (Shefchek et al., 2020; Sinner et al., 2019). TM = tracheomalacia-associated genes. (D) Genome browser views of Gli3-3xFlag
(GSE133710), Foxf1 (GSE77159), and H3K4me3 (GSE119885) ChiP-seq data, as well as ATAC-seq data (GSE119885) on Foxf1, Sox9, Rspo2, and Notum loci. Gli3 and Foxf1 bind the Foxf1, Sox9, and Notum loci, but only Foxf1 shows direct binding of the Rspo2 locus along with significant ATAC and H3K4me3 peaks suggesting active transcription. Statistically significant ChIP peaks are underlined in red.
Figure 6: Expression of Wnt pathway genes Rspo2 and Notum are reduced in Gli and Foxf1 Mutants

(A-B) RNAscope in situ hybridization of E11.5 control, Foxg1Cre;Gli3T\textsuperscript{Flag/+}, and Foxg1Cre;Foxf1\textsuperscript{f/f} embryos reveals decreases in (A) Rspo2 and (B) Notum in the ventral-lateral tracheal mesenchyme. These results suggest that HH/Gli-Foxf1 signaling is upstream of Rspo2 and Notum during tracheal development.

All scale bars, 100 µm. e=esophagus, t=trachea.
Figure 7: Model of a HH/Gli-Foxf1-Wnt signaling network controlling Sox9+ chondrogenesis.

HH/Gli signals from the epithelium (yellow) result in more Gli2/3 activator (GliA) than Gli repressor (GliR). Activated Gli2/3 directly stimulates Foxf1 expression, which in turn supports growth and survival of the tracheal mesoderm (pink). Foxf1 and Gli TFs cooperate to directly promote Sox9 transcription. In addition Gli and Foxf1 promotes the expression of a number of Wnt-pathway components in the mesenchyme including Rspo2, Wnt4, Wnt11 and Notum, which act in concert with epithelial Wnt7b and Wls to further enhance and maintain Sox9 expression. Wnt/β-catenin signaling in the ventral tracheal mesoderm is essential for tracheal chondrogenesis. Notum, another direct Gli and Foxf1 target, attenuates Wnt/β-catenin signaling possibly to regulate chondrocytes maturation. In the dorsal tracheal mesenchyme (green), which is thought to have lower Wnt and Bmp signaling, Gli-Foxf1 does not activate Sox9+ chondrogenesis and the tissue adopts a smooth muscle fate.
Figure S1: Kinetics of Foxg1Cre and Dermo1Cre Recombination

(A-B) GFP and Foxf1 immunostaining of (A) Foxg1Cre;mTmG and (B) Dermo1Cre;mTmG foreguts and E8.5, E9.5, and E10.5. The Foxg1Cre line starts to recombine at E8.5 with robust recombination at E9.5, whereas the Dermo1Cre starts to recombine at E9.5 with robust recombination by E10.5. (C) Immunostaining of E10.5 Foxg1Cre;mTmG embryos showing GFP-/Sox9+/Foxf1+/Tubb3- neural crest cells in surrounding the esophagus are distinct from the GFP+/Sox9+/Foxf1-/Tubb3+ neural crest cells surrounding the ventral trachea. All scales bars 100 µm. N=2-3 embryos/ genotype/stage.
Figure S2: Foxg1Cre, Dermo1Cre, and Wnt1Cre Lineage Tracing in Tracheoesophageal Development

(A) GFP and DsRed immunostaining of E13.5 Foxg1Cre;mTmG, Dermo1Cre;mTmG, and Wnt1Cre;mTmG foreguts show that as expected the Foxg1Cre and Dermo1Cre lineages give rise to tracheoesophageal mesenchyme, while the Wnt1 neural crest cell lineage gives rise to neurons that innervate the esophageal and trachealis muscles. (B-D) Immunostaining of GFP and DsRed with Foxf1, Sox9, or Acta2 in (B) Foxg1Cre;mTmG embryos, (C) Dermo1Cre;mTmG embryos and (D) Wnt1Cre;mTmG embryos at E13.5. Insets show that the Foxg1Cre and Dermo1Cre lineage trace the lateral plate mesoderm that gives rise to GFP+/Foxf1+/Acta2+ smooth muscle and GFP+/Sox9+ chondrocytes, but not the GFP-/Sox9+ enteric neurons between the esophageal smooth muscle. In contrast neural crest specific Wnt1Cre lineage traces the Sox9+ cells that are likely to be enteric neurons innervating the esophageal and tracheal smooth muscle. All scale bars, 100 µm. e=esophagus, t=trachea. N=3 embryos / genotype.
Figure S3: Foxf1 and Sox9 Expression in Sox2 and Nkx2-1 Mutants

(A-C) Immunostaining of Foxf1 and Sox9 at E13.5 in (A) control, (B) Nkx2-1<sup>GFP/GFP</sup> (or Nkx2-1<sup>-/-</sup>) and (C) FoxA2CreER;Sox2<sup>f/f</sup>. Nkx2-1 and Sox2 mutants both have a single unseparated foregut tube that exhibits appropriate dorsal-ventral patterning. However, Nkx2-1 mutants have fewer Sox9+ chondrocytes whereas Sox2 mutants have fewer Foxf1+ smooth muscle progenitors. Scale bar = 100 µm. e = esophagus, t = trachea. N = 3 embryos / genotype.
Figure S4: Expression of HH Pathway Components

(A) Immunostaining of ShhCre<sup>GFP/+</sup> heterozygous embryos (Cre-GFP fusion knocked into the endogenous Shh loci). GFP immunostaining shows a shift in Shh expression from trachea at E10.5 to esophageal at E11.5. (B) RNAscope <i>in situ</i> hybridization of Ihh at E10.5 and E11.5. (C) RNAscope <i>in situ</i> hybridization of Gli1, Shh, and Smo in E11.5 control and Foxg1Cre;Gli3<sup>Tgrep</sup> embryos. Scale bar = 100 µm. e = esophagus, t = trachea. N = 3-5 embryos / genotype.
Figure S5: Cell Proliferation and Apoptosis in HH/Gli and Foxf1 Mutants

(A-B) Immunostaining of cell proliferation marker phospho-Histone H3 (pHH3) or cell apoptosis marker cleaved caspase-3 (CC3) in (A) E11.5 control and Gli3\textsuperscript{Flag+/} and Smo\textsuperscript{f/f} mutants or (B) in E11.5 control and Foxf1 mutants. There is no substantial difference in proliferation or cell death in the any mutants compared to control embryos. N=3-5 embryos/genotype.
Figure S6: Additional Data on Gli3 Target Genes.
(A-B) GO enrichment analysis of (A) downregulated and (B) upregulated genes in Foxg1Cre;Gli3T<sup>Flag/+</sup> mutants (E10.5 and E11.5 combined). (C-D) Histogram of RNA-seq transcript levels in E11.5 trachea tissue showing (C) selected mediators of tracheal chondrogenesis and (D) Wnt-pathway genes. Control expression in black and mutant expression in grey. Asterisks indicate differential expression of Log<sub>2</sub>TPM>|1|, p<0.05 in E11.5 Foxg1Gli3T<sup>Flag/+</sup> tracheas. (E) Genome browser views of Gli3-3xFlag (GSE133710), Foxf1 (GSE77159), and H3K4me3 (GSE119885) ChiP-seq data, as well as ATAC-seq data (GSE119885) in the Wnt4 and Wnt11 loci. Wnt4 appears to be a direct Foxf1 target, while Wnt11 may be directly regulated by both Gli3 and Foxf1. (F) RNAscope in situ hybridization of Wnt4 in E11.5 control and Foxg1Cre;Gli3T<sup>Flag/+</sup> tracheas.
Table S1

Click here to Download Table S1

Table S2

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