

1 **Bidirectional Wnt signaling between endoderm and mesoderm confer tracheal**  
2 **identity in mouse and human.**

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23 **Abstract (150 words)**

24 The periodic cartilage and smooth muscle structures in mammalian trachea are derived from  
25 tracheal mesoderm, and tracheal malformations result in serious respiratory defects in neonates.  
26 Here we show that canonical Wnt signaling in mesoderm is critical to confer trachea  
27 mesenchymal identity in human and mouse. Loss of  $\beta$ -catenin in fetal mouse mesoderm caused  
28 loss of Tbx4<sup>+</sup> tracheal mesoderm and tracheal cartilage agenesis. The Tbx4 expression relied on  
29 endodermal Wnt activity and its downstream Wnt ligand but independent of known Nkx2.1-  
30 mediated respiratory development, suggesting that bidirectional Wnt signaling between endoderm  
31 and mesoderm promotes trachea development. Repopulating *in vivo* model, activating Wnt, Bmp  
32 signaling in mouse embryonic stem cell (ESC)-derived lateral plate mesoderm (LPM) generated  
33 tracheal mesoderm containing chondrocytes and smooth muscle cells. For human ESC-derived  
34 LPM, SHH activation was required along with Wnt to generate proper tracheal mesoderm.  
35 Together, these findings may contribute to developing applications for human tracheal tissue  
36 repair.

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45 **Main paragraph (3,080 words)**

46 The mammalian respiratory system is crucial for postnatal survival, and defects in the development  
47 of the respiratory system cause life-threatening defects in breathing at birth<sup>1</sup>. The trachea is a large  
48 tubular air path that delivers external air to the lung. Abnormal development of the tracheal  
49 mesenchyme, including cartilage and smooth muscle (SM), is associated with congenital defects in  
50 cartilage and SM such as tracheoesophageal fistula (TEF) and tracheal agenesis (TA)<sup>2, 3</sup>. Thus,  
51 understanding [trachea](#) development is crucial to better understand TEF/TA and establish a protocol to  
52 reconstruct trachea from pluripotent stem cells for human tissue repair.

53 Trachea/lung organogenesis is coordinated by endodermal-mesodermal interactions during  
54 embryogenesis. The primordial tracheal/lung endoderm appears at the ventral side of the anterior  
55 foregut at embryonic day 9 to 9.5 (E9.0–9.5) in mouse (Fig. 1a). Previous studies have revealed that  
56 development of tracheal/lung endoderm is initiated by graduated expression of mesodermal Wnt2/2b  
57 and Bmp4 expression along the dorsal-ventral axis<sup>4-7</sup>. This mesodermal-to-endodermal Wnt and Bmp  
58 signaling drives expression of Nkx2.1, the key transcription factor of tracheal/lung lineages<sup>8</sup>, at the  
59 ventral side of the anterior foregut, which in turn suppresses Sox2 to segregate these Nkx2.1<sup>+</sup>  
60 endodermal cells from the esophageal lineage. The Nkx2.1<sup>+</sup> endoderm then invaginates into the ventral  
61 mesoderm to form the primordial trachea and lung buds. At the same time, the Sox2<sup>+</sup> endoderm at the  
62 dorsal side develops into the esophagus by E10.5 (Fig. 1a). By recapitulating developmental processes  
63 *in vitro*, [trachea](#)/lung endodermal cells and differentiated epithelial populations have been generated  
64 from both mouse and human pluripotent stem cells<sup>9-11</sup>, and can also be used for disease modeling<sup>12-14</sup>.  
65 However, an established protocol for inducing tracheal/lung mesoderm and differentiated  
66 mesenchymal tissue from pluripotent cells has not yet been reported because developmental signaling  
67 pathways coordinating the mesodermal development are still undefined.

68 The tracheal mesoderm originates from [ventral fold of the](#) lateral plate mesoderm (LPM)

69 surrounding the anterior foregut endoderm. At the same time as endodermal Nkx2.1 induction,  
70 tracheal/lung mesoderm is also defined, expressing Tbx4/5 by E10.5, which are markers for  
71 tracheal/lung mesoderm and required for proper mesenchymal development (Fig. 1a)<sup>15</sup>. In contrast to  
72 Tbx5 which is also expressed in LPM and cardiac mesoderm<sup>16, 17</sup>, Tbx4 expression is restricted to  
73 respiratory tissue. At E9.5, Tbx4 is only detected in lung buds but not tracheal mesoderm  
74 (Supplementary Fig. S1). Tbx4 expression is then detected in tracheal mesoderm from E10.5. Tbx4  
75 and Tbx5 cooperate to steer normal trachea development. Both genes are required for mesodermal  
76 development of the trachea, particularly for cartilage and smooth muscle differentiation as well as  
77 morphogenesis. The crucial functions of these genes are validated by Tbx4, 5 double mutants  
78 exhibiting the phenotypes of tracheal stenosis<sup>15</sup>. We previously reported that synchronized polarization  
79 of mesodermal cells and temporal initiation of cartilage development regulates tracheal tube  
80 morphogenesis by coordinating the length and diameter of the mouse trachea, respectively<sup>18, 19</sup>.  
81 However, the mechanism underlying the initial induction of tracheal mesoderm is still unclear.  
82 To study the initiation of the mesodermal development of the trachea, we validated the involvement  
83 of Nkx2.1 in mesodermal Tbx4 expression because endodermal-mesodermal interactions orchestrate  
84 organogenesis throughout development in general. Nkx2.1 is an endodermal transcription factor  
85 necessary for tracheal and lung development and its genetic ablation results in TEF<sup>8</sup>. We examined  
86 *Nkx2.1*<sup>null</sup> mouse embryos and confirmed the TEF phenotype with a single tracheoesophageal (Tr-E)  
87 tube (Fig. 1b). Interestingly, *Nkx2.1*<sup>null</sup> embryos retained Tbx4 expression in the ventrolateral  
88 mesoderm of a single Tr-E tube, although the segregation was defective (Fig. 1b), indicating that  
89 mesodermal induction of the trachea is independent of endodermal Nkx2.1. We compared the  
90 phenotype of *Nkx2.1*<sup>null</sup> with that of *Shh*<sup>Cre</sup>, *Ctnnb1*<sup>fllox/fllox</sup> embryos which also show anterior foregut  
91 endoderm segregation defect and loss of Nkx2.1 expression (Fig. 1c and d)<sup>4, 5</sup>. In contrast to *Nkx2.1*<sup>null</sup>  
92 embryos, *Shh*<sup>Cre</sup>, *Ctnnb1*<sup>fllox/fllox</sup> embryos did not express Tbx4, suggesting the activation of endodermal

93 Wnt signaling, but not Nkx2.1, is required for following mesodermal Tbx4 expression. Thus, the initial  
94 induction of tracheal mesoderm is independent of known Nkx2.1-mediated respiratory endoderm  
95 development, but dependent on Wnt signaling at the ventral anterior foregut endoderm.

96 To further study the spatiotemporal regulation of canonical Wnt signaling during tracheoesophageal  
97 segregation at E9.5 to E11.5, we used a reporter line LEF1<sup>EGFP</sup> and examined the distribution of EGFP  
98 in the canonical Wnt signaling response (Figs. 2a and b)<sup>20</sup>. At E9.5, EGFP was detected in the ventral  
99 half of the anterior foregut endoderm where trachea endodermal cells appear and express Nkx2.1 (Figs.  
100 2a and b, *arrowheads*) and then **decreased temporally at E10.5**. After E10.5, the EGFP reporter was  
101 activated in the surrounding mesoderm and its intensity increased at E11.5 (Figs. 2a and b,  
102 *arrowheads*), **which was similar to the patterning of Axin2-LacZ, another reporter line for the response**  
103 **of canonical Wnt signaling<sup>21</sup>. We further conducted RNAscope *in situ* hybridization against Axin2, an**  
104 **endogenous Wnt target gene, to confirm activation of Wnt signaling in mesoderm. Axin2 was highly**  
105 **expressed in surrounding mesoderm at E10.5 compared to endoderm, similar to the pattern observed**  
106 **in the reporter line (Fig. 2c). Because these Wnt-responsive mesodermal cells expressed Tbx4 (Fig.**  
107 **2b), we hypothesized that Wnt signaling in the early mesoderm is involved in the initiation of the**  
108 **tracheal mesoderm.**

109 To validate the role of mesodermal Wnt signaling, we genetically ablated Ctnnb1, also known as  
110  $\beta$ -Catenin, which is a core component of canonical Wnt signaling, from embryonic mesoderm. We  
111 employed the Dermo1-Cre line which targets embryonic mesoderm, including tracheal/lung  
112 mesoderm, and generated *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>flox/flox</sup> mice<sup>22-25</sup>. In the mutant embryos, Tbx4 expression  
113 was absent in the mesoderm at E10.5 (Fig. 2d), indicating that mesodermal canonical Wnt signaling  
114 is necessary for Tbx4 expression. In contrast, endodermal Nkx2.1 expression and tracheoesophageal  
115 segregation were not affected, implying that mesodermal Wnt signaling and Tbx4 is dispensable for  
116 endodermal development. **Supporting the observation of lung buds in *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>flox/flox</sup>**

117 embryos<sup>26</sup>, the mutant lung buds still expressed Tbx4 in mesoderm (Supplementary Fig. S2a).  
118 Disruption of Wnt signaling in the mesoderm altered Tbx4 expression in the tracheal but not lung  
119 mesoderm, suggesting that Wnt-mediated mesodermal Tbx4 induction is a unique system in trachea  
120 development but not lung development.

121 We further found that the *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>flox/flox</sup> mutant exhibits tracheal cartilage agenesis. In  
122 the mutants, a periodic cartilage ring structure labeled with Sox9 failed to develop at E16.5, and  
123 circumferential SM bundles labeled with smooth muscle actin (SMA) were also malformed (Figs. 2e  
124 and e') Therefore, mesodermal Wnt signaling is crucial for trachea mesenchymal development,  
125 particularly for tracheal cartilage development.

126 To determine whether Tbx4 is a direct or indirect target of canonical Wnt signaling in respiratory  
127 mesoderm, we explored Tcf/Lef binding sequences in the Tbx4 lung mesenchyme element (Tbx4-  
128 LME)<sup>27, 28</sup>. We identified 5 repeats of the Tcf/Lef binding sequence by using University of California  
129 Santa Cruz (UCSC) Genome browser and JASPAR (Fig. 2f)<sup>29, 30</sup>. These Tcf/Lef binding sequences  
130 were well conserved in all vertebrates except for fishes. These sequences are active cis-regulatory  
131 regions for H3K27Ac, H3K4me1 and p300 as determined by ChIP-seq and by chromatin accessibility.

132 Next, we sought to identify a source of Wnt ligands that initiates mesodermal Tbx4 expression.  
133 Due to the essential role of Wnt2 at early tracheal/lung development<sup>4</sup>, we conducted *in situ*  
134 hybridization for *Wnt2* and revealed transient expression of *Wnt2* in the ventrolateral mesoderm of the  
135 anterior foregut at E9.5, which was obviously reduced by E10.5 when Tbx4 was expressed (Figs. 2b  
136 and 3a). Wnt2 is most likely not involved in Tbx4 expression after E10.5. This observation prompted  
137 us to hypothesize that an endodermal-to-mesodermal interaction but not mesodermal autonomous  
138 induction is required for Tbx4 expression. To test this hypothesis, we generated *Shh*<sup>Cre</sup>, *Wls*<sup>flox/flox</sup> mice,  
139 in which endodermal Wnt ligand secretion is inhibited by targeting *Wntless* (*Wls*) gene, which is  
140 essential for exocytosis of Wnt ligands<sup>31</sup>. This endoderm-specific deletion of *Wls* resulted in loss of

141 Tbx4 expression in the mesoderm, but retained Nkx2.1 expression in the endoderm and *Wnt2* in the  
142 mesoderm (Figs. 3b and c)<sup>31</sup>, making these mice a phenocopy to *Dermo1<sup>Cre</sup>*, *Ctnnb1<sup>flox/flox</sup>* mice (Fig.  
143 2d). *Shh<sup>Cre</sup>*, *Wls<sup>flox/flox</sup>* embryos also formed lung buds and expressed Tbx4 in the distal lung mesoderm  
144 (Supplementary Fig. S2b), supporting our idea that Wnt signaling in mesoderm mainly contributes to  
145 initiation of mesodermal development of the trachea, but not of the lung. These findings indicate that  
146 the endodermal Wnt ligands are sufficient for trachea mesodermal development. From these  
147 observations, we conclude that mesodermal Wnt2 activates endodermal canonical Wnt signaling  
148 which activates endodermal Wnt ligand expression independent of Nkx2.1. These Wnt ligands then  
149 induce endodermal-to-mesodermal canonical Wnt signaling to initiate mesodermal Tbx4 expression  
150 (Fig. 3d). These results also suggest that specification in the trachea endodermal lineage is not  
151 necessary for the initial induction of the tracheal mesoderm.

152 In the developing mouse trachea, several Wnt ligands are expressed in the endoderm between  
153 E11.5 to E13.5, such as Wnt3a, 4, 5a, 6, 7b, 11 and 16<sup>26, 31</sup>. Current single cell RNA-seq data have  
154 shown the presence of several Wnt ligands including Wnt4, 5a, 5b, 6 and 7b in the respiratory  
155 endoderm of mouse embryos at E9.5 (Han et al., back-to-back). We performed *in situ* hybridization  
156 (ISH) against these Wnt ligands at E10.5 to determine the particular ligand inducing Tbx4 expression  
157 in trachea development (Fig. 3e, Supplementary Fig. S4). Wnt4 was expressed in esophageal  
158 mesoderm and barely detected in tracheal endoderm. Wnt5a, 5b and 6 were detected in both the  
159 endoderm and mesoderm of the trachea. More importantly, Wnt7b was abundantly expressed in  
160 tracheal endoderm, suggesting that Wnt7b might be responsible for the ensuing induction of  
161 mesodermal Tbx4 expression

162 To examine whether Wnt signaling is capable of initiating the differentiation of naïve  
163 mesodermal cells to Tbx4<sup>+</sup> trachea mesodermal cells *in vitro*, we established a protocol for lateral plate  
164 mesoderm (LPM) induction from mouse ESCs by refining the published protocol for LPM induction

165 from human pluripotent stem cells<sup>32</sup>. Because mouse and human ESCs show different states called  
166 naïve and primed, which correspond to pre- and post-implantation epiblasts, respectively, we  
167 converted mouse ESCs (mESCs) into an epiblast ‘primed’ state that led to middle-primitive streak  
168 (mid-PS) cells<sup>33</sup>. These mid-PS cells were then differentiated into LPM cells (Fig. 4a). At day 5, LPM  
169 induction was confirmed by immunocytochemistry (ICC) for Foxf1 and Gata4 which are known to be  
170 expressed in LPM including splanchnic mesoderm<sup>34, 35</sup> (Fig. 4b), and showed that 89% of total cells  
171 were Foxf1<sup>+</sup> LPM. qRT-PCR also showed obvious upregulation of LPM marker genes such as Foxf1,  
172 Gata4, Hoxb6, Prrx1, and Bmp4 (Figs. 4c and e). Given that previous mouse genetic studies have  
173 identified Bmp4 as a crucial regulator of trachea development<sup>6, 36</sup>, we tested whether canonical Wnt  
174 and Bmp4 signaling are sufficient to direct the differentiation of LPM into the tracheal mesoderm  
175 (Foxf1<sup>+</sup>/Tbx4<sup>+</sup>). mESC-derived LPM cells were cultured with CHIR99021, a GSK3 $\beta$  inhibitor to  
176 stabilize  $\beta$ -catenin and activate canonical Wnt signaling, and Bmp4. At day 6, 89% of total cells  
177 became double positive for Foxf1<sup>+</sup> and Tbx4<sup>+</sup>. qRT-PCR further demonstrated elevated expression of  
178 tracheal marker genes such as Tbx5, Wnt2, Bmp4 in addition to Tbx4 (Figs. 4d and e). To further  
179 confirm the respiratory characteristics of these cells, we took advantage of the 5 repeats of Tcf/lef  
180 binding sequences in Tbx4-LME, which we described in Fig. 2f. We established a luciferase reporter  
181 assay by reporter plasmids that express luciferase under the control of Tbx4-LME (Fig. 4f). The  
182 reporter plasmid was transfected into mESC-derived LPM and luciferase activity was assessed during  
183 differentiation. After 24 hours (at day 6), the luciferase activity increased in the presence of  
184 CHIR99021 (Fig. 4g). Importantly, the mutated reporter, in which all Tcf/Lef binding sequences were  
185 changed to random sequences (Figs. 2f and 4f), did not respond to CHIR99021. These results  
186 determined that the mESC-derived cells were differentiated into proper tracheal mesoderm at day 6.

187 Because tracheal mesenchyme includes cartilage and smooth muscle, we wondered whether our  
188 protocol induces mESC to differentiate into these tissues. At day 12, Sox9<sup>+</sup> aggregated cell masses



189 positive for Alcian blue staining appeared on the dish, indicative of chondrocytes (Figs. 4h-j). Smooth  
190 muscle cells (SMA<sup>+</sup> cells) concurrently appeared to show fibroblastic morphology and filled the spaces  
191 not filled by the Sox9<sup>+</sup> cells (Figs. 4h and i). Other chondrogenic markers (Aggrecan, Collagen2a1,  
192 Sox5/6, Epiphygan) and smooth muscle markers (Tagln, Collagen1a1) were also present in the  
193 differentiated cells (Figs. 4k, l and Supplementary Fig. S6a). These data suggest that the mESC-  
194 derived tracheal mesoderm is able to develop into tracheal mesenchyme, including chondrocytes and  
195 smooth muscle cells.

196 Finally, we tested the role of Wnt signaling in the human tracheal mesoderm using human ESCs  
197 (hESCs). Human LPM induction was performed by following an established protocol<sup>32</sup> (Fig. 5a).  
198 Subsequently, the cells were directed to tracheal mesoderm by using CHIR99021 and BMP4. For  
199 validating hESC-derived LPM, we checked the common LPM markers at day 2 and confirmed that  
200 these markers were abundantly expressed in the LPM (Figs. 5b, c and Supplementary Fig. S7j).  
201 Immunostaining determined that 95% of the total cells expressed FOXF1 at day 2 (Fig. 5b). Because  
202 Tbx4 is also expressed in the limbs and other fetal mouse tissues<sup>28</sup>, we sought additional genetic  
203 markers for the tracheal mesoderm. We searched the single-cell transcriptomics dataset of the  
204 developing splanchnic mesoderm at E9.5 and identified *Nkx6.1* as a marker for mesodermal cells  
205 surrounding the trachea, lung and esophagus (Han et al., co-submitted). We performed  
206 immunostaining and found that Nkx6.1 was expressed in tracheal and esophageal mesenchyme  
207 throughout development (Supplementary Fig. S5). Of note, Nkx6.1 was expressed in esophageal and  
208 dorsal tracheal mesenchyme but not ventral trachea, which enabled us to define three subtypes of  
209 tracheal-esophageal mesenchyme based on the combination of Tbx4 and Nkx6.1 expression (i.e.  
210 Tbx4<sup>+</sup>/Nkx6.1<sup>+</sup>; dorsal tracheal mesenchyme, Tbx4<sup>+</sup>/Nkx6.1<sup>-</sup>; ventral tracheal mesenchyme, Tbx4<sup>-</sup>  
211 /Nkx6.1<sup>+</sup>; esophageal mesenchyme) (Supplementary Fig. S5). Having characterized the subtypes of  
212 tracheal-esophageal mesoderm *in vivo*, the expression of *TBX4* and *NKX6.1* in the hESC-derived

213 tracheal mesoderm was examined by ICC and qRT-PCR. Although *TBX4* was induced in a Wnt agonist  
214 dose-dependent manner, *NKX6.1* expression was not significantly elevated (Supplementary Figs. S7a,  
215 b), suggesting that human trachea mesodermal development requires an additional factor to become  
216 more *in vivo*-like. Because the ventral LPM is exposed to SHH in addition to Wnt and Bmp4 during  
217 tracheoesophageal segregation<sup>37, 38</sup>, we assessed whether the SHH agonist (PMA; purmorphamine)  
218 can improve differentiation from hESC-derived LPM cells into the tracheal mesoderm. As expected,  
219 both *TBX4* and *NKX6.1* expression was upregulated by the SHH agonist (Supplementary Figs. S7c,  
220 d). After day 5, the differentiating cells also expressed respiratory markers such as *TBX4*, *TBX5*,  
221 *WNT2*, *BMP4* and *NKX6.1* (Figs. 5d-h, Supplementary Fig. S7j). In this culture condition,  
222 CHIR99021 enhanced the expression of *TBX4* and *NKX6.1* genes in a dose-dependent manner  
223 (Supplementary Figs. S7e, f). We further estimated the efficiency of the induction by immunostaining  
224 for *TBX4* and *FOXF1*, and then confirmed that 83% of total cells were *TBX4*<sup>+</sup>/*FOXF1*<sup>+</sup> double  
225 positive cells at day 5 (Fig. 5d). At day 10, *NKX6.1* expression was clearly elevated, and 30.3% of the  
226 total cells became *TBX4*<sup>+</sup>/*NKX6.1*<sup>+</sup> double positive (Figs. 5e-g) while 18.0% were *TBX4*<sup>+</sup>/*NKX6.1*<sup>-</sup>  
227 (Fig. 5g). These data suggest that the half of the cells induced with our protocol are trachea  
228 mesodermal cells. Further extended culture induced *SOX9*<sup>+</sup> aggregates which were positive for Alcian  
229 blue staining in a Wnt activity-dependent manner (Figs. 5h-l). Likewise in mESC-derived cells,  
230 *ACTA2*<sup>+</sup> smooth muscle-like fibroblastic cells occupied *Sox9*<sup>-</sup> region (Fig. 5i). These cells also  
231 expressed chondrogenic markers and smooth muscle cell markers (Figs. 5k, l and Supplementary Fig.  
232 S6b).

233 In this culture system, the removal of BMP4 from the growth factor cocktail did not affect  
234 differentiation, implying that exogenous BMP4 activation is dispensable (Supplementary Fig. S7g-i).  
235 Because of the obvious upregulation of the endogenous *BMP4* gene in the hESC-derived LPM by day  
236 2, endogenous BMP4 may be enough to induce tracheal mesoderm and chondrocytes (Supplementary

237 Fig. S7j). Taken together, these data suggest that Wnt signaling plays a unique role in driving  
238 differentiation into tracheal mesoderm and chondrocytes from the LPM, which is conserved between  
239 mice and human.

240 This study demonstrated that endodermal-to-mesodermal canonical Wnt signaling is the cue  
241 that initiates trachea mesodermal development in developing mouse embryos, which is independent  
242 of the previously known Nkx2.1-mediated respiratory tissue development. Based on our knowledge  
243 of developmental biology, we successfully generated tracheal mesoderm and chondrocytes from  
244 mouse and human ESCs. In our protocol, we stimulated ESC-derived LPM with Wnt, Bmp and SHH  
245 signaling to mimic spatial information of the ventral anterior foregut. For induction of respiratory  
246 endoderm, Wnt, Bmp and Fgf signaling are required to direct cells in anterior foregut to differentiate  
247 into the respiratory lineage<sup>9-11</sup>. Thus, Wnt and Bmp signaling are conserved factors that provide spatial  
248 information, while Fgf and SHH are required in endoderm and mesoderm induction, respectively,  
249 reflecting the unique signaling pathways in each tissue. Mesoderm induction may need fewer  
250 exogenous growth factors because the mesodermal cells themselves are a source of spatial information,  
251 such as BMP4 in our protocol.

252 In this study, we were unable to perform tissue-specific targeting for trachea endodermal or  
253 mesodermal cells because of multiple Cre-expression patterns in *Shh-Cre* and *Dermo1-Cre* mouse  
254 lines. For example, *Shh* is also expressed in the notochord and ventral neural tube<sup>39</sup>. Future studies  
255 with analyses of respiratory tissue-specific Cre lines would strengthen the evidence demonstrating that  
256 mutual interaction between respiratory endoderm and mesoderm is required for the induction of  
257 trachea development.

258 *Dermo1<sup>Cre</sup>*, *Ctnnb1<sup>flox/flox</sup>* mutants display a tracheal cartilage agenesis phenotype. Due to the  
259 multiple functions of *Ctnnb1* in transcriptional regulation and cellular adhesion, however, it is possible  
260 that *Ctnnb1* knockout affects not only Wnt-mediated transcriptional regulation but also mesenchymal

261 cell-cell adhesion<sup>24</sup>. To exclude this possibility, we examined the distribution of CDH2 as an adhesive  
262 molecule in tracheal mesoderm. CDH2 expressions in the ventral half of tracheal mesoderm were  
263 indistinguishable between control and *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>fllox/fllox</sup> embryos (Supplementary Fig. S3).  
264 Furthermore, our luciferase assay revealed that respiratory mesenchyme specific cis-regulatory region  
265 of *Tbx4* is stimulated by CHIR99021 through Tcf/Lef binding elements in the developing tracheal  
266 mesoderm. These findings suggest that Wnt signaling-mediated transcriptional regulation is important  
267 for the induction of tracheal mesoderm.

268           Recently, Han et al. delineated mesodermal development during organ bud specification  
269 using single-cell transcriptomics analyses of mouse embryos from E8.5 to E9.5 (Han et al., co-  
270 submitted to *Nature communications*). Based on the trajectory of cell fates and signal activation, this  
271 group also generated organ-specific mesoderm, including respiratory mesoderm, from hESCs, thereby  
272 determining that Wnt, BMP4, SHH and retinoic acid direct differentiation of hESC-derived splanchnic  
273 mesoderm to respiratory mesoderm, supporting our current findings.

274 These culture methods could be a strong tool to study human organogenesis and the aetiology of TEA  
275 and TA, as well as to provide cellular resource for human tracheal tissue repair.

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285 **Methods**

286 *Mice*

287 All mouse experiments were approved by the Institutional Animal Care and Use Committee of RIKEN  
288 Kobe Branch. Mice were handled in accordance with the ethics guidelines of the institute. *Nkx2.1<sup>null</sup>*,  
289 *Shh<sup>Cre</sup>*, *Dermo1<sup>Cre</sup>*, *Ctnnb1<sup>flox/flox</sup>*, *Wls<sup>flox/flox</sup>* mice were previously generated<sup>18, 23, 40-42</sup>.

290 In all experiments, at least 3 embryos from more than 2 littermates were analyzed. All attempts for  
291 replicate were successful. Sample size was not estimated by statistical methods. No data was excluded  
292 in this study. All control and mutant embryos were analyzed. No blinding was done in this study.

293

294 *Immunostaining*

295 Mouse embryos were fixed by 4% Paraformaldehyde/PBS (PFA) at 4°C overnight. Specimens  
296 were dehydrated by ethanol gradient and embedded in paraffin. Paraffin sections (6-µm) were de-  
297 paraffinized and rehydrated for staining. Detailed procedure and antibodies of each staining were listed  
298 in [Supplementary Table 1](#).

299

300 *In situ hybridization*

301 Mouse embryos were fixed with 4%PFA/PBS at 4°C overnight, and then tracheas were dissected.  
302 Specimens were incubated in sucrose gradient (10, 20, 30%) and embedded in OCT compound. Frozen  
303 sections (12-µm) were subjected to *in situ* hybridization. For *Wnt2*, *4*, *5a*, *7b* probe construction,  
304 cDNA fragments were amplified by primers listed in [Supplementary Table 2](#). These cDNA fragments  
305 were subcloned into pBluscript SK+ at *EcoRI* and *SalI* sites. For *Wnt5b* and *6* probes, pSPROT1-  
306 *Wnt5b* (MCH085322) and pSPROT1-*Wnt6* (MCH000524) were linearized at *SalI* sites, The NIA/NIH  
307 [Mouse 15K and 7.4K cDNA Clones](#) were provided by the RIKEN BRC<sup>43-45</sup>. Antisense cRNA  
308 transcripts were synthesized with DIG labeling mix (Roche Life Science) and T3 or SP6 RNA

309 polymerase (New England Biolabs Inc.). Slides were permeabilized in 0.1% Triton-X100/PBS for  
310 30min and blocked in acetylation buffer. After pre-hybridization, slides were hybridized with  
311 500ng/ml of DIG-labeled cRNA probes overnight at 65°C. After washing with SSC, slides were  
312 incubated with anti-DIG-AP antibodies (1:1000, Roche Life Science, 11093274910). Sections were  
313 colored with BM-purple (Roche Life Science, 11442074001).

314 For RNAscope experiments, the RNAscope Multiplex Fluorescent v2 assays (Advanced Cell  
315 Diagnostics, 323110) were used. The detailed procedure and probes were listed on Supplementary  
316 Table 3

317

#### 318 *Cell culture*

319 For mesodermal differentiation from mES cells, [C57BL/6J-Chr 12A/J/NaJ AC464/GrsJ mES cells](#)  
320 [\(The Jackson Laboratory\)](#) and EB3 cells (AES0139, RIKEN BioResource Center) were used.  
321 [C57BL/6J-Chr 12A/J/NaJ AC464/GrsJ mES cells](#) were kindly provided by Kentaro Iwasawa and  
322 Takanori Takebe (Center for Stem Cell & Organoid Medicine (CuSTOM), Perinatal Institute, Division  
323 of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital, Cincinnati). EB3 was  
324 kindly provided by Dr. Hitoshi Niwa (Department of Pluripotent Stem Cell Biology, Institute of  
325 Molecular Embryology and Genetics in Kumamoto University)<sup>46, 47</sup>. Cells were maintained in 2i +  
326 leukemia inhibitory factor (LIF) media (1,000 units/ml LIF, 0.4µM PD0325901, 3µM CHIR99021 in  
327 N2B27 medium) on ornithine-laminin coated-dishes<sup>33</sup>. For mesodermal differentiation of mouse ES  
328 cells, cells were digested by TrypLE express (Thermo Fisher Scientific, 12604013) and seeded onto  
329 Matrigel-coated 12 well plate. EpiLC were induced by EpiLC differentiation medium (1% knockout  
330 serum, 20ng/ml Activin A, 12ng/ml FGF2, and 10µM Y27632 in N2B27 Medium)<sup>33</sup> for 2 days.  
331 Lateral plate mesoderm was established by Loh's protocol with some modification<sup>32</sup>. EpiLC cells were  
332 digested by TrypLE express to single cells and seeded onto Matrigel-coated 12 well plate at the density

333 of  $6 \times 10^5$  cells/well. The cells around middle primitive streak was induced by LPM D2 medium  
334 composed of 2% B27 Supplement Serum free (Thermo Fisher Scientific, 17504044), 1 x GlutaMax  
335 (Thermo Fisher Scientific, 35050061), 20ng/ml basic FGF (Peprotech, AF-100-18B),  $6 \mu\text{M}$   
336 CHIR99021 (Sigma Aldrich, SML1046), 40ng/ml BMP4 (R&D Systems, 5020-BP-010), 10ng/ml  
337 Activin A (Peprotech, PEP-120-14-10),  $10 \mu\text{M}$  Y27632 (Sigma Aldrich, Y0503) in Advanced DMEM  
338 (Thermo Fisher Scientific, 12491015) for 48 hours. After that, LPM was induced by LPM D4 medium  
339 composed of 2% B27 Supplement Serum free, 1 x GlutaMax,  $2 \mu\text{M}$  XAV939 (Sigma Aldrich, X3004),  
340  $2 \mu\text{M}$  SB431542 (Merck, 616461), 30ng/ml human recombinant BMP4 in Advanced DMEM for 24  
341 hours. At Day 5, respiratory mesenchyme was induced by Day5 medium composed of 2% B27  
342 Supplement Serum free, 1 x GlutaMax,  $1 \mu\text{M}$  CHIR99021, 10ng/ml BMP4. Medium were freshly  
343 renewed every day.

344 H1 (NIHhESC-10-0043 and NIHhESC-10-0062), human embryonic stem cell, was provided by  
345 Cincinnati children's hospital medical center Pluripotent Stem Cell Facility. Cells were maintained in  
346 mTeSR1 medium (Stem Cell Technologies) on Matrigel-coated plate. For differentiation of H1 cells  
347 to mesodermal cells, confluent cells were digested by Accutase to single cells and seeded onto Geltrex-  
348 coated 12well plate at the dilution of 1:20 – 1:18 in mTeSR1 with  $1 \mu\text{M}$  Thiazovivin (Tocris). Next day,  
349 the cells around middle primitive streak were induced by cocktails of  $6 \mu\text{M}$  CHIR99021 (Sigma  
350 Aldrich, SML1046), 40ng/ml BMP4 (R&D Systems, 5020-BP-010), 30ng/ml Activin A (Cell  
351 Guidance Systems), 20ng/ml basic FGF (Thermo Fisher Scientific) and 100nM PIK90 (EMD  
352 Millipore) in Advanced DMEM/F12 including 2% B27 Supplement minus vitamin A, 1% N2  
353 Supplement, 10uM Hepes, 100UI/mL Penicillin/Streptomycin, 2mM L-glutamine for 24 hours. After  
354 that, LPM was induced by LPM D2 medium composed of  $1 \mu\text{M}$  Wnt C59 (Cellagen Technologies),  
355  $1 \mu\text{M}$  A83-01 (Tocris), 30ng/ml human recombinant BMP4 in Advanced DMEM/F12 including 2%  
356 B27 Supplement minus V. A., 1 x N2 Supplement, 10uM Hepes, 100UI/mL Penicillin/Streptomycin,

357 2mM L-glutamine for 24 hours. To generate respiratory mesenchyme, we combined 3uM CHIR99021,  
358 2uM Purmorphamine (Tocris), and 10ng/ml Bmp4 in Advanced DMEM/F12 medium including 2%  
359 B27 Supplement Serum free, 1 x N2 Supplement, 10uM HEPES, 100UI/mL Penicillin/Streptomycin,  
360 2mM L-glutamine from Day 2 to Day10. Medium was freshly renewed everyday

361

### 362 *Immunocytochemistry*

363 At differentiating process, cells were fixed by 4% PFA for 10 minutes at room temperature. For  
364 intracellular staining, cells were permeabilized by 0.2% TritonX-100/PBS for 10 minutes at room  
365 temperature. After blocking the cells with 5% normal donkey serum, cells were incubated with  
366 primary antibodies overnight at 4°C. Then, cells were incubated with secondary antibodies for 1hr at  
367 room temperature. Detailed procedure and antibodies of each staining were listed in [Supplementary](#)  
368 [Table 4](#).

369

### 370 *Luciferase reporter assay*

371 The fraction of mouse *Tbx4*-lung mesenchyme specific enhancer (LME) (mm10, chr11:85,893,703-  
372 85,894,206, GenScript, ID U3154EL200-3)<sup>27</sup> or *Tbx4*-LME containing putative Tcf/Lef sites mutated  
373 (GenScript, ID U3154EL200-6) were synthesized and cloned into pGL4.23 (luc2/minP) vector  
374 (promega).

375 mESC-derived LPM cells were transfected at day 5 in 150µl of Opti-MEM (Thermo Fisher Scientific,  
376 31985088) with 2µl of Lipofectamine Stem (Thermo Fisher Scientific, STEM00003) and 1µg of  
377 pGL4.23 (luc2/minP) containing a fraction of mouse *Tbx4*-LME or *Tbx4*-LME containing mutated  
378 Tcf/Lef sites. Four hours after transfection the tracheal mesenchyme was induced using day5 medium  
379 in presence or absence of Wnt agonist (3µM CHIR99021) and cells were cultured for 24 hours and  
380 then lysed and assayed using Dual-Luciferase Reporter Assay System (Promega, E1980).



381

382 *Alcian blue staining*

383 Cells were fixed in 4% PFA/PBS for 10 minute at room temperature. After washing with PBS,  
384 cells were incubated with 3% acetic acid for 3 minutes and then stained with 1% alcian blue/3% acetic  
385 acid for 20 minutes.

386

387 *Quantitative RT-PCR*

388 Total mRNA was isolated by using the Nucleospin kit (TaKaRa, 740955) according to  
389 manufacturer's instruction. cDNA was synthesized by Super<sup>TM</sup>Script<sup>TM</sup> VILO cDNA synthesis kit  
390 (Thermo Fisher Scientific, 11754050). qPCR was performed by PowerUp<sup>TM</sup> SYBR<sup>TM</sup> Green Master  
391 Mix on QuantStudio 3 or 6. Primer sequences were listed on [Supplementary Table 5 and 6](#). Data are  
392 expressed as a Fold Change and were normalized with undifferentiated cells expression.

393

394 *Statistical analyses*

395 Statistical analyses were performed with Excel2013 (Microsoft) or PRISM8 (GraphPad software).  
396 For multiple comparison, one-way ANOVA and Tukey's methods were applied. For paired comparison,  
397 statistic significance was determined by F-test and Student's or Welch's two-tailed t test.

398

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403 [C57BL/6 mouse ES cell line](#). We are grateful to [Debora Sinner](#) for the help on RNAscope experiment.  
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413

#### 414 **Author contributions**

415 K.K., and M.M. designed the project and performed experiments with the aid of A.L.M., A.Y., C.  
416 M., and K.T.F. AMZ analysed single cell transcriptomics for definitive endoderm and splanchnic  
417 mesoderm. A.L.M. [performed enhancer analyses of Tbx4 gene and](#) supported human ES cell  
418 experiments, A.Y. supported mouse experiments. K.K., K.T. F. and C.M. performed mouse ES cell  
419 experiment. C. A. and M.H. contributed to mouse and human embryonic-stem cell-based lateral plate  
420 mesoderm induction and differentiation experiments.

421 K.K. and M.M. wrote the manuscript with the contribution of all authors.

422

#### 423 **Competing interests**

424 The authors declare no competing interests.

425

#### 426 **Data availability**

427 The authors declare that all data supporting the findings of this study are available within the article  
428 and its Supplementary Information files or from the corresponding author upon reasonable request.  
429 [The Source data underlying Figs. 4b-e, 4g, 4i, 5b-e, 5g, 5i and Supplementary Figs. S6 and S7 were](#)

430 [provided as a Source data file.](#)

431 The datasets generated during the current studies are available in the System Science of Biological  
432 Dynamics (SSBD) database (<http://ssbd.qbic.riken.jp/>).

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455 **References**

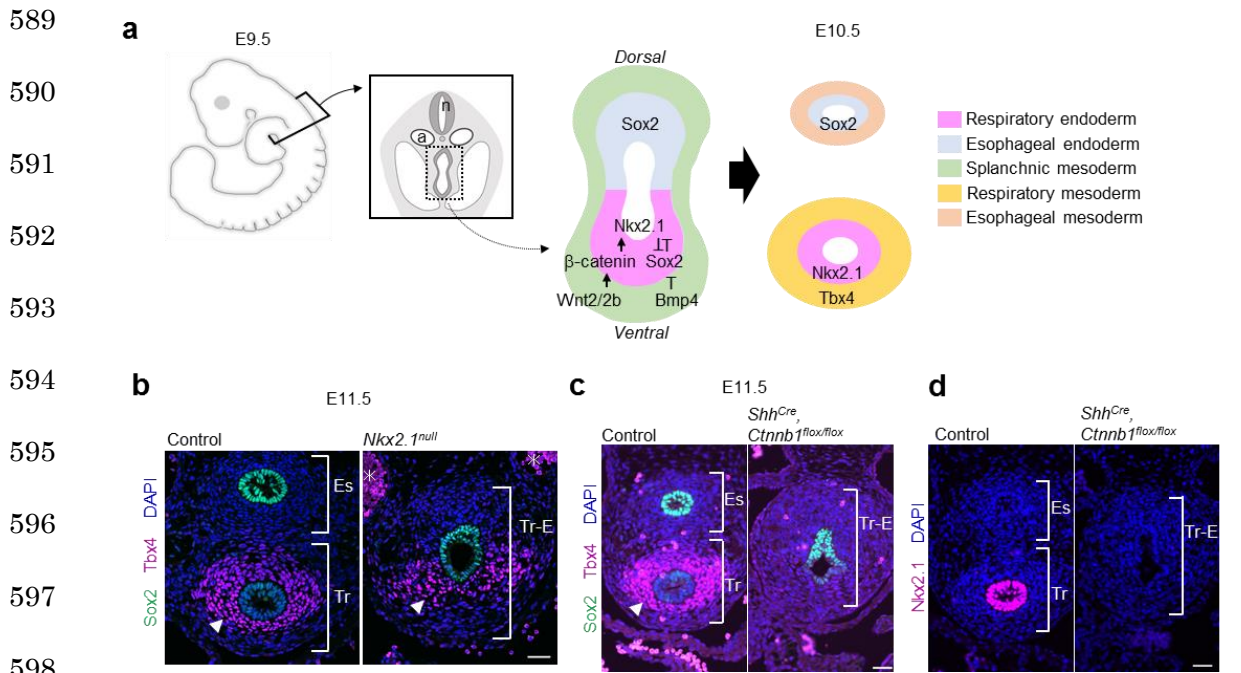
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**Figure 1 Activation of Wnt signaling in endoderm, but not Nkx2.1 expression, is activated to promote mesodermal development of the mouse trachea.**

**a**, Schematic model of tracheoesophageal segregation.

**b**, Transverse sections of *Nkx2.1*<sup>null</sup> mouse embryos and littermate controls. Sections were stained for Sox2 (green), Tbx4 (magenta), and DAPI (blue). Arrowheads indicate Tbx4<sup>+</sup> tracheal mesoderm. Asterisks indicate non-specific background signal of blood cells in dorsal aorta.

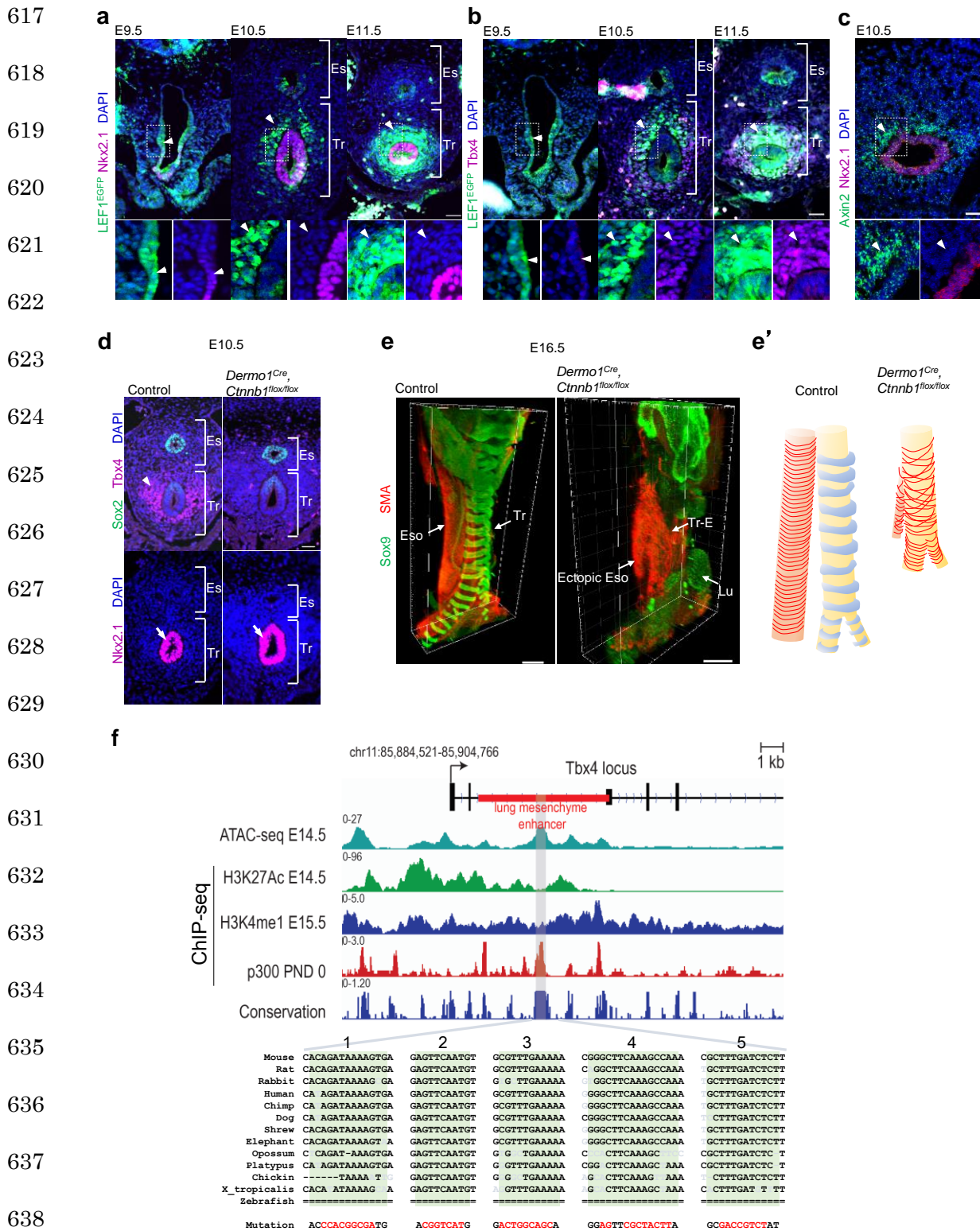
**c**, Transverse sections of *Shh*<sup>Cre</sup>, *Ctnnb1*<sup>flox/flox</sup> mouse embryos and littermate controls. Sections were stained by Sox2 (green), Tbx4 (magenta), and DAPI (blue). Arrowheads indicate Tbx4<sup>+</sup> tracheal mesoderm.

**d**, Transverse sections of *Shh*<sup>Cre</sup>, *Ctnnb1*<sup>flox/flox</sup> mouse embryos and littermate controls. Sections were stained by Nkx2.1 (magenta) and DAPI (blue).

n; neural tube, a; aorta, Es; Esophagus, Tr; Trachea, Tr-E; Trachea-Esophageal tube

Scale bar, 40μm





641 **Figure 2 Wnt signaling is activated to promote mesodermal development of the mouse trachea.**

642 **a**, Transverse sections of LEF1<sup>EGFP</sup> reporter mouse embryos at E9.5 to E11.5. Sections were stained for EGFP (*green*), Nkx2.1  
643 (*magenta*), and DAPI (*blue*). Arrowheads indicate GFP<sup>+</sup> cells.

644 **b**, Transverse sections of LEF1<sup>EGFP</sup> reporter mouse embryos at E9.5 to E11.5. Sections were stained for EGFP (*green*), Tbx4 (*magenta*),  
645 and DAPI (*blue*). Arrowheads indicate GFP<sup>+</sup> cells.

646 **c**, Transversal section of mouse embryo at E10.5. Section were stained for Axin2 (*green*), Nkx2.1 (*magenta*) and DAPI (*blue*) by  
647 RNAscope experiment. Arrowheads indicate Axin2<sup>+</sup> cells.

648 **d**, Transverse sections of *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>lox/lox</sup> mouse embryos and littermate controls at E10.5. Upper panels show sections stained  
649 for Sox2 (*green*), Tbx4 (*magenta*), and DAPI (*blue*). Lower panels show sections stained for Nkx2.1 (*magenta*) and DAPI (*blue*).

650 Arrowhead indicates Tbx4<sup>+</sup> cells. Arrows indicate Nkx2.1<sup>+</sup> cells.

651 **e**, Three-dimensional imaging of whole trachea and esophagus tissue at E16.5. Cartilage morphology and smooth muscle architecture  
652 in the tracheas of *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>lox/lox</sup> mouse embryos and control littermates. Whole trachea and esophagus were stained for  
653 Sox9 (*green*) and SMA (*red*).

654 **e'**, Model of tracheal architecture in *Dermo1*<sup>Cre</sup>, *Ctnnb1*<sup>lox/lox</sup> mouse embryos and control littermates based on (**e**). Eso; Esophagus,

655 **f**, Integrative Genomics Viewer (IGV) snapshot of mm10 (chr11:85,884,521-85,904,766) showing mouse *tbx4* lung mesenchyme  
656 specific enhancer (LME) and compiled ENCODE data of ATAC-seq E14.5 (ENCSR335VJW), H3K27Ac E14.5 (ENCSR452WYC),  
657 H3K4me1 E15.5 (ENCFF283EBS), EP300 post-natal day (PND) 0 and vertebrate conservation (Phastcons). Numbers indicate fold  
658 enrichment over input (ChIP-seq). CisBP and Jaspar predicted Tcf/Lef-binding sites (highlighted in green, region: mm10,  
659 chr11:85,893,703-85,894,206) are localized at the ATAC-seq and p300 peaks that are conserved among most vertebrates. Sequence  
660 in red shows the Tcf/Lef-binding sites mutated.

661 Lu; Lung, Tr; Trachea, Tr-E; Tracheoesophageal tube.

662 Scale bar: 40  $\mu$ m (a, b), 50  $\mu$ m (c, d), 300  $\mu$ m (e).

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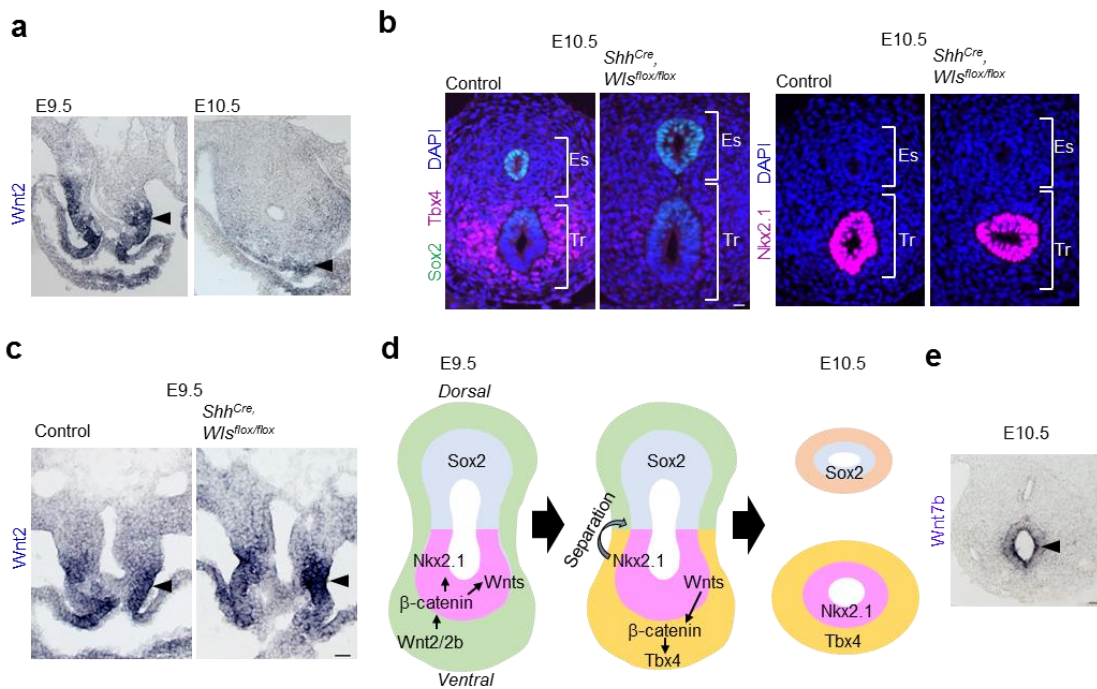
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688 **Figure 3 Endodermal Wnt ligands induce Tbx4 expression for tracheal mesoderm development of mouse trachea.**

689 a, *In situ* hybridization for *Wnt2* mRNA during tracheoesophageal segregation. Arrowheads indicate *Wnt2* expression in the  
690 ventrolateral mesoderm at E9.5 and E10.5.

691 b, Transverse sections of *Shh<sup>Cre</sup>, Wls<sup>flox/flox</sup>* mouse embryos and littermate controls at E10.5. Left panels show sections stained with  
692 Sox2 (green), Tbx4 (magenta), and DAPI (blue). Right panels show sections stained for Nkx2.1 (magenta) and DAPI (blue).

693 c, *In situ* hybridization for *Wnt2* mRNA in *Shh<sup>Cre</sup>, Wls<sup>flox/flox</sup>* mouse embryos and littermate controls at E9.5. Arrowheads indicate *Wnt2*  
694 expression in the ventrolateral mesoderm.

695 d, Refined model of tracheoesophageal segregation and tracheal mesodermal differentiation

696 e, *In situ* hybridization for *Wnt7b* mRNA in mouse embryo at E10.5. Arrowhead indicates *Wnt7b<sup>+</sup>* cells.

697 Eso; Esophagus, Lu; Lung, Tr; Trachea.

698 Scale bar; 40 μm (a-c), 50 μm (e).

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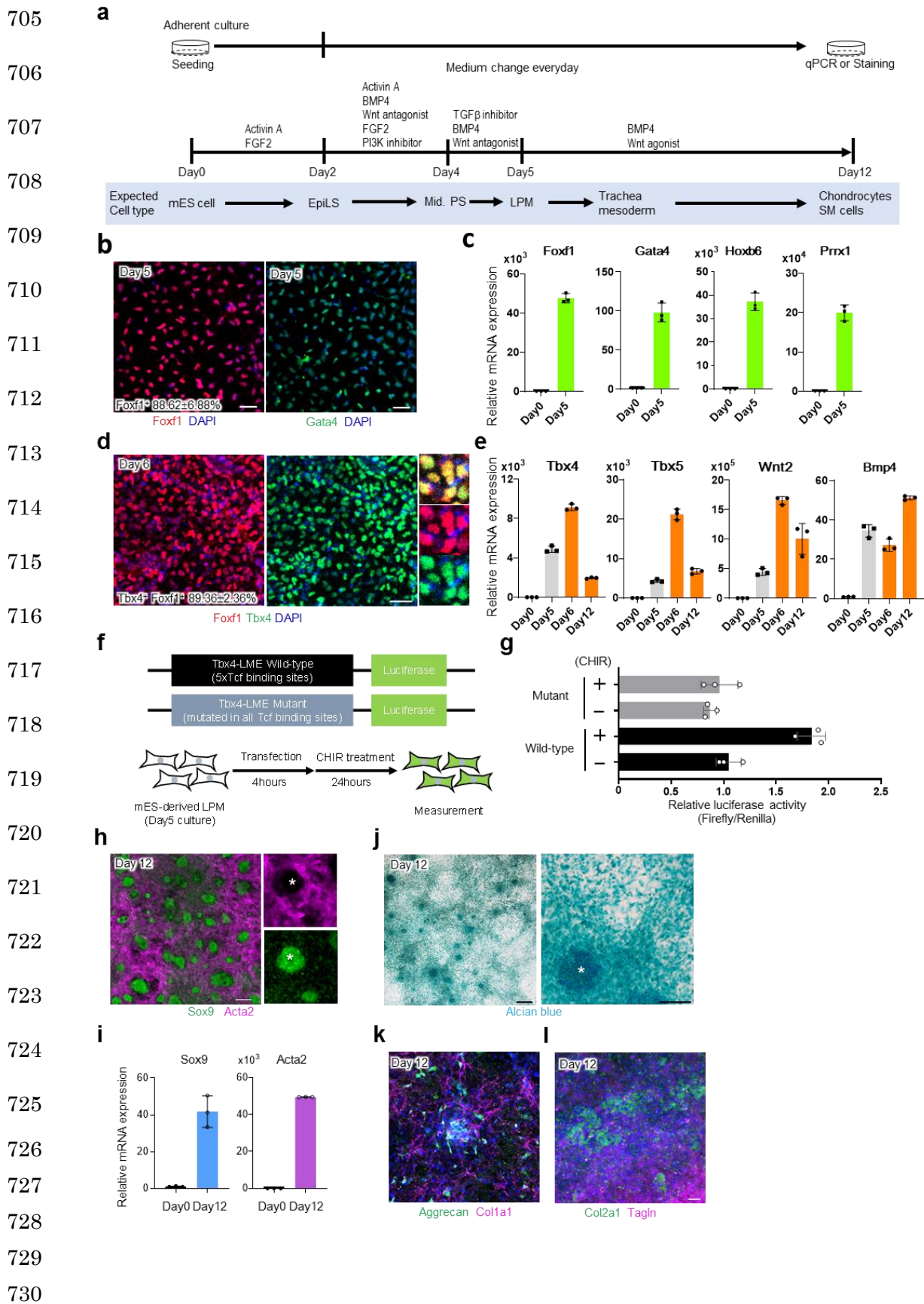
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731 **Figure 4 Generation of trachea mesodermal cells and chondrocytes from mouse ESCs *in vitro*.**

732 **a**, Experimental design to generate tracheal mesoderm from mESCs.

733 **b**, Differentiating cells from mESCs at day 5. Cells were stained for Foxf1 (*red*) and Gata4 (*green*), respectively.

734 **c**, Results of qRT-PCR for LPM marker expression of mESC-derived LPM at day 0 and 5.

735 **d**, Differentiating cells from mESCs at day 6. Cells were stained for Tbx4 (*green*) and Foxf1 (*red*).

736 **e**, Results of qRT-PCR for respiratory mesoderm marker expression of mESC-derived tracheal mesodermal cells during differentiation.

737 **f**, Diagram showing the constructs utilized in luciferase experiments containing *Tbx4* LME wild-type containing five Tcf/Lef-binding

738 sites (5xTcf) and *Tbx4*-LME Mutant. mESC-derived LPM cells transfected with wild type or mutant *Tbx4* LME during 4 hrs following

739 by respiratory induction in presence or absence of CHIR99021.

740 **g**, Luciferase assay examining the activation of *Tbx4* LME wt and mutant in response to 3 $\mu$ M CHIR99021

741 **h** Differentiating cells from mESCs at day 12. Cells were stained for *Acta2* (*magenta*) and *Sox9* (*green*). The asterisk indicates

742 *Sox9*<sup>+</sup>/*SMA*<sup>-</sup> chondrocyte aggregates.

743 **i**, Results of qRT-PCR for *Sox9* and *Acta2* expression of hESC-derived tracheal mesodermal cells at day0 and 12.

744 **j**, Differentiating cells from mESCs at day 12. Chondrocytes were stained with Alcian blue. The asterisk indicates one of the

745 chondrocyte aggregates.

746 **k**, Differentiating cells from mESCs at day 12. Cells were stained for *Col1a1* (*magenta*) and Aggrecan (*green*).

747 **l**, Differentiating cells from mESCs at day 12. Cells were stained for *Tagln* (*magenta*) and *Col2a1* (*green*).

748 Each column shows the mean with S.D. (n=3). Scale bar; 50 $\mu$ m.

749 Source data for b, c, d, e, g, i are provided in Source data file.

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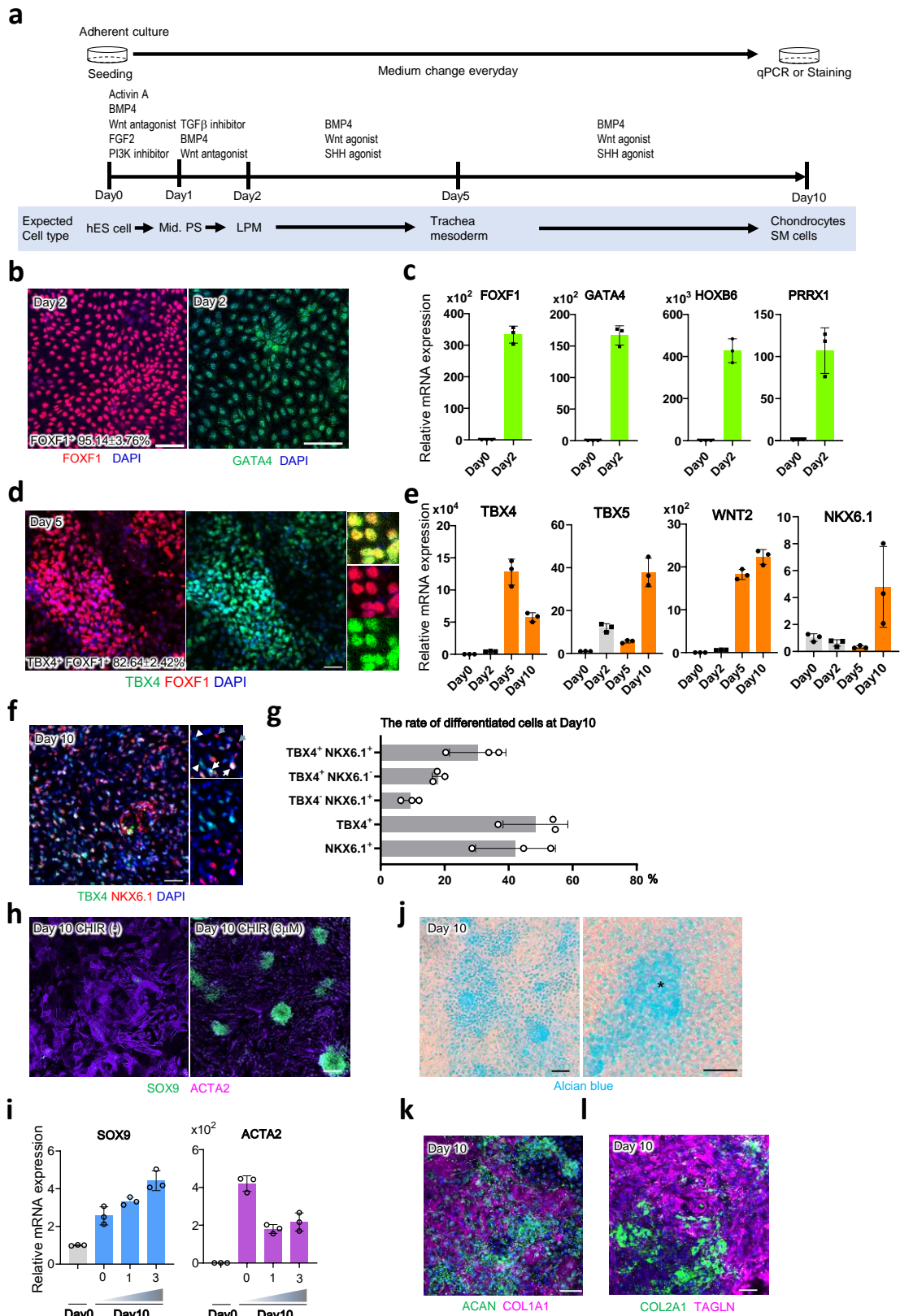
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803 **Figure 5 Generation of trachea mesodermal cells and chondrocytes from human ESCs *in vitro*.**

804 **a**, Experimental design to generate tracheal mesoderm from hESCs

805 **b**, Differentiating cells from hESCs at day 2. Cells were stained for FOXF1 (*red*) and GATA4 (*green*), respectively.

806 **c**, Results of qRT-PCR for LPM marker expression of hESC-derived LPM at day2

807 **d**, Differentiating cells from hESCs at day5. Cells were stained for TBX4 (*green*) and FOXF1 (*red*).

808 **e**, Results of qRT-PCR for respiratory mesoderm marker expression of hESC-derived tracheal mesodermal cells during differentiation.

809 **f**, Differentiating cells from hESCs at day10. Cells were stained for TBX4 (*green*) and NKX6.1 (*red*). White arrows indicate

810 TBX4<sup>+</sup>/NKX6.1<sup>+</sup> mesodermal cells. White arrowheads indicate TBX4<sup>+</sup>/NKX6.1<sup>-</sup> mesodermal cells. Grey arrows indicate TBX4<sup>-</sup>

811 /NKX6.1<sup>+</sup> mesodermal cells.

812 **g**, The rate of differentiated cells at day10.

813 **h**, Differentiating cells from hESCs with or without CHIR99021 treatment at day 10. Cells were stained for ACTA2 (*magenta*) and

814 SOX9 (*green*).

815 **i**, Results of qRT-PCR for *SOX9* and *ACTA2* expression of hESC-derived trachea mesodermal cells at day 10 with different doses of

816 CHIR99021.

817 **j**, Differentiating cells from hESCs at day 10. Chondrocytes were stained with Alcian blue. The asterisk indicates one of the

818 chondrocyte aggregates.

819 **k**, Differentiating cells from hESCs at day 10. Cells were stained for COL1A1 (*magenta*) and AGGRECAN (*green*).

820 **l**, Differentiating cells from hESCs at day 10. Cells were stained for TAGLN (*magenta*) and COL2A1 (*green*).

821 Each column shows the mean with S.D. (n=3). Scale bar; 50µm (d, f, h, j, k, l), 100µm (b)

822 Source data for b, c, d, e, g, i are provided in Source data file.