

# **TGF- $\beta$ modulates cell fate in human ES cell-derived foregut endoderm by inhibiting multiple endogenous signaling pathways**

by

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1 **Summary**

2

3 Genetic differences between pluripotent stem cell lines causes variable activity of extra-cellular  
4 signaling pathways, which limits the reproducibility of directed differentiation protocols. Here we  
5 used human embryonic stem cells (hESCs) to interrogate how exogenously provided factors  
6 modulate endogenous signaling events during specification of foregut endoderm lineages.  
7 We find that TGF- $\beta$ 1 activates an *OTX2/LHX1* gene regulatory network that promotes anterior fate  
8 by antagonizing endogenous Wnt signaling. In contrast to Porcupine inhibition, the effects of TGF- $\beta$ 1  
9 cannot be reversed by exogenous Wnt ligands, suggesting that induction of SHISA proteins and  
10 intracellular accumulation of Fzd receptors make TGF- $\beta$ 1 treated cells refractory to Wnt signaling.  
11 Subsequently, TGF- $\beta$ 1-mediated inhibition of Bmp- and Wnt-signaling suppresses liver- and  
12 promotes pancreas fate. However, pancreas differentiation is delayed by TGF- $\beta$ 1-induced CYP26A1  
13 expression and inhibition of RA signaling. Our study thus identifies multiple mechanisms of crosstalk  
14 between major developmental signaling pathways during foregut patterning.

15

16 **Keywords** Stem cells, Signaling, Patterning, TGF- $\beta$ , Wnt, Bmp, RA, Pancreas, Liver

17

## 18 **Introduction**

19 Directed differentiation of human embryonic stem cells (hESC) seeks to recapitulate the  
20 signaling events that govern cell lineage decisions, from germ layer specification during  
21 gastrulation, to subsequent regionalization along the anterior-posterior (A-P) and dorso-  
22 ventral (D-V) axes. In vertebrates, a combination of Wnt and high Nodal signaling triggers  
23 formation of the definitive endoderm (DE) layer that emerges from the primitive streak.  
24 Closure of the DE along the anterior- and caudal intestinal portals leads to formation of the  
25 primitive gut (PG). The PG is patterned along the A-P and D-V axes to form three broad  
26 regions: foregut, midgut and hindgut that are further patterned into distinct organ  
27 territories, including thyroid, lung, liver, pancreas and the different subdivisions of the  
28 gastro-intestinal tract (Arnold and Robertson, 2009; Grapin-Botton, 2005; Tam and Loebel,  
29 2007). As is also the case for meso- and ectoderm, posterior endoderm fates are promoted  
30 by high levels of Wnt, BMP and retinoic acid (RA) signaling (Bayha et al., 2009; Deimling and  
31 Drysdale, 2009; Spence et al., 2011; Stevens et al., 2017), whereas ligand-sequestering  
32 antagonists such as Dkk1, Sfrp, Noggin and Chordin, secreted from anterior visceral  
33 endoderm and anterior mesendoderm (ME), protect anterior tissues from Wnt and BMP  
34 signaling and thus permit the development of anterior fates (McLin et al., 2007; Rankin et  
35 al., 2011). Similarly, the retinoic acid (RA) catabolizing enzyme, Cyp26a1, expressed in the  
36 anterior epiblast and the anterior half of the newly formed germ layers, protects anterior  
37 structures from RA exposure (Abu-Abed et al., 2001; Abu-Abed et al., 2003; Ribes et al.,  
38 2007). Later, these signaling pathways are repurposed and act in a stage-dependent manner  
39 to promote distinct organ lineages including liver and pancreas (Rankin et al., 2018).  
40 Liver and pancreas arise from common multipotent progenitors in the ventral foregut under  
41 the influence of FGF, BMP and Wnt signals emanating from adjacent, mesodermal tissues  
42 (Zaret, 2008). Dose-dependent FGF signaling from cardiogenic mesoderm and BMP-signaling  
43 from the septum transversum mesenchyme induces hepatic fate and suppresses pancreatic  
44 fate (Deutsch et al., 2001; Jung et al., 1999; Rossi et al., 2001; Serls et al., 2005). Accordingly,  
45 most hESC pancreas differentiation protocols use BMP signaling inhibitors to reduce liver  
46 specification (Nostro et al., 2011; Pagliuca et al., 2014; Reznia et al., 2014). Conversely,  
47 TGF- $\beta$  signaling has been shown to favor pancreatic over hepatic specification in hESC  
48 cultures (Loh et al., 2014). However, it is not clear how TGF- $\beta$  and BMP signaling interact  
49 during these fate decisions.

50 How Wnt signaling acts on the multipotent foregut progenitors to specify organ fate is not  
51 fully understood (reviewed in Zaret, 2008). Ectopic Wnt signaling in *Xenopus* foregut  
52 progenitors inhibits the development of foregut organ buds, including pancreas, liver and  
53 lung, while repression of Wnt signaling cause an expansion of liver and pancreas buds  
54 (McLin et al., 2007). However, this can be interpreted as Wnt repression being required for  
55 specification of anterior endoderm, and thus foregut progenitors. In zebrafish, loss of  
56 canonical Wnt2/Wnt2bb signaling prevents liver and swim bladder specification and the  
57 pancreas and anterior intestines expand (Ober et al., 2006; Poulain and Ober, 2011). More  
58 recent studies in mouse and human systems indicate that non-canonical Wnt signaling is  
59 able to promote pancreatic over liver fate choice, while contradictory results were found  
60 regarding the ability of canonical Wnt signaling to promote liver fate (Mahaddalkar et al.,  
61 2020; Rodriguez-Seguel et al., 2013). Thus, the interaction between signals that regulate  
62 segregation of liver and pancreas primordia are still not fully understood.

63 Similarly, we only have a limited understanding of how exogenously supplied growth factors  
64 affect endogenous signaling pathways at the various steps of hESC differentiation protocols.  
65 Although differences in endogenous signaling activity associated with different hESC lines  
66 (Ortmann et al., 2020) is an obvious source of variation in differentiation outcomes, the role  
67 of endogenous signaling pathways has not received a lot of attention. Generally,  
68 endogenously active pathways are inhibited by addition of antagonists, if considered  
69 detrimental to the desired differentiation outcome, often determined by trial-and-error  
70 approaches. Conversely, pathways whose activity is considered desirable are stimulated by  
71 addition of agonists. Yet, studies of embryonic development have uncovered numerous  
72 examples of crosstalk among signaling pathways and it is to be expected that variability of  
73 endogenous signaling, as well as addition of exogenous factors, will affect such crosstalk and  
74 thus the outcome of in vitro PSC differentiation in a cell line dependent manner.

75 Here we investigated how exogenously provided growth factors and inhibitors affect  
76 endogenous signaling events during early pancreatic lineage specification in differentiating  
77 hESC cultures. By screening major developmental signaling pathways we identified TGF- $\beta$ 1  
78 and the porcupine inhibitor IWP-L6 as potent inducers of pancreatic fate when present  
79 during development of foregut endoderm and early pancreatic progenitors. We confirm the  
80 anteriorizing effect of Wnt inhibition and further show that TGF- $\beta$ 1 anteriorizes endoderm  
81 by inhibiting the expression and/or function of components in the Wnt signaling pathway.

82 Concurrently, TGF- $\beta$ 1 and IWP-L6 both stimulate proliferation of OTX2<sup>+</sup> anterior endoderm  
83 cells. We next show that Wnt/ $\beta$ -catenin signaling promotes liver fate and suppress pancreas  
84 fate in hESC cultures and that TGF- $\beta$ 1 suppresses liver fate by dual inhibition of BMP and  
85 Wnt signaling in foregut progenitors. Unexpectedly, we find that TGF- $\beta$ 1-induced CYP26A1  
86 inhibits RA signaling to delay pancreas specification and maintain foregut progenitors in an  
87 OTX2<sup>+</sup> state.

88 Our work uncovers mechanisms that underlie an extensive crosstalk between  
89 developmental signaling pathways in foregut progenitors as they develop towards liver and  
90 pancreas during hESC differentiation. Knowledge about such signaling dynamics and their  
91 effect on progenitor proliferation will enable more efficient in vitro generation of insulin-  
92 producing beta cells.

93

## 94 **Results**

95 **TGF- $\beta$ 1 and Wnt inhibitors promote expression of pancreatic progenitor markers.** When  
96 subjecting a *PDX1*<sup>EGFP/+</sup> HUES4 reporter cell line (Ameri et al., 2017) to a control pancreas  
97 differentiation protocol (Rezania et al., 2014) we noted a disappointingly low expression of  
98 *PDX1* at the end of stage 5 (S5), where endocrine differentiation commences (Figure 1A and  
99 1B). As induction of definitive endoderm (DE) appeared efficient at S1 (Figure S1A), we  
100 reasoned that subsequent anterior-posterior (A-P) patterning and/or specification of  
101 pancreatic endoderm (PE) was suboptimal in our cultures. We therefore added agonists or  
102 antagonists of selected pathways active in AP-patterning and pancreas specification to  
103 differentiating *PDX1*<sup>EGFP/+</sup> HUES4 cells from S2 to S5 and monitored GFP expression at the  
104 end of S5 (D13). FACS analysis showed a significant increase in GFP<sup>+</sup> positive cells when  
105 blocking Wnt-secretion with the porcupine inhibitor IWP-L6 or adding either recombinant  
106 TGF- $\beta$ 1 or Activin A, while perturbation of Notch signaling and relevant receptor tyrosine  
107 kinase pathways had only minimal effects (Figure 1B). Consistently, a prominent increase in  
108 PDX1 protein expression was seen by immunofluorescent (IF) staining and western blotting  
109 following treatment with IWP-L6 or TGF- $\beta$ 1 (Figure S1B and S1D). Gene expression analysis  
110 by qRT-PCR at D3-D13 showed that *PDX1* and *NKX6-1* transcripts increased from S3 and S5,  
111 respectively, after IWP-L6 treatment, while the effect of TGF- $\beta$ 1 on *PDX1* expression was  
112 only evident at S4. We also noted that expression of the anterior marker *OTX2* was  
113 significantly higher in both IWP-L6- and TGF- $\beta$ 1-treated samples than in controls at S2 and

114 S3. Conversely, expression of the posterior marker *CDX2* and the early liver lineage marker  
115 alpha fetoprotein (*AFP*) was suppressed from S2 and onwards by both treatments (Figure  
116 1C). These results suggest that IWP-L6 inhibits the secretion of posteriorizing Wnt proteins  
117 in our cultures, consistent with how Wnt proteins act *in vivo* (Loh et al., 2014; McLin et al.,  
118 2007). This notion is supported by IWP-L6 being effective when added selectively at S2/3,  
119 while later addition had no effect on pancreatic marker expression at S5 (Figure S1C).  
120 Analysis of OTX2 protein expression confirmed a marked increase at S2 after treatment with  
121 IWP-L6 or the Tankyrase inhibitor XAV-939, supporting that inhibition of Wnt signaling  
122 anteriorizes the cells (Figure 1D and Figure S1D). Notably, TGF- $\beta$ 1 treatment increased the  
123 number of OTX2<sup>+</sup> cells at S2 to the same extent as IWP-L6 (Figure 1D). Further IF analysis  
124 showed an increase in PDX1<sup>+</sup> cells already at S3 following IWP-L6 treatment and at S4  
125 following TGF- $\beta$ 1 treatment, both at the expense of AFP<sup>+</sup> cells that were present in  
126 remarkably high numbers in control cells at both time points (Figure 1E and Figure S1D).  
127 Taken together, these results suggest that endogenous Wnt proteins posteriorize hESC-  
128 derived DE cultures while exogenous TGF- $\beta$ 1 has the opposite effect.

129

130 **TGF- $\beta$ 1 anteriorize endoderm by antagonizing Wnt signaling.** To gain a better  
131 understanding of how IWP-L6 and TGF- $\beta$ 1 affect hESC-derived DE cultures, we performed  
132 RNA-seq analysis of cells treated with IWP-L6, TGF- $\beta$ 1 or both at the end of S2 (D5) and S3  
133 (D7) in the human ES cell lines HUES4 and H1 (Table S1). Principle component analysis (PCA)  
134 showed that the samples clustered according to both cell line and treatment. Importantly,  
135 the cells demonstrated a clear transcriptional response to the different treatments and the  
136 trajectories of the clusters along PC1 and PC2 were similar in both cell lines (Figure 1F).  
137 DESeq2 analysis found 92, 811 and 1417 genes differentially expressed (adjusted p-value <  
138 0.05) in D5 cells treated with IWP-L6, TGF- $\beta$ 1, or both, respectively, compared to control  
139 cells. Similarly, 732, 1481, and 1804 genes were differentially expressed at D7. 56, 484, and  
140 717 genes were upregulated ( $\log_2$  FC > 1) and 36, 327, and 700 genes were downregulated  
141 ( $\log_2$  FC < -1) at D5. At D7 303, 791, and 911 genes were upregulated and 429, 690, and  
142 1043 genes were downregulated (Table S2). Analysis by k-means clustering revealed that  
143 the majority of the regulated genes overlapped between the conditions (Figure 1G, 1H and  
144 Table S2). Genes upregulated by TGF- $\beta$ 1 and TGF- $\beta$ 1 + IWP-L6 at D5 were found in clusters 1  
145 and 6. Strikingly, IWP-L6 treatment also markedly upregulated cluster 1 genes, while cluster

146 6 genes were weakly stimulated or unchanged (Figure 1G and Table S2). Cluster 1 and 6  
147 included members of the gene regulatory network (GRN) responsible for anterior  
148 specification downstream of Smad2/3 such as *EOMES*, *GSC*, *HHEX*, *LHX1* and *OTX2* as well as  
149 downstream targets *CER1*, *NOG*, *FZD5* and *SHISA2* (from here on termed the *OTX2/LHX1*  
150 GRN). Similarly, genes downregulated by TGF- $\beta$ 1 and TGF- $\beta$ 1 + IWP-L6 at D5 (clusters 2 – 5)  
151 were typically also downregulated by IWP-L6, albeit more moderately. These included the  
152 posterior markers *EVX1* and *CDX2* as well as the Wnt and BMP agonists and target genes  
153 *WNT11*, *RSPO3*, *LEF1*, *RNF43* and *BMP5/6*, *BAMBI* and *ID2/4*, respectively (Figure 1G and  
154 Table S2). Gene set enrichment analysis (GSEA) was dominated by enrichment of Gene  
155 Ontology (GO) categories relating cell cycle (see below), but also terms related to A-P  
156 pattern specification and negative regulation of Wnt signaling were enriched (Figure 1I,  
157 Table S3 and Table S4). Leading edge analysis of the latter two gene sets identified genes in  
158 the *OTX2/LHX1* GRN, including *OTX2*, *HHEX*, *LHX1*, *SIX3*, *FZD5* and *CER1* (Figure 1J) and  
159 known Wnt pathway genes *SHISA2*, *SFRP1/2*, *ROR2*, *TCF7L2*, *KREMEN2* following TGF- $\beta$ 1  
160 treatment (Figure 1K).

161  
162 **TGF- $\beta$ 1 and IWP-L6 stimulate proliferation of anterior foregut progenitors.** Addition of  
163 TGF- $\beta$ 1 and/or IWP-L6 during S2-3 resulted in a marked increase in cell density from D5 and  
164 onwards (Figure 2A and 2F). Consistently, proliferation markers such as *MKI67*, *PCNA*,  
165 *CCNA2*, *CCNB1*, *CDCA8*, *CDC20*, *CDC25A/B*, *E2F1*, and *MCM6* were upregulated at D5 by  
166 TGF- $\beta$ 1 and IWP-L6 treatment (Figure 2B and Table S2). The top ten GO terms at D5 all  
167 related to processes involved in cell division and GSEA of IWP-L6 and/or TGF- $\beta$ 1-treated D5  
168 samples showed a significant enrichment of cell cycle related genes (Figure 2C, 2D, Table S3  
169 and S4). We therefore assayed cell proliferation at D5 and D7 by EdU incorporation. An  
170 increase in proliferation was observed by FACS-analysis of EdU-incorporation at both  
171 timepoints (Figure 2E) and blocking TGF- $\beta$  signaling with the ALK4/5 inhibitor SB505124  
172 significantly reduced EdU-incorporation at D7 (Figure 2F). The increase in EdU-percentage  
173 following IWP-L6 was also reversed with SB505124, indicating that TGF- $\beta$ 1 and inhibition of  
174 Wnt-signaling might promote proliferation through a common mechanism. To test whether  
175 IWP-L6 and TGF- $\beta$ 1 promote proliferation of bona fide PFG cells and not simply favor the  
176 emergence of a new proliferating cell type, we quantified the percentage of *OTX2*<sup>+</sup>EdU<sup>+</sup> cells

177 at D5. IF analysis confirmed that both IWP-L6 and TGF- $\beta$ 1 showed a significant increase in  
178 the percentage of OTX2<sup>+</sup>EdU<sup>+</sup> cells (Figure 2G and 2H).

179 We next examined whether other growth factors present in the media during S2-3 were  
180 providing proliferative cues under control conditions. We quantified EdU incorporation after  
181 depletion of single factors from the control S2 and S3 media. We found that depletion of  
182 FGF7, but not any other factor, from the media caused a two-fold decrease in the  
183 percentage of EdU<sup>+</sup> cells at D5 and almost 10-fold reduction D7 (Figure 2I), consistent with  
184 the role of mesenchymal FGF10 as a proliferative signal for multipotent pancreatic  
185 progenitor cells in mice (Bhushan et al., 2001). We then tested whether FGF7 was required  
186 for the proliferative effects of IWP-L6 and TGF- $\beta$ 1. Strikingly, both IWP-L6 and TGF- $\beta$ 1 was  
187 capable of inducing proliferation in the absence of FGF7, albeit IWP-L6 was slightly less  
188 potent compared to TGF- $\beta$ 1 (Figure 2J).

189 As we found TGF- $\beta$ 1 induced the OTX2/LHX1 GRN and OTX2 is able to directly activate cell  
190 cycle genes (Bunt et al., 2012), we examined publicly available ATAC-seq and ChIP-seq data  
191 for chromatin accessibility, Smad2 binding, and binding of selected TFs from the *OTX2/LHX1*  
192 GRN to cell cycle genes in human ES cell cultures differentiating towards endoderm (Geusz  
193 et al., 2021; Tsankov et al., 2015). This analysis revealed that cell cycle genes upregulated by  
194 TGF- $\beta$ 1 all showed binding of OTX2 in their promoter regions at the DE stage (Figure S2),  
195 suggesting that OTX2 could be directly activating cell cycle genes in proliferating foregut  
196 progenitors. Taken together, these findings suggest that Wnt-inhibition and TGF- $\beta$ 1  
197 stimulate proliferation of foregut progenitors via OTX2-mediated activation of the cell cycle.

198

199 **TGF- $\beta$ 1 activate an *OTX2/LHX1* GRN that antagonize Wnt signaling at multiple levels.** To  
200 better understand how TGF- $\beta$ 1 antagonized Wnt signaling, we first analyzed expression of  
201 Wnt pathway agonists, antagonists and target genes in response to TGF- $\beta$ 1 treatment. We  
202 noted high expression of *WNT11* and moderate expression of *WNT3*, *WNT5A* and *WNT5B* in  
203 control cultures, while *RSPO3* was the only R-Spondin expressed (Figure S3A and Table S1).  
204 Notably, TGF- $\beta$ 1 treatment caused a significant reduction in expression of *WNT11* and  
205 *RSPO3* as well as the target genes *LEF1* and *RNF43*, while similar reductions after IWP-L6  
206 treatment only reached significance for *LEF1* and *RSPO3* (Figure 3A). As expected, blocking  
207 TGF- $\beta$ 1 action with SB431542 rescued expression of *RSPO3* and *LEF1* at D5, but had no  
208 effect on IWP-L6-induced changes (Figure S3B). TGF- $\beta$ 1 treatment stimulated expression of



209 both soluble and intracellular Wnt antagonists, including *SFRP1*, *SHISA2* and *SHISA3* at D5,  
210 while *SFRP5* was suppressed. Similarly, we found induction of *SFRP1*, *SFRP2*, *SHISA2* and  
211 *SHISA4* at D7 (Figure 3B and Table S2). *CER1* was also strongly induced at both timepoints,  
212 but may not antagonize Wnt signaling in humans (see discussion).  
213 Since TGF- $\beta$ 1 treated D5 cells fail to activate Wnt target genes in response to exogenous  
214 Wnt3a/RSPO3 (Figure 3C), the induction of *SHISA2/3* caught our attention as the encoded  
215 proteins antagonize Wnt signaling cell autonomously by sequestering Frizzled (Fzd)  
216 receptors in the endoplasmic reticulum (ER) (Onishi and Zou, 2017; Yamamoto et al.,  
217 2005). We therefore analyzed Fzd mRNA and protein expression in control, IWP-L6 and TGF-  
218  $\beta$ 1-treated cultures. The four highest expressed Fzd transcripts were *FZD4*, -5, -7 and -8  
219 (RPKM > 10; Figure S3A and Table S1). Consistent with mouse *Fzd2*, *Fzd5*, *Fzd7* and *Fzd8*  
220 being members of the *Otx2/Lhx1* GRN (Costello et al., 2015; Sibbritt et al., 2018), treatment  
221 with IWP-L6 or TGF- $\beta$ 1 increased *FZD2* and *FZD5* expression significantly, with smaller, non-  
222 significant increases in *FZD7* and *FZD8* (Figure S3C and Table S2). To visualize FZD receptors  
223 and to address whether TGF- $\beta$ 1-induced *SHISA2/3* expression correlated with FZD  
224 subcellular localization, we permeabilized control and TGF- $\beta$ 1-treated cells and labeled  
225 them with a human-Fc-tagged version of the next-generation surrogate Wnt; DRPB-Fz7/8,  
226 which recognizes Fzd1, -2, -5, -7, and -8 (Dang et al., 2019; Miao et al., 2020). Confocal  
227 imaging showed prominent intracellular accumulation of FZD-receptors in TGF- $\beta$ 1 treated  
228 samples compared to controls (Figure 3D), suggesting that *SHISA2/3*-mediated retention of  
229 FZD receptors in the ER may contribute to TGF- $\beta$ 1-induced suppression of Wnt signaling.  
230 When examining Wnt co-receptor expression we find a tendency to increased *ROR2* mRNA  
231 in the RNA-seq data in response to IWP-L6 and TGF- $\beta$ 1 (Figure S3D). We therefore analyzed  
232 *ROR2* expression by qRT-PCR and found a twofold increase (Figure 3E). Conversely, *LRP4*  
233 was downregulated (Figure S3D), while the more highly expressed *LRP5/6* remained  
234 unchanged (Table S2). Overall, these changes may bias any residual Wnt signaling towards  
235 the non-canonical pathway.  
236 Studies in mice, fish and frogs have found that genes encoding signaling antagonists such as  
237 *Cer1*, *Sfrp1* and *Shisa2* as well as the Wnt receptors *Fzd2*, *Fzd5*, *Fzd7* and *Fzd8* are activated  
238 by the concerted action of *Otx2* and *Lhx1* (Costello et al., 2015; Fossat et al., 2015; Sibbritt  
239 et al., 2018), while ligands such as *Wnt8* and *Wnt11* are repressed by *Gsc* in concert with  
240 *Otx2* (Seilliez et al., 2006; Yao and Kessler, 2001; Yasuoka et al., 2014). Since these TFs were

241 strongly induced by TGF- $\beta$ 1 (Figure S3E), we assessed chromatin accessibility and TF binding  
242 for these Wnt pathway genes in human ES cell cultures differentiating towards endoderm  
243 (Geusz et al., 2021; Tsankov et al., 2015). This revealed enhanced chromatin accessibility  
244 and binding of *EOMES*, *FOXA2* and *OTX2* at two putative *cis*-acting regions located ~65 and  
245 ~130 kb downstream of the *SHISA2* gene at the DE stage (Figure 3F). Data for human LHX1  
246 were not available, but *Otx2* and *Lhx1* have previously been shown to bind the mouse  
247 *Shisa2* gene (Costello et al., 2015). Similarly, two regions located ~30 and ~117 kb  
248 downstream of the *SHISA3* gene and multiple regions within an ~80 kb region upstream of  
249 *SFRP1* showed enhanced chromatin accessibility at the DE stage and binding of *EOMES*,  
250 *FOXA2* and *OTX2*, while *CER1* showed enhanced chromatin accessibility and binding of  
251 *SMAD2*, *EOMES*, *FOXA2* and *OTX2* at the promoter region at both ME and DE stages (Figure  
252 3F). Taken together, these findings suggest that TGF- $\beta$ 1 treatment at S2 acts by maintaining  
253 expression of a conserved *OTX2/LHX1/GSC* GRN that suppresses Wnt/ $\beta$ -catenin signaling via  
254 multiple mechanisms including ligand downregulation, induction of antagonists, and  
255 changes in co-receptor expression.

256

### 257 **TGF- $\beta$ 1 induced inhibition of BMP- and Wnt-signaling promotes pancreas over liver**

258 **specification.** The strong suppression of *AFP* expression by IWP-L6 and TGF- $\beta$ 1 prompted us  
259 to further investigate pancreas versus liver differentiation in our cultures. In addition to *AFP*,  
260 other early liver-specific genes such as, *APOA1*, *FABP1* and *TTR* were all reduced at D7  
261 (Figure 4A). Furthermore, GSEA of the RNA-seq datasets showed that both IWP-L6 and TGF-  
262  $\beta$ 1 strongly reduced expression of liver specific genes (Figure 4B and S4A) and hepatocyte  
263 signatures were the most reduced among single cell RNA-seq-based cell type signatures  
264 during S2-3 (Figure S4B, Table S4 and S5). Furthermore, GSEA of our RNA-seq data  
265 compared to RNA-seq data from LASER-capture micro dissected dorsal pancreatic buds (DP)  
266 and hepatic cords (HC) of Carnegie stage 13 human embryos (Jennings et al., 2017)  
267 confirmed that expression of genes that were induced or reduced in IWP-L6-treated cells D7  
268 corresponded to genes seen in the dorsal pancreas (DP high) and hepatic cord (HC high)  
269 gene sets, respectively (Figure 4C, S4C and Table S6). Notably, IWP-L6 or TGF- $\beta$ 1 treatment  
270 at S2 alone (D4-5) was sufficient to suppress *AFP* at D7, indicating that Wnt and TGF- $\beta$   
271 signaling modulate lineage segregation between liver and pancreas already at the primitive

272 gut tube stage (Figure S4D). Taken together, these results indicate that canonical Wnt  
273 signaling promotes human liver specification, as it does in zebrafish (Ober et al., 2006;  
274 Poulain and Ober, 2011) and that TGF- $\beta$  signaling has an opposing effect.  
275 To understand how TGF- $\beta$  signaling inhibits liver specification we interrogated our RNA-seq  
276 data for potential changes in BMP and Wnt signaling, two known liver promoting pathways.  
277 First, we found that the classical BMP ligands *BMP2*, *BMP4*, *BMP5*, *BMP6* and *BMP7* were  
278 expressed at moderate to high levels in control conditions (Figure S4E). TGF- $\beta$ 1 significantly  
279 suppressed expression of *BMP5* and *BMP6* and strongly induced the BMP antagonists *CER1*,  
280 *FST* and *NOG* (Figure 4D and S2E). In contrast, IWP-L6 had no effect on these genes with the  
281 exception of *BMP5*. Consistently, the downstream target genes *ID2*, *ID4* and *BAMBI* were  
282 downregulated by TGF- $\beta$ 1 but not by IWP-L6 (Figure 4E). Together, these findings suggest  
283 that TGF- $\beta$ 1-induced suppression of liver markers may be mediated, at least partly, through  
284 suppression of BMP signaling.  
285 To test to whether reduced Wnt ligand availability might account for the IWP-L6- and TGF-  
286  $\beta$ 1-mediated suppression of liver markers, we co-treated cells with recombinant Wnt3a  
287 and/or RSPO3 during S2-3 and assayed *AFP* and *FABP1* expression. As expected, IWP-L6-  
288 mediated inhibition of liver markers D7 was fully reversed by co-treatment with Wnt3a with  
289 or without additional RSPO3 but not by RSPO3 alone (Figure 4F and 4G). However, Wnt3a,  
290 with or without RSPO3, did not prevent TGF- $\beta$ 1-induced inhibition of *AFP* and *FABP1*  
291 expression, indicating that suppression of Wnt ligand expression cannot fully account for  
292 the effect of TGF- $\beta$ 1 on liver differentiation seen at D7. As also seen at D5, treatment with  
293 Wnt3a and RSPO3 failed to induce expression of Wnt target genes in the presence of TGF- $\beta$ 1  
294 (Figure 4H), showing that TGF- $\beta$ 1 blocks Wnt-signaling downstream of ligand availability.  
295 This may be due to increased expression of the antagonists *SFRP1/2* and *SHISA2/4* at D7  
296 (Figure 3B).  
297 Lastly, we asked whether BMP signaling influenced TGF- $\beta$ 1-mediated suppression of Wnt  
298 signaling and liver differentiation. Notably, BMP-signaling increased expression of Wnt11  
299 and Wnt downstream targets *LEF1*, *RSPO3* and *RNF43*, and both IWP-L6- and TGF- $\beta$ 1-  
300 mediated suppression of these genes at D7 was partially rescued by replacing LDN193189  
301 with BMP4 (Figure 4I). Strikingly, omitting the BMP receptor inhibitor LDN193189 and/or  
302 adding exogenous BMP4 or BMP6 counteracted IWP-L6- and TGF- $\beta$ 1-induced suppression of  
303 *AFP* and *FABP1* expression at D7, albeit the effect was most pronounced for IWP-L6 (Figure

304 4J-L). These data suggest that TGF- $\beta$ 1-mediated suppression of BMP signaling may  
305 contribute to reduced Wnt signaling by attenuating Wnt ligand expression.

306

307 **TGF- $\beta$ 1 delays pancreatic differentiation by attenuating RA signaling.** To begin to unravel  
308 the mechanism responsible for the delayed induction of PDX1 following TGF- $\beta$ 1 treatment  
309 we interrogated our RNA-seq datasets for changes in signaling pathways known to be  
310 important for *PDX1* gene expression. We noted a strong induction of *CYP26A1* expression at  
311 D5 in TGF- $\beta$ 1 treated cells, which was augmented by IWP-L6 co-treatment, while IWP-L6  
312 alone had no effect (Figure 5A). At D7 *CYP26A1* expression was high regardless of TGF- $\beta$ 1  
313 addition, most likely due to the presence of 1  $\mu$ M RA in S3 medium (Rezania et al., 2014).  
314 Nevertheless, we did observe reduced expression of *ALDH1A1* (encoding the RA-  
315 synthesizing enzyme RALDH1) at D7 in response to TGF- $\beta$ 1 treatment (Figure 5A). These  
316 data suggest that TGF- $\beta$ 1-treatment during S2 might desensitize the cells to RA added at S3.  
317 To test this hypothesis, we examined expression of pancreatic markers in control, TGF- $\beta$ 1  
318 and IWP-L6-treated cells at D7, after co-treatment with the *CYP26A1*-inhibitor, R115866 at  
319 S2 (Figure 5B). Notably, addition of R115866 during S2 prevented the TGF- $\beta$ 1-induced  
320 suppression of *PDX1*, *SOX9*, *ONECUT1* and *HNF1B* expression and blunted the induction of  
321 *OTX2* expression at D7 (Figure 5C). However, inhibition of *CYP26A1* did not prevent TGF- $\beta$ 1-  
322 mediated suppression of *AFP* expression. The effect of R115866 on *OTX2* and *PDX1*  
323 expression was also evident on the protein level (Figure 5D and 5E). To begin to understand  
324 how anterior *CYP26A1* expression is activated we again examined publicly available ATAC-  
325 seq and ChIP-seq data for chromatin accessibility and binding of Smad2 and TFs from the  
326 *Otx2/Lhx1* GRN to the *CYP26A1* locus. This analysis revealed regions of increased chromatin  
327 accessibility at D3 where cells have been exposed to Activin A and again at D7 where cells  
328 have been exposed to RA (Figure 5F). Notably, the immediate 5'-flanking region was bound  
329 by SMAD2 and EOMES at the ME stage and by OTX2 at the DE stage (Figure 5F). Taken  
330 together, these results suggest that TGF- $\beta$ 1 signaling delays pancreatic differentiation via  
331 SMAD2-induced *CYP26A1* expression, which desensitizes foregut cells to RA exposure.

332

## 333 Discussion

334 Here we show that TGF- $\beta$  signaling anteriorize hESC-derived definitive endoderm and  
335 subsequently promote pancreas fate over liver ditto via inhibition of Wnt/ $\beta$ -catenin and  
336 Bmp signaling in foregut progenitors. Our results uncover mechanisms used by TGF- $\beta$  to  
337 modulate the activity of other signaling pathways operating in differentiating hESCs. We  
338 observed a very similar response to TGF- $\beta$ 1 and the Porcupine inhibitor IWP-L6, for which  
339 the most parsimonious explanation is suppression of Wnt signaling by TGF- $\beta$ 1. Remarkably,  
340 we also noted a strong stimulation of proliferation of S2 and S3 progenitors with these  
341 treatments. The exact mechanism still needs to be elucidated, but may involve direct  
342 activation of cell cycle genes by OTX2. Notably, the proliferative effect is independent of  
343 exogenous FGF. Together these observations should prove useful in relation to improving  
344 the efficacy of directed differentiation protocols for hESCs.

345 TGF- $\beta$ /Nodal-induced Smad2/3 signaling promotes anterior fate in mice by inducing  
346 expression of Eomes that directly activates Foxa2 and the LIM domain homeobox  
347 transcription factor Lhx1, which in partnership with Otx2, activates numerous anterior ME  
348 genes as well as negative regulators of Bmp-, Nodal-, and Wnt/ $\beta$ -catenin signaling (Costello  
349 et al., 2015; Fossat et al., 2015; Ip et al., 2014; Sibbritt et al., 2018). We observed strong,  
350 induction of *EOMES*, *GSC*, *LHX1* and *OTX2* and other anterior TFs after TGF- $\beta$ 1 treatment at  
351 S2, indicating anteriorization of the endoderm formed at S1. Many of these markers were  
352 also induced by IWP-L6 treatment, as expected when inhibiting the posteriorizing effect of  
353 Wnt signaling. Together, these observations are best explained by suppression of Bmp-,  
354 Nodal and Wnt-signaling. This suppression appears to occur at several levels including  
355 repression of ligand expression and activation of antagonists such as *CER1*, *FST*, *LEFTY1/2*,  
356 *SFRP1* and *SHISA2/3*. The antagonists are well known targets of the Otx2/Lhx1 GRN in  
357 mouse and *Xenopus* (Costello et al., 2015; Fossat et al., 2015; Sibbritt et al., 2018) and our  
358 analysis of published ChIP-seq data (Tsankov et al., 2015) indicate that this may also be the  
359 case in humans. Future ChIP-seq analysis of LHX1 in human cells could bolster this notion.

360 The mechanism underpinning the observed suppression of BMP and WNT ligand expression  
361 is unresolved, but GSC is a strong candidate as a direct repressor of ligand genes as it is  
362 induced by TGF- $\beta$ 1 in our cells and serve such a role in vivo, in concert with OTX2 (Yasuoka  
363 et al., 2014). Indeed, this study suggests that co-binding of OTX2 with either LHX1 or GSC  
364 likely determines activation or repression of OTX2 target genes, respectively, in *Xenopus*

365 anterior development. SIX3, which in addition to anterior neural plate is expressed in hESC-  
366 derived ME and in early mouse DE (Shim et al., 2020), may also be involved. It is induced,  
367 albeit at low levels, by TGF- $\beta$ 1 in our cells and has been shown to repress BMP and WNT  
368 ligand expression in anterior neural plate (Gestri et al., 2005; Lagutin et al., 2003). Again,  
369 future ChIP-seq analyses of GSC and SIX3 in hESC-derived DE will help test these notions.  
370 The identity of the endogenous Wnt ligand(s) that act as posteriorizing factors in our  
371 cultures is not fully resolved by our data. However, we propose that Wnt11 is the key ligand  
372 for several reasons. First, Wnt11 is by far the highest expressed ligand in our culture with 6-  
373 10-fold higher RPKM values than any other Wnt ligand. Second, we find that Wnt11,  
374 together with RSPO3, is strongly suppressed by TGF- $\beta$ 1 treatment. Third, Wnt11 has been  
375 shown to be involved in foregut patterning in *Xenopus*, where Wnt11 activity must be  
376 suppressed by Sfrp5 in the anterior endoderm in order to maintain anterior foregut  
377 endoderm identity (Li et al., 2008; McLin et al., 2007). These studies found Sfrp5 expressed  
378 in surface cells of the foregut epithelium, in close proximity to the deep endoderm that  
379 expresses Wnt11, which activated both the Wnt/ $\beta$ -catenin and the Wnt/PCP pathways.  
380 Notably, morpholino-mediated depletion of Sfrp5 or Wnt11 overexpression in *Xenopus*  
381 embryos both caused a loss of foregut identity.

382  
383 Looking at organ-specific markers, we found that liver markers were suppressed by both  
384 TGF- $\beta$ 1 and IWP-L6 and this was evident from D5 and onwards. Conversely, differentiation  
385 towards the pancreatic lineage was promoted, but only from D10 and onwards after TGF- $\beta$ 1  
386 treatment. Analysis of endogenous signaling activity based on expression of well-known  
387 target genes at D5 and D7 revealed the expected suppression of Wnt signaling by IWP-L6  
388 and suppression of both Wnt and Bmp signaling in response to TGF- $\beta$ 1. The TGF- $\beta$  and BMP  
389 pathways often cross repress each other, through mechanisms that are not fully resolved,  
390 but may in some cases involve sequestration of a limited pool of Smad4 (Candia et al., 1997;  
391 Galvin et al., 2010). A reciprocal relationship between TGF- $\beta$  and BMP in relation to  
392 induction of liver versus pancreas lineages has previously been reported in hESCs (Loh et  
393 al., 2014), but a mechanism was not described. Our results are thus consistent with previous  
394 observations and our RNA-seq data suggest that TGF- $\beta$ 1 may suppress Bmp signaling in  
395 foregut progenitors by repressing *BMP6* expression and inducing Bmp antagonists such as  
396 *NOG*, *FST* and *CER1*. *CER1* was strongly induced at both D5 and D7, while *NOG* and *FST* were

397 induced at D5 and D7, respectively. While Bmp signaling is well known to promote liver  
398 development in vivo (Chung et al., 2008; Rossi et al., 2001), a mechanism involving  
399 suppression of BMP signaling may still appear surprising at first glance as the Bmp receptor  
400 inhibitor LDN193189 is present in S3 medium. However, as LDN193189 is only added at S3,  
401 it may leave time for endogenously produced BMPs to act at S2. Furthermore, LDN193189  
402 only inhibits BMPR1B ~50% at the concentration used here (Sanvitale et al., 2013).

403  
404 Notably, we found that blocking Wnt secretion via Porcupine inhibition promoted pancreas  
405 differentiation, while suppressing liver differentiation and that TGF- $\beta$ 1 also suppressed Wnt  
406 signaling at D7. In zebrafish, the ligands Wnt2 and Wnt2bb act via Fzd5 to activate Wnt/ $\beta$ -  
407 catenin signaling and promote liver development (Ober et al., 2006; Poulain and Ober,  
408 2011), but whether and how Wnt/ $\beta$ -catenin signaling promotes mammalian liver  
409 development has been less clear. Non-canonical Wnt signaling has been reported to favor  
410 pancreas over liver fate, but no evidence of Wnt/ $\beta$ -catenin signaling promoting liver fate  
411 was found in the same study (Rodriguez-Seguel et al., 2013). However, recent work on  
412 hESC-derived foregut progenitors did find that Wnt/ $\beta$ -catenin signaling promoted liver fate  
413 (Mahaddalkar et al., 2020), and in agreement with this finding, we could rescue liver fate in  
414 IWP-L6 treated cultures by addition of Wnt3a. In our hESC cultures, the two most highly  
415 expressed *WNT* genes are *WNT5A* and *WNT11*, making these candidates for inducing liver  
416 fate, likely augmented by *RSPO3*. Like Wnt5a, Wnt11 is often considered a non-canonical  
417 ligand but both ligands can also activate the canonical pathway if the responding cells  
418 express the proper receptors (Li et al., 2008; Mikels and Nusse, 2006; Tao et al., 2005).  
419 However, the identity of a putative liver specifying Wnt ligand in vivo remains unknown, but  
420 candidates are Wnt2, Wnt2b and Wnt5a, which are all expressed in the mesoderm proximal  
421 to the developing liver (McMahon and McMahon, 1989; Monkley et al., 1996; Rodriguez-  
422 Seguel et al., 2013; Zakin et al., 1998). Mouse *Wnt2/Wnt2b* double mutants display normal  
423 liver and pancreas development (Goss et al., 2009), but additional Wnt ligands (e.g. Wnt5a)  
424 have been suggested to fulfill the liver specifying role in mammals (Poulain and Ober, 2011).  
425 Notably, the canonical co-receptor Lrp5 is enriched in liver progenitors compared to  
426 pancreas further suggesting that Wnt/ $\beta$ -catenin signaling may promote liver fate. A  
427 contributing factor to TGF- $\beta$ 1-mediated suppression of liver fate may be the upregulation of  
428 *ROR2* expression. In mouse foregut progenitors *Ror2* is expressed in pancreas-, but not liver

429 progenitors and, as mentioned above, non-canonical signaling favors pancreas over liver  
430 fate (Rodriguez-Seguel et al., 2013).

431 As also seen at D5, TGF- $\beta$ 1 suppressed Wnt activity at D7 even in the presence of exogenous  
432 Wnt ligand, possibly by stimulating *SFRP1/2* and *SHISA2/4* expression at D7. *CER1* is also  
433 strongly induced, but human CER1, which is ~69% identical to mouse Cer1, may not  
434 antagonize Wnt signaling (Belo et al., 2000; Piccolo et al., 1999). Importantly, omitting  
435 LDN193189 from in the S3 medium, or replacing it with Bmp ligand, reactivated *WNT11*-  
436 and Wnt target gene expression and partially restored liver differentiation in the presence  
437 of TGF- $\beta$ 1. This suggests that Bmp signaling acts upstream of Wnt signaling and that TGF- $\beta$ 1-  
438 induced suppression of Wnt signaling could be mediated, at least partly, by inhibition of  
439 Bmp stimulated Wnt activity. Consistent with this notion, Bmp2b acts prior to Wnt2bb and  
440 Wnt2 in zebrafish liver development (Chung et al., 2008; Poulain and Ober, 2011). Taken  
441 together, our findings show that BMP- and Wnt/ $\beta$ -catenin signaling coordinate induction of  
442 liver lineage in human progenitors and suggest mechanisms for TGF- $\beta$ 1-mediated  
443 suppression of both pathways that ultimately promotes pancreatic over liver fate.

444

445 One notable difference between the effect of TGF- $\beta$ 1 and IWP-L6 was the delay in onset of  
446 pancreatic marker expression in TGF- $\beta$ 1 treated samples. Intriguingly, a previous study  
447 found suppression of Pdx1 expression in the developing pancreas after treatment of 3-4 and  
448 5-6 somite stage half-embryo cultures with TGF- $\beta$ 2 and an increase after treatment of 5-6S  
449 half-embryo cultures with the ALK4,5,7 inhibitor SB431542. The underlying mechanism was  
450 not elucidated, but it was shown that changes in proliferation rate or apoptosis were not  
451 involved. (Wandzioch and Zaret, 2009). Importantly, our RNA-seq data showed that addition  
452 of TGF- $\beta$ 1 during S2 induced a profound increase in the expression of the RA-degrading  
453 enzyme CYP26A1. This induction is likely a reflection of the normal anterior expression  
454 pattern observed for *Cyp26a1* in mouse embryos (Abu-Abed et al., 2001; Abu-Abed et al.,  
455 2003; Ribes et al., 2007), and may be a direct induction by SMAD2, possibly in conjunction  
456 with EOMES. As RA is well-known inducer of Pdx1 and pancreatic fate (Kraus and Grapin-  
457 Botton, 2012; Micallef et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002),  
458 elevated CYP26A1 levels could explain the delay in the onset of pancreatic marker  
459 expression. Indeed, the delay was prevented by adding the CYP26A1 inhibitor R115866. This  
460 finding may partly explain why many pancreas differentiation protocols rely on ALK4,5,7



461 inhibitors during later stages (Nostro et al., 2011; Pagliuca et al., 2014; Rezania et al., 2014;  
462 Rezania et al., 2011).

463 In summary, our study suggests several mechanisms by which Bmp, TGF- $\beta$  and Wnt signaling  
464 interact to control differentiation of hESC-derived foregut progenitors. Our findings improve  
465 the understanding of human foregut patterning and organ development and provide  
466 valuable new avenues for improving directed differentiation protocols for hESCs in order to  
467 obtain clinically relevant cell types in unlimited quantities.

468

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477

478 **Author Contributions.** N.S.F. and P.S. conceived the study, designed and interpreted  
479 experiments and wrote the manuscript. N.S.F, M.S.H. and J.v.C.K. carried out experiments,  
480 while N.S.F and K.H.L. performed the bioinformatics analyses. K.B.J., Y.M. and K.C.G. designed  
481 and produced the NGS detection reagent. All authors revised and approved the manuscript.

482

483 **Declaration of Interests.** The authors declare no competing interests.

484

#### 485 **Figure legends**

#### 486 **Figure 1. TGF- $\beta$ 1 and IWP-L6 anteriorize endoderm and promote pancreas over liver fate**

487 (A) Schematic overview of the basic differentiation protocol. The stages when compounds  
488 being screened were added are indicated. DE: definitive endoderm; PGT: primitive gut tube;  
489 PP1: pancreatic progenitor 1; PP2: pancreatic progenitor 2; EP: endocrine precursor.

490 (B) FACS analyses of *PDX1*<sup>EGFP/+</sup> HUES4 cells showing percent GFP<sup>+</sup> cells at D13 after  
491 treatment with indicated factors during S2-S5. Mean  $\pm$  SD, N=3. \*  $p < 0.05$ , \*\*  $p < 0.005$ ,  
492 \*\*\*\*  $p < 0.0001$ .

493 (C) qRT-PCR analyses of *OTX2*, *CDX2*, *AFP*, *PDX1* and *NKX6-1* expression in differentiating  
494 HUES4 cells at the indicated time points after treatment with the indicated factors during  
495 S2-S3. Data are shown relative to undifferentiated HUES4 cells. Mean  $\pm$  SD, N=3, \*  $p < 0.05$ ,  
496 \*\*  $p < 0.005$ , \*\*\*  $p < 0.0005$ , \*\*\*\*  $p < 0.0001$ .

497 (D) IF staining for OTX2 in differentiating HUES4 cells at D5 after treatment with the  
498 indicated factors during S2. The cells were counterstained with DAPI. Scale bar, 50  $\mu$ m.

499 (E) IF staining for PDX1 (green) and AFP (red) in differentiating HUES4 cells at D7 and D10  
500 after treatment with the indicated factors during S2-S3. The cells were counterstained with  
501 DAPI. Insets show higher magnifications of boxed areas. Scale bars, 50  $\mu$ m.

502 (F) Principle component analysis (PCA) showing clustering along PC1 and PC2 for *PDX1*<sup>EGFP/+</sup>  
503 HUES4 (n=3) and H1 (n=2) cells treated with IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during  
504 S2 (D5) or S2-S3 (D7).

505 (G) Heatmaps scaled by row to show patterns of regulated genes in *PDX1*<sup>EGFP/+</sup> HUES4 cells  
506 (n=3) by k-means clustering analysis of RNA-seq data. Signature genes are indicated for each  
507 cluster.

508 (H) Venn diagram showing overlap of all deregulated genes at D5 and D7 with an FDR <0.1  
509 and fold change >1.5.

510 (I) GSEA plots comparing gene expression data from each treatment at D5 (n=3) with the  
511 indicated gene sets. Normalized Enrichment Scores (NES) and p-values are indicated.

512 (J-K) GSEA plots comparing D5 gene expression data from each treatment (J) or TGF- $\beta$ 1 (K)  
513 with gene sets for anterior-posterior pattern specification or negative regulation of Wnt  
514 signaling, respectively (N=3). Signature genes from leading and trailing edge analyses are  
515 shown in red and blue boxes, respectively. Normalized Enrichment Scores (NES) and p-  
516 values are shown.

517 See also Figure S1.

518

## 519 **Figure2. TGF- $\beta$ 1 and IWP-L6 stimulate proliferation of anterior foregut progenitors**

520 (A) DAPI staining showing increased cellular density at D7 and D10 following treatment with  
521 IWP-L6 or TGF- $\beta$ 1 during S2-S3. Scalebar, 100 $\mu$ m.

522 (B) Expression of cell cycle related genes by RNA-seq in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with  
523 IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during S2 (D5) or S2-S3 (D7). Mean  $\pm$  SEM, N=3, \*  
524 padj < 0.05, \*\* padj < 0.005, \*\*\* padj < 0.0005, \*\*\*\* padj < 0.0001.  
525 (C) NES-scores for the ten most enriched biological process GO terms for genes enriched at  
526 D5 in cells treated with IWP-L6 or TGF- $\beta$ 1 during S2.  
527 (D) GSEA plots comparing gene expression data from each treatment at D5 with a gene set  
528 for cycling genes. Normalized Enrichment Score (NES) and p-values are indicated.  
529 (E) FACS analysis of EdU incorporation and DNA content in differentiated *PDX1*<sup>EGFP/+</sup> HUES4  
530 cells at D5 and D7 after treatment with vehicle, IWP-L6 or TGF- $\beta$ 1 during S2 (D5) or S2-S3  
531 (D7).  
532 (F) Percentage of EdU<sup>+</sup> cells in differentiated *PDX1*<sup>EGFP/+</sup> HUES4 cells at D5 and D7 after  
533 treatment with the indicated factors. Mean  $\pm$  SD, N=3, \* p < 0.05, \*\* p < 0.005, \*\*\*\* p <  
534 0.0001.  
535 (G) IF analysis of OTX2<sup>+</sup>/EdU<sup>+</sup> co-expression following treatment with vehicle, IWP-L6 or  
536 TGF- $\beta$ 1. Scalebar, 100 $\mu$ m.  
537 (H) Quantification of OTX2<sup>+</sup>/EdU<sup>+</sup> co-expression following treatment with vehicle, IWP-L6 or  
538 TGF- $\beta$ 1. Mean  $\pm$  SD, N=3, \* p < 0.05, \*\* p < 0.005, \*\*\*\* p < 0.0001.  
539 (I) Percentage of EdU<sup>+</sup> cells following depletion of individual factors from the control  
540 medium. Mean  $\pm$  SD, N=3, \* p < 0.05, \*\* p < 0.005, \*\*\* p < 0.0005.  
541 (J) Percentage of EdU<sup>+</sup> cells following withdrawal of FGF7 in cells treated with IWPL6 or TGF-  
542  $\beta$ 1. Mean  $\pm$  SD, N=3, \* p < 0.05, \*\* p < 0.005, \*\*\* p < 0.0005, \*\*\*\* p < 0.0001.  
543 See also Figure S2.

544

### 545 **Figure3. TGF- $\beta$ 1 activates an *OTX2/LHX1* GRN that antagonize Wnt signaling**

546 (A) Expression of *WNT11*, *RSPO3*, *LEF1* and *RNF43* by RNA-seq in *PDX1*<sup>EGFP/+</sup> HUES4 cells  
547 treated with vehicle (Ctrl), IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during S2 (D5) or S2-S3  
548 (D7). Mean  $\pm$  SEM, N=3, \* padj < 0.05, \*\* padj < 0.005, \*\*\*\* padj < 0.0001.  
549 (B) Differential expression of Wnt-signaling antagonists by DESeq2 analysis of RNA-seq data  
550 from *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 during S2 (D5) or S2-S3  
551 (D7). Log2 fold change (log2FC) relative to vehicle controls is shown as are adjusted p-values  
552 (padj).

553 (C) qRT-PCR analysis of *LEF1*, *RNF43* and *RSPO3* expression at D5 in response to Wnt3a +  
554 *RSPO3* stimulation during S2 in control cells (Ctrl) or cells treated with IWP-L6 or TGF- $\beta$ 1.  
555 Mean  $\pm$  SD, N=3, \*  $p < 0.05$ .

556 (D) Confocal microscopy of D5 *PDX1*<sup>EGFP/+</sup> HUES4 cells stained for  $\beta$ -catenin (green) and Fzd  
557 receptors (red) after treatment with vehicle (Ctrl) or TGF- $\beta$ 1 during S2.

558 (E) qRT-PCR analysis of *ROR2* expression at D5 in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with IWP-  
559 L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during S2. Mean  $\pm$  SD, N=3, \*  $p < 0.05$ .

560 (F) Signal tracks of ATAC-seq from Geusz et al. (2021) and SMAD2, EOMES, FOXA2 and OTX2  
561 ChIP-seq data from Tsankov et al. (2015) at the *SHISA2*, *SHISA3*, *SFRP1* and *CER1* loci.

562 Dashed lines are used to mark areas with differential chromatin accessibility at the DE stage,  
563 and TF binding at the ME and/or DE stages. ES: ES cells; DE: definitive endoderm; GT:

564 primitive gut tube; PP1: pancreatic progenitor 1, ME: mesendoderm.

565 See also Figure S3.

566

567 **Figure 4. TGF- $\beta$ 1 promotes pancreas over liver fate by inhibiting BMP- and Wnt-signaling**

568 (A) Expression of *AFP*, *APOA1*, *FABP1* and *TTR* by RNA-seq in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated  
569 with IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during S2 (D5) or S2-3 (D7). Mean  $\pm$  SEM, N=3,  
570 \*  $p_{adj} < 0.05$ , \*\*  $p_{adj} < 0.005$ , \*\*\*\*  $p_{adj} < 0.0001$ .

571 (B) GSEA plots comparing gene expression data from each treatment at D7 with a gene set  
572 for liver specific genes (Hsiao et al., 2001). Normalized Enrichment Score (NES) and p-values  
573 are shown.

574 (C) GSEA plots comparing gene expression data from each treatment at D7 with genes  
575 highly expressed in human Carnegie Stage (CS)13 dorsal pancreas (DP) vs hepatic cords (HC)  
576 and genes highly expressed in HC vs DP (Jennings et al., 2017).

577 (D-E) Expression of BMP ligands, secreted BMP antagonists (D) and BMP target genes (E) by  
578 RNA-seq in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T)  
579 during S2 (D5) or S2-S3 (D7). Mean  $\pm$  SEM, N=3, \*\*  $p_{adj} < 0.005$ , \*\*\*  $p_{adj} < 0.0005$ , \*\*\*\*  
580  $p_{adj} < 0.0001$ .

581 (F) IF analysis of AFP<sup>+</sup> cells at D7 in response to Wnt3a, *RSPO3* or Wnt3a + *RSPO3*  
582 stimulation during S2-S3 in cells treated with vehicle, IWP-L6 or TGF- $\beta$ 1.

583 (G-H) qRT-PCR analysis of *AFP*, *FABP1* (G), *LEF1* and *RSPO3* (H) expression at D7 in response  
584 to Wnt3a, RSPO3 or Wnt3a + RSPO3 stimulation during S2-S3 in *PDX1*<sup>EGFP/+</sup> HUES4 cells  
585 treated with vehicle (Veh), IWP-L6 or TGF- $\beta$ 1. Mean  $\pm$  SD, N=3, \*  $p < 0.05$ .

586 (I) Expression of *WNT11*, *LEF1*, *RSPO3* and *RNF43* by qRT-PCR in response to replacement of  
587 LDN193189 with BMP4 during S3 in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with vehicle (Veh), IWP-  
588 L6 or TGF- $\beta$ 1. Mean  $\pm$  SD, N=3, \*  $p < 0.05$ , \*\*  $p < 0.005$ .

589 (J) IF analysis of AFP<sup>+</sup> cells at D7 in response to replacement of LDN193189 with BMP6  
590 during S3 in *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with vehicle (Veh), IWP-L6 or TGF- $\beta$ 1.

591 (K) Western blot showing AFP and PDX1 expression in *PDX1*<sup>EGFP/+</sup> HUES4 cells cultured in  
592 standard S3 medium (Ctrl), S3 medium without LDN193189 (w/o LDN) or with replacement  
593 of LDN193189 with either BMP4 or BMP6 during S3, in cells treated with vehicle (Veh), IWP-  
594 L6 (IWP) or TGF- $\beta$ 1 (TGF). Also shown are hESC and DE stages. Vinculin is used as internal  
595 control.

596 (L) Expression of *AFP* and *FABP1* by qRT-PCR in standard S3 medium (Ctrl), S3 medium  
597 without LDN193189 (w/o LDN) or with replacement of LDN193189 with BMP4 during S3, in  
598 cells treated with vehicle (Veh), IWP-L6 or TGF- $\beta$ 1. Mean  $\pm$  SD, N=3, \*  $p < 0.05$ , \*\*  $p < 0.005$ .  
599 See also Figure S4.

600

### 601 **Figure 5. TGF- $\beta$ 1 delays pancreatic differentiation by attenuating RA signaling**

602 (A) Expression of *CYP26A1* and *ALDH1A1* measured by RNA-seq in *PDX1*<sup>EGFP/+</sup> HUES4 cells  
603 treated with IWP-L6, TGF- $\beta$ 1 or IWP-L6 + TGF- $\beta$ 1 (I+T) during S2 (D5) or S2-S3 (D7). Mean  $\pm$   
604 SEM, N=3, \*\*  $p_{adj} < 0.005$  and \*\*\*\*  $p_{adj} < 0.0001$ .

605 (B) Schematic overview of protocol using a CYP26-selective inhibitor, R115866 with TGF- $\beta$ 1.

606 (C) Expression of *PDX1*, *SOX9*, *ONECUT1*, *HNF1B*, *OTX2* and *AFP* measured by qRT-PCR in D7  
607 *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with vehicle (DMSO), TGF- $\beta$ 1 and/or R115866 as indicated.

608 (D) Western blot showing OTX2, PDX1 and AFP expression in D7 *PDX1*<sup>EGFP/+</sup> HUES4 cells  
609 treated with vehicle (Veh), IWP-L6, TGF- $\beta$ 1 and/or R115866 as indicated. Also shown are  
610 hES and DE stages. Vinculin is used as internal control.

611 (E) IF analysis of PDX1 expression in D7 *PDX1*<sup>EGFP/+</sup> HUES4 cells treated with vehicle (Veh),  
612 IWP-L6, TGF- $\beta$ 1 and/or R115866 as indicated.

613 (F) Signal tracks of ATAC-seq from Geusz et al. (2021) and SMAD2, EOMES, FOXA2 and OTX2  
614 ChIP-seq data from Tsankov et al. (2015) at the *CYP26A1* locus. Dashed lines are used to  
615 mark areas with differential chromatin accessibility at the DE and PP1 stages, and TF binding  
616 at the ME and/or DE stages. ES: ES cells; DE: definitive endoderm; GT: primitive gut tube;  
617 PP1: pancreatic progenitor 1, ME: mesendoderm.

618

## 619 **STAR METHODS**

### 620 ***CONTACT FOR REAGENT AND RESOURCE SHARING***

#### 621 **Lead contact**

622 Further information and requests for resources and reagents may be directed to, and will be  
623 fulfilled by the lead contact, Palle Serup ([palle.serup@sund.ku.dk](mailto:palle.serup@sund.ku.dk))

624

#### 625 **Materials availability**

626 This study did not generate new unique reagents.

627

#### 628 **Data and code availability**

629 Sequencing datasets generated in this paper are available at ArrayExpress: E-MTAB-10715.

630

## 631 ***EXPERIMENTAL MODEL AND SUBJECT DETAILS***

### 632 **Cell lines and culture conditions**

633 The human PSC lines H1 (WA01, WiCell; RRID: CVCL\_9771) and *PDX1*<sup>EGFP/+</sup> HUES4 obtained  
634 from our facility (Ameri et al., 2017) were maintained in DEF-CS culture media (Takara  
635 Biosciences) following manufacturer's instructions with daily media change and passaged  
636 every 3-4 days with TrypLE Express Enzyme (Thermo Fisher). All cells were cultured in a  
637 humidified 37°C, 5% CO<sub>2</sub> incubator.

638

### 639 ***METHOD DETAILS***

#### 640 **Differentiation of hESCs in chemically defined conditions**

641 The hESCs were differentiated to pancreatic progenitor cells by a previously described  
642 protocol (Rezania et al., 2014) with minor modifications. 150.000cells/cm<sup>2</sup> were seeded in  
643 6- or 24-well multi-well plates or in 8-well Ibidi chamber slides after single cell suspension  
644 for 3-5 min at 37 °C in TrypLE Express Enzyme (Thermo Fisher). After 48h, at day 0 (~90%

645 confluency), the cells were washed once in 1× DPBS without Mg<sup>2+</sup> and Ca<sup>2+</sup> (Thermo Fisher)  
646 before addition of S1 basal media (MCDB131, Thermo Fisher) supplemented with Sodium  
647 Bicarbonate (1.5g/l, Sigma-Aldrich), Glucose (10mM, Sigma-Aldrich), GlutaMax (1x, Thermo  
648 Fisher), BSA (0,5%, Proliant Biologicals), CHIR-99021 (3µM, Axon Medchem) and Activin A  
649 (100 ng/ml, Peprotech). S1 cells (D3) were washed once in DPBS without Mg<sup>2+</sup> and Ca<sup>2+</sup> and  
650 further supplemented for 2d with S2 media including Sodium Bicarbonate (1.5g/l), Glucose  
651 (10mM), GlutaMax (1x), BSA (0,5%), L-Ascorbic Acid (0.25mM, Sigma-Aldrich) and  
652 recombinant human FGF7 (KGF) (50ng/ml, Peprotech). During S3-S4, the media was  
653 supplemented with Sodium Bicarbonate (2.5g/l), Glucose (10mM), GlutaMax (1x), BSA (2%),  
654 L-Ascorbic Acid (0.25mM), KGF (S3 50ng/ml and S4 2ng/ml), RA (S3 1µM and S4 0.1µM,  
655 Sigma-Aldrich), SANT-1 (0,25µM, Sigma-Aldrich), TPB (S3 200nM and S4 100nM, Sigma-  
656 Aldrich), LDN-193189 (S3 100nM and S4 200nM, Stemgent) and ITS-X (1:200, Thermo  
657 Scientific). During S5, the media was supplemented with Sodium Bicarbonate (1.5g/l),  
658 Glucose (20mM), GlutaMax (1x), BSA (2%), RA (0.05µM), SANT-1 (0.25mM), LDN-193189  
659 (100nM), T3 (1µM, Sigma-Aldrich), ALK5i-II (10µM, Millipore), Zinc sulfate (10µM, Sigma-  
660 Aldrich), Heparin (10µM, Sigma-Aldrich) and ITS-X (1:200). In the initial screen, the media  
661 was further supplemented with the following factors during S2-4; Activin A (100ng/ml), TGF-  
662 β1 (10ng/ml, Peprotech), SB505124 (10 µM, Sigma-Aldrich), DAPT (10 µM, Selleckchem),  
663 CHIR-99021 (3µM), IWP-L6 (5µM, Axon Medchem), PD98059 (1 µM, Selleckchem), HGF (50  
664 ng/ml, Peprotech), EGF-L7 (50ng/ml, Peprotech), Wnt3a (100 ng/ml, RnD Systems) or EGF  
665 (50ng/ml, Peprotech). In the following experiments, IWP-L6 (5µM) and/or TGF-β1 (10ng/ml)  
666 were added during S2-3 unless otherwise indicated. When applicable, SB431542 (10 µM,  
667 Sigma-Aldrich), XAV939 (1µM, Sigma-Aldrich), R115866 (10 µM, Sigma-Aldrich), BMP4/6  
668 (50ng/ml, Peprotech), Wnt3a (100ng/ml) or RSPO3 (100ng/ml, Peprotech) were added  
669 during S2-3 according to experimental setup.

670

## 671 **Flow cytometry**

672 *PDX1*<sup>EGFP/+</sup> HUES4 hESCs were differentiated S1-S4 and dissociated at D13 with TrypLE  
673 Express Enzyme and washed twice in DPBS without Mg<sup>2+</sup> and Ca<sup>2+</sup> + 2%BSA. Resuspended  
674 cells were further dissociated in tubes with cell strainer caps (Fisher Scientific), stained with

675 DAPI and analyzed on an LSR Fortessa flow cytometer. FACS gating was determined using  
676 undifferentiated cells and non-GFP expressing cells.

677

### 678 **Immunofluorescence analysis, Fzd detection and Imaging**

679 Cells destined for immunofluorescence and confocal microscopy were grown on 24-well  
680 plates or 8-well Ibidi  $\mu$ -slides (Ibidi), respectively. The cells were washed once in DPBS and  
681 fixed in 4% Formaldehyde (VWR) for 30 minutes, then permeabilized using 0.5% Triton-X in  
682 DPBS for 10 min at room temperature and blocked in SuperBlock (Thermo Fisher) for 30  
683 minutes.

684 Primary antibodies (see Key Resources Table) were diluted in 0.1% Triton X-100 in DPBS.

685 Incubation was done at 4°C overnight followed by 3x5 minutes wash in DPBS. Secondary

686 antibodies (1:500, raised in Donkey) conjugated to either Alexa Fluor 488, Cy3 or Cy5 (all

687 Jackson ImmunoResearch) were incubated for 45 minutes at room temperature and then

688 washed 3x5min in DPBS followed by nuclear staining with DAPI (Thermo Fisher). FZD

689 receptor expression was detected using a human Fc-tagged version of the NGS Wnt ligand:

690 DRPB-Fz7/8 (Miao et al., 2020). Control and TGF- $\beta$ 1 treated cells, grown on 8-well Ibidi  $\mu$ -

691 slides (Ibidi), were fixed, permeabilized and incubated for 1h in media conditioned with cells

692 expressing DRPB-Fz7/8 together with a primary antibody against  $\beta$ -catenin (BD Biosciences).

693 Detection of DRPB-Fz7/8 was done with a PE-tagged anti-human Fc antibody (Jackson

694 ImmunoResearch).

695 Images were captured and processed on a Zeiss Axioobserver using Plan-Apochromat

696 10x/0.45 and Plan-Apochromat 20x/0.8 objectives and ZEN software. Confocal images were

697 captured on a Zeiss LSM780 confocal microscope using a Plan-Apochromat 63x/1.40 Oil

698 objective. Figures were prepared using Adobe Photoshop CS6 and Adobe Illustrator CS6

699 (Adobe Systems, San Jose, CA, USA).

700

### 701 **RNA extraction and quantitative real-time PCR**

702 Total RNA was extracted with the RNeasy Plus Mini kit (Qiagen) and reverse-transcribed

703 using the SuperScript III First-Strand synthesis kit (Invitrogen/Thermo Fisher). Quantitative

704 real-time PCR experiments were performed using the StepOnePLUS system (Applied

705 Biosystems) and PowerUP SYBR Green Master Mix (Applied Biosystems/ThermoFisher). See



706 Table S7 for primers. Relative changes in gene expression was compared to undifferentiated  
707 hESCs using the  $\Delta\Delta C_t$  method.

708

### 709 **Western blotting**

710 Harvested cells were lysed in RIPA buffer containing 1x phosphatase inhibitor cocktail  
711 (Sigma-Aldrich) and cOmplete Ultra Protease inhibitor (Thermo Scientific/Roche) on ice for  
712 10min. Cell lysates were sonicated 5x30sec ON/OFF on a Diagenode BioRuptor in 1.5mL  
713 eppendorff tubes followed by centrifugation at 21 000g for 30 minutes at 4°C and saving the  
714 supernatant. Pierce BCA protein kit (ThermoFisher) was used to measure protein  
715 concentration on a Nanodrop 2000 (ThermoFisher). Lysates were boiled for 5 minutes in  
716 Laemmli sample buffer and 20-40  $\mu$ g protein was separated by electrophoresis on NuPage  
717 4-12% BisTris SDS-PAGE gels in MOPS buffer (Thermo Fisher) and transferred to PVDF  
718 membranes (Bio-Rad) using the BioRad Mini-Protean transfer system. Membranes were  
719 blocked in SuperBlock (Thermo Fisher) for 1 hour at room temperature and incubated with  
720 primary antibodies (see Key Resources Table) overnight at 4 °C. After three washes with  
721 TBS-T (0.1% Tween-20 in 1x Tris-buffered saline), the blot was incubated with respective  
722 secondary HRP antibodies at room temperature for 30 minutes. ECL Prime Western Blotting  
723 Detection Reagent was used for detection according to the manufacturer's instruction  
724 (Sigma-Aldrich). For re-blotting, antibodies were stripped by Restore Western Blot Stripping  
725 Buffer (Thermo Fisher).

726

### 727 **EdU-incorporation, analysis and quantification**

728 *PDX1*<sup>EGFP/+</sup> HUES4 cells were labeled with EdU and detected according to the Click-iT EdU  
729 Alexa Fluor 594 Flow Cytometry Assay kit (Thermo Fisher) protocol at D5 and D7 of  
730 differentiation. In brief, 10  $\mu$ M EdU was added to the differentiation culture medium and  
731 cells were harvested and dissociated in to a single-cell suspension after 3h. The cells were  
732 fixed and permeabilized followed by EdU detection with the Click-iT EdU reagent and  
733 nuclear staining with DAPI. FACS gating was determined using non-EdU treated cells and  
734 EdU-treated cells without detection reagent. Cells were analyzed using a LSR Fortessa flow  
735 cytometer. Alternatively, EdU-treated cells were fixed, permeabilized and stained according  
736 to the Click-iT Plus EdU Cell Proliferation Kit for Imaging, 647 dye (ThermoFisher).

737 OTX2<sup>+</sup> and OTX2<sup>+</sup>EdU<sup>+</sup> cells were quantified from confocal images acquired on an LSM780  
738 confocal microscope as described above. Cells were identified using the Spot function in  
739 Imaris™ (Bitplane) with the diameter set to 4.15 μm and the Co-localization function with  
740 the maximum distance between spots set to ≤2 μm to identify co-expressing cells.

741

#### 742 **RNA-seq**

743 RNA-seq libraries were built using biological triplicates from *PDX1*<sup>EGFP/+</sup> HUES4 cells and  
744 duplicates from H1 cells with 1μg of RNA using the NEB NEXT Ultra II RNA Library Prep Kit  
745 (NEB #E7770) and mRNA magnetic isolation module for poly(A) purification (NEB #E7490)  
746 with 5 cycles of amplification. Quality of the RNA and subsequently of the libraries were  
747 measured on a Fragment Analyser and the libraries were loaded accordingly and barcoded  
748 with NEB Next multiplex Oligos for Illumina (E7335 and E7500) on Illumina NextSeq 500 with  
749 Hi-output 1x75bp kit.

750 FASTQ-files were generated using bcl2Fastq (Illumina) and aligned to hg38 human genome  
751 using STAR (Dobin et al., 2013) with standard alignment settings resulting in >90% alignment  
752 of reads. Quantified gene count matrix with STAR (flag: --quantMode GeneCounts) and  
753 loaded into R using DESEQ2 package for differential expression analysis on gene level (Love  
754 et al., 2014). We performed quality control assessment including Principal Component  
755 Analysis after regularized logarithmic transformation. For the differential expression analysis  
756 for each cell line on each day we tested control (ctrl) vs. IWP-L6, TGF-β and IWP-L6+TGF-β  
757 (I+T). From these comparisons, scaled counts for all deregulated genes with  
758 log<sub>2</sub>FoldChange>1 and adjusted p-value <0.05 are shown in Figure 2 to provide an overview  
759 of effects and heterogeneity. Clusters were obtained with kmeans clustering using the  
760 superheat package. Gene lengths were obtained with EDAsseq package used to provide gene  
761 length normalised read counts (RPKM) for supplementary files (Risso et al., 2011). Used  
762 ggplot for plotting except for heatmaps. Code for differential expression analysis available  
763 upon request.

764 Gene Set Enrichment Analysis (GSEA) was done using a GSEA software downloaded from the  
765 Broad Institute (Mootha et al., 2003; Subramanian et al., 2005). DESeq2 results were  
766 imported based on Wald Statistics as preranked lists and enrichment was calculated with  
767 the classic setting and 1000 permutations. Gene sets were downloaded from the Molecular  
768 Signature Database; mSigDB v7.2 (Chang et al., 2004; Hsiao et al., 2001; Liberzon et al.,

769 2011). We performed GSEA against the entire C5: ontology gene sets (Table S3) and C8: cell  
770 type signature gene sets (Table S5) from mSigDB v7.2 as well as against selected gene sets  
771 representing cycling genes, liver-specific genes and genes enriched in developing human  
772 pancreas and liver. RNA-seq data generated in this study are available at the ArrayExpress  
773 database under the accession number E-MTAB-10715.

774

#### 775 **Public datasets**

776 The ATAC-seq data (GSE149148, Geusz et al., 2021) and the ChIP-seq data (GSE61475,  
777 Tsankov et al., 2015) used in this study were downloaded from Gene Expression Omnibus  
778 (GEO) as processed data in bigwig and bed formats.

779

#### 780 **Statistical analysis and reproducibility**

781 All statistics were performed using GraphPad Prism 8 software (GraphPad). Data sets with  
782 two groups having equal variances were analyzed by a two-tailed Student's t test. For data  
783 with unequal variances, two-tailed Welch's t tests were applied. Comparison of three or  
784 more groups was performed by one-way analysis of variance (ANOVA) followed by either  
785 Dunnett's test using ctrl samples as reference, or Tukey's test for comparison of all means.  
786 Statistics for differential expression of RNA-seq are provided as padj values from the DESeq2  
787 analysis. *P* values are displayed in the figures and sample sizes are provided in the figure  
788 legends. Statistical significance is defined as  $p < 0.05$  for qRT-PCR and GSEA data as well as  
789 FACS and IF image quantifications, while the DESeq2 analysis used an adjusted p-value  
790 (padj) cut-off set to 0.1 (default).

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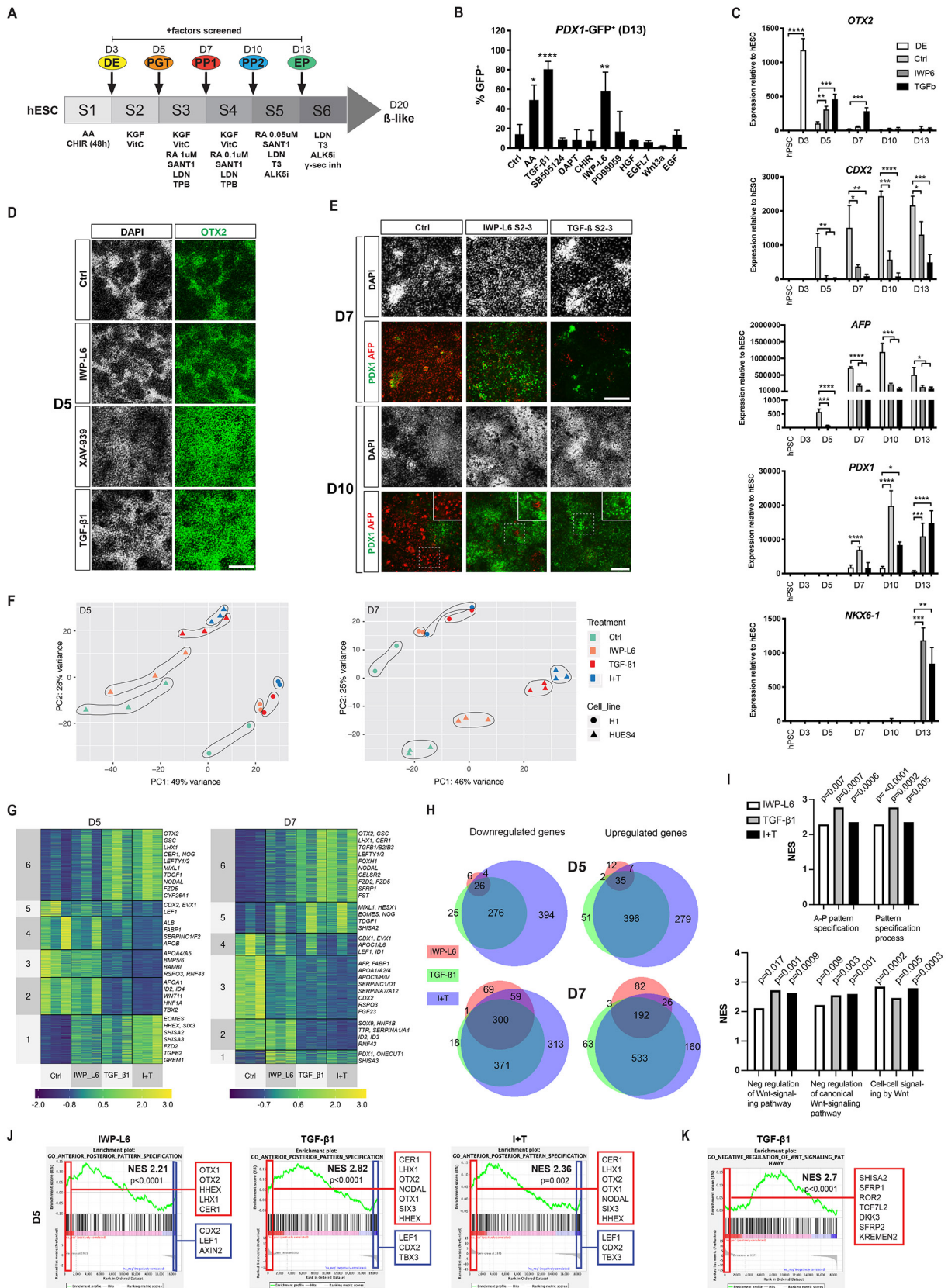


Figure 1

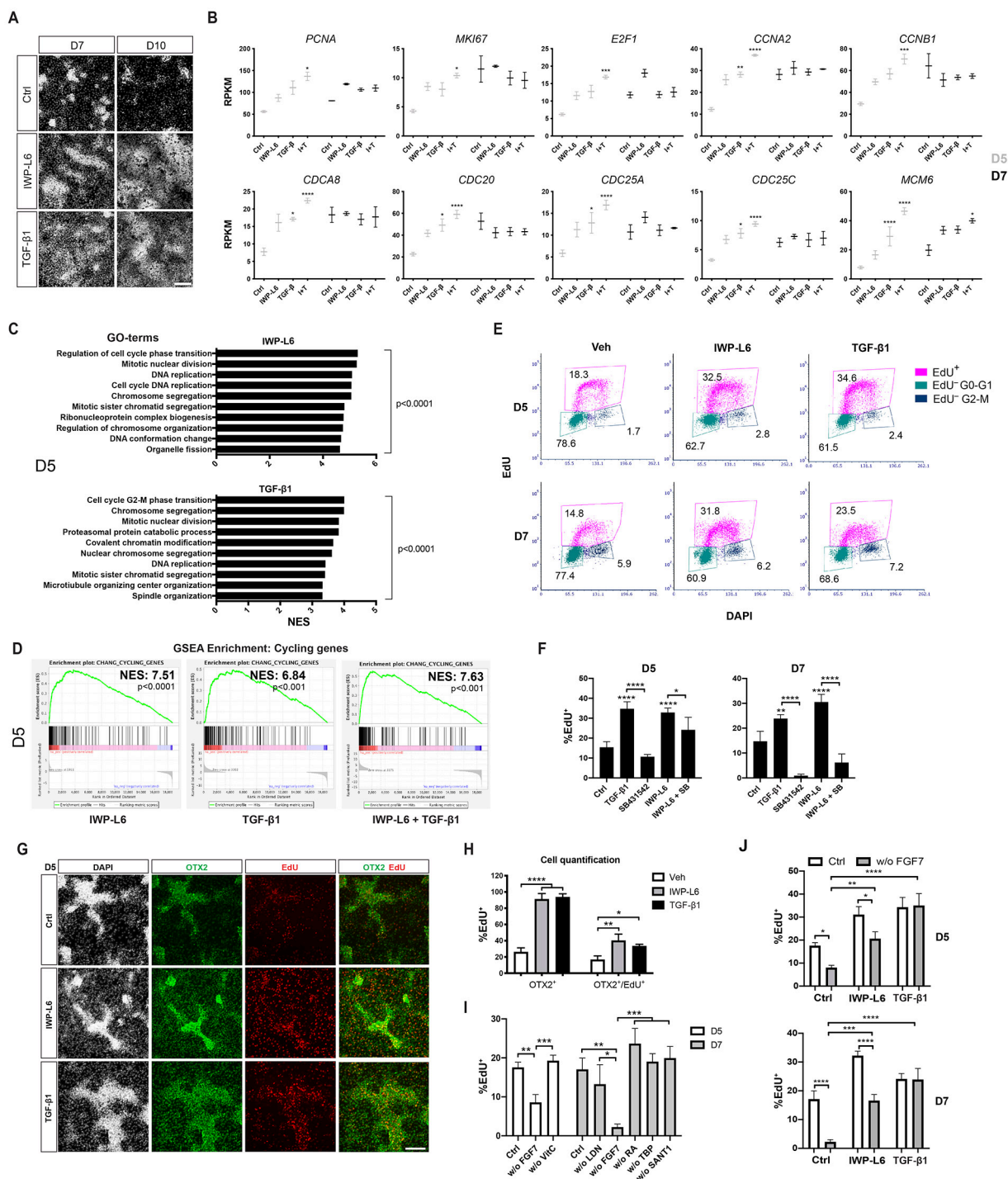


Figure 2

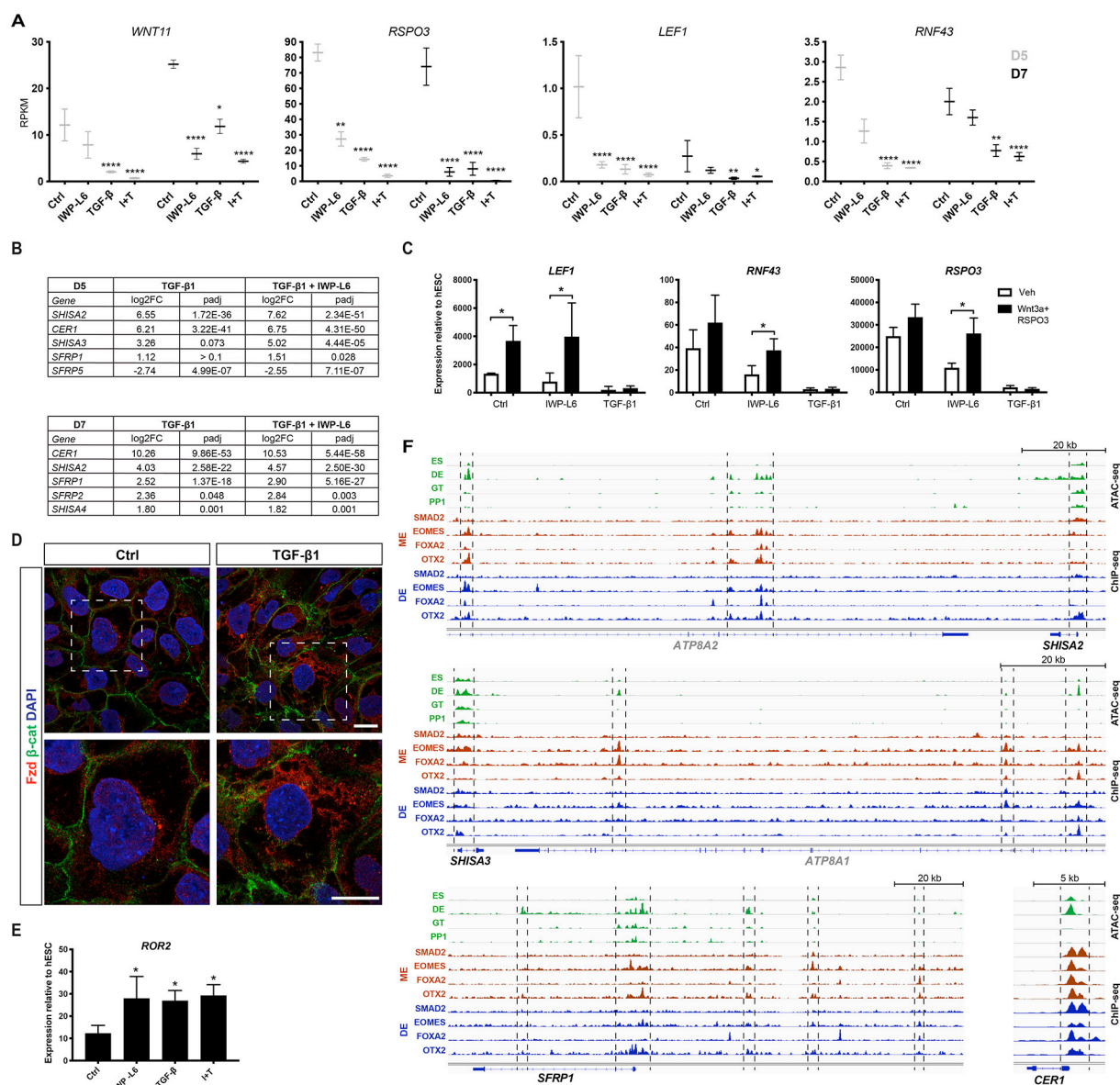


Figure 3

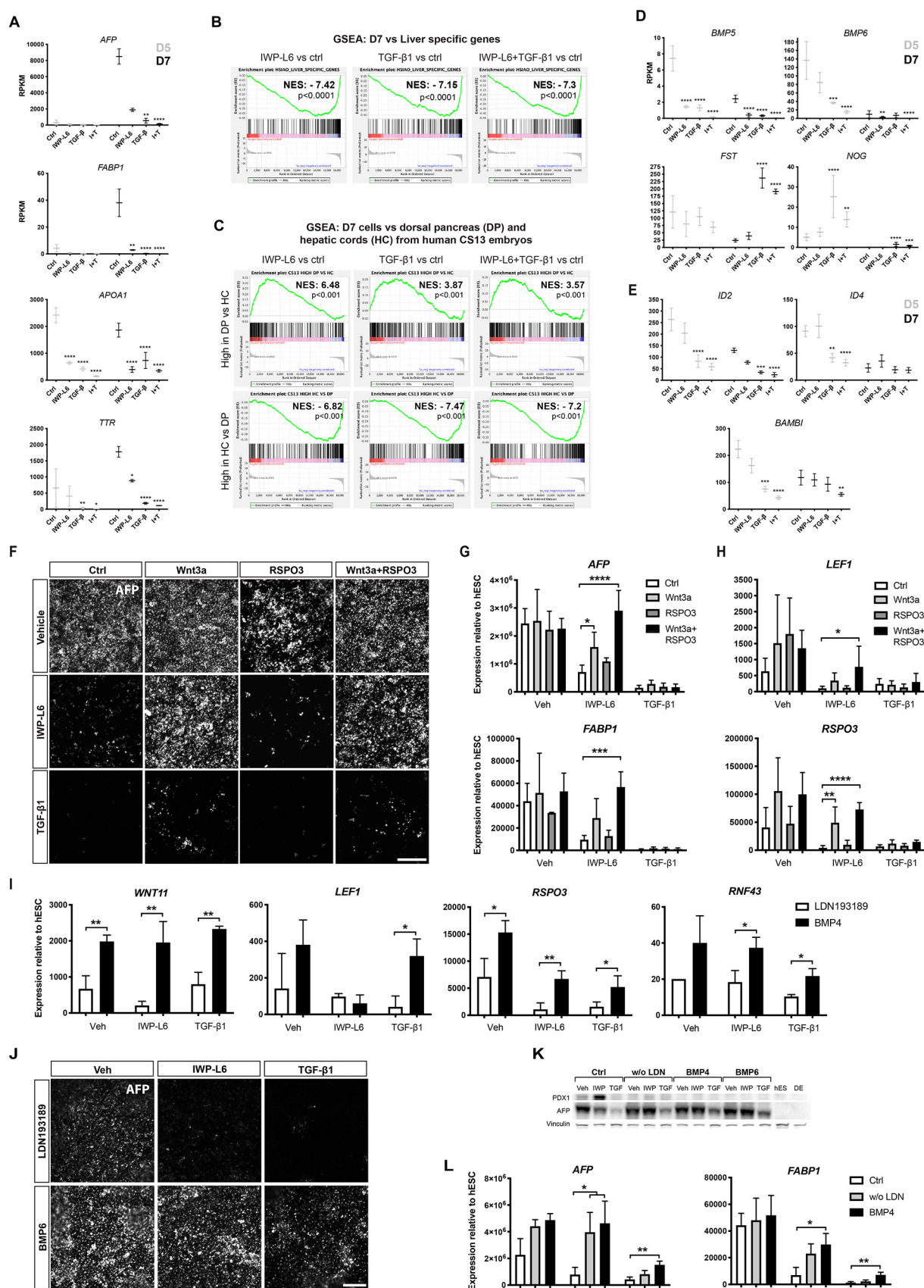


Figure 4

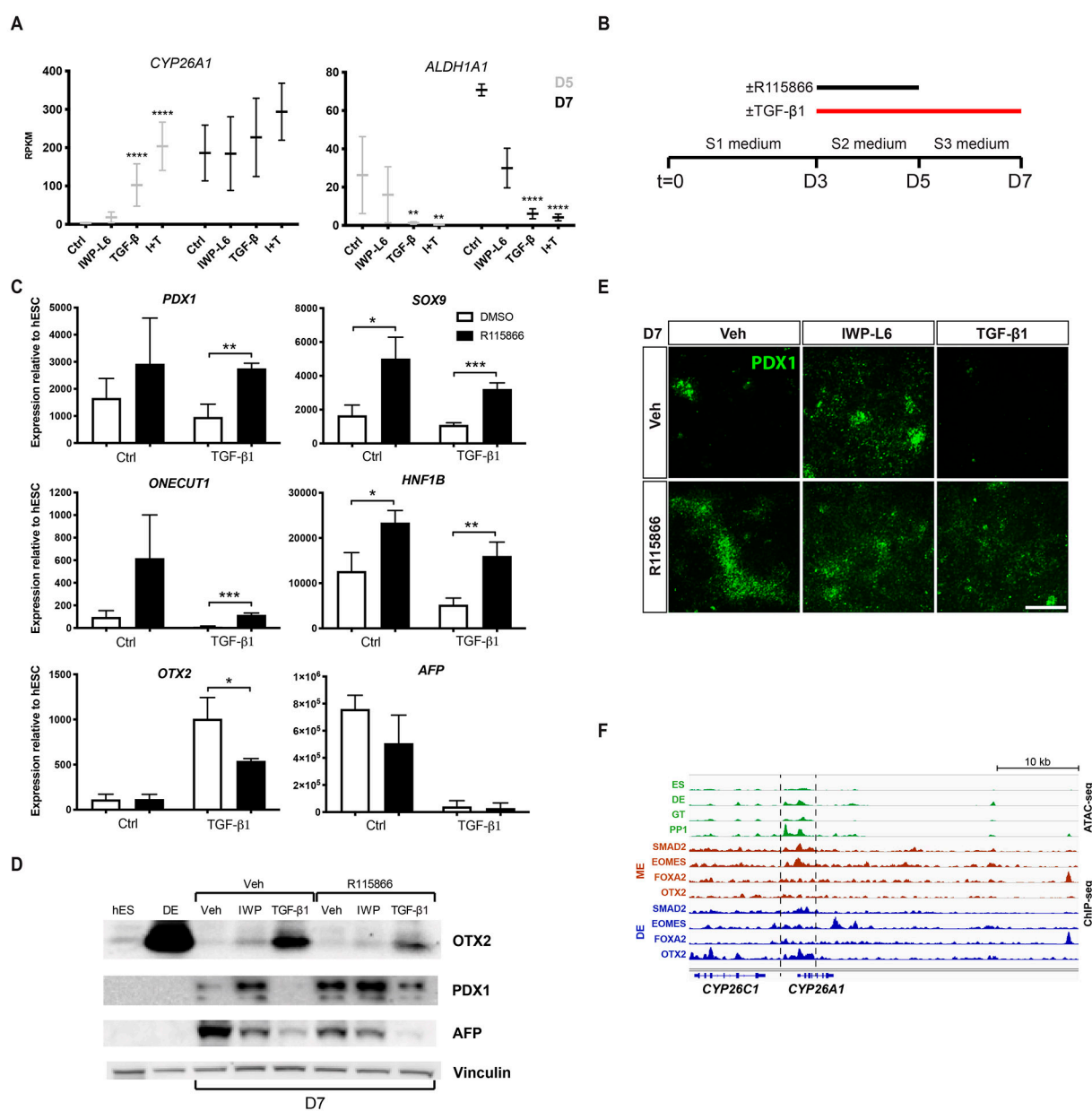


Figure 5