

Defective HNF1A hinders GLI3 processing favoring duodenal versus pancreatic fate, thus leading to intestinal elongation in vivo

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Regulatory circuits driving regional cell fate specification and lineage restriction decisions are not fully understood. The molecular mechanisms by which Hedgehog signaling controls lineage segregation in the posterior foregut remain unclear. Here, we employed bulk and single-cell transcriptomics, microscopy, physiology, and genetic cell tracing in differentiating human induced pluripotent stem cells and mouse transgenic models to uncover an essential autoregulatory loop between *Hnf1a* and Hedgehog signaling. *Hnf1a* abrogation initiates a domino effect leading to a drift in foregut cell specification toward duodenal cell identity instead of pancreatic fate. This was replicated in vivo in mice. We show that a common dominant negative *Hnf1a* mutation disrupts GLI3 processing by cilium proteins in the posterior foregut, thus impeding its own upregulation in response to Hedgehog signaling inhibition. In the context of this defective loop, we identified a network of deregulated selector genes responsible for the lineage segregation changes. These may be relevant for changes in the liver, pancreas, and gut seen in patients with mutations in *HNF1A*.

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The identification of the regulatory networks driving cell fate specification of equipotent cells during development and regeneration is a key prerequisite for controlling cell fate decisions. While significant progress has been recorded in the past decade, the sequence of molecular events controlling lineage choices remains a subject of active investigation in most organs, including the pancreas (Yang et al. 2022; Moghe et al. 2025). During development, the formation of the primitive gut tube occurs concomitantly with the definition of the anterior–posterior axis (Bouwmeester et al. 1996; Thomas et al. 1998; Wells and Melton 1999; Scheibner et al. 2021). This polarity further progresses into defined, usually mutually exclusive, discrete gene expression domains that will give rise to specific organs (Dessimoz et al. 2006). The regional specification and stringent border demarcation are essential for proper or-

gan development, with expression domain changes leading to homeotic transformations (Silberg et al. 2002; Gao et al. 2009; Raghoebir et al. 2012; Francis et al. 2019) in the gut tube. The commitment toward specific cell fates is progressively achieved via the cross-talk between the gut mesoderm and endoderm (Grapin-Botton and Melton 2000), with the anterior region giving rise to the esophagus, stomach, duodenum, liver, and pancreas, while the midgut will form the ileum and jejunum segments of the small intestine. The pancreas arises in the duodenal region from pancreatic progenitors, which share a common origin and a contiguous expression domain with the duodenum and liver progenitors (Zaret and Grompe 2008; Willnow et al. 2021; Yang et al. 2022).

Hnf1a is a transcription factor with a key role in cell fate commitment and islet identity during pancreas and liver development (Boj et al. 2001; Ng et al. 2024; Unger et al. 2024). Homozygous *Hnf1a*-null mice present a wide range

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of deleterious phenotypes with stunted growth, reduced pancreatic islet size and fitness, hepatomegaly (Lee et al. 1998), elongated intestine (Lussier et al. 2010), kidney complications (Pontoglio et al. 1996), and defective glucose regulation, among others (Pontoglio et al. 1998; Servitja et al. 2009; Mathisen et al. 2024). In humans, variants in the *HNF1A* gene can be stratified along a dysfunction phenotype continuum spanning from normoglycemia to type 2 diabetes and monogenic HNF1A-MODY (maturity-onset diabetes of the young) (Althari et al. 2020).

HNF1A-MODY, the most prevalent subtype of monogenic diabetes (Yamagata et al. 1996), is characterized by a large repertoire of mutations (Bjørkhaug et al. 2003; Behl et al. 2022), with at least 17 being shown to cause some type of important β -cell dysfunction (Wang et al. 2000; Eide et al. 2008; Servitja et al. 2009; Legøy et al. 2020; Kind et al. 2022; Li et al. 2022; Svalastoga et al. 2023). Despite the common denominator of impaired glycemic control, the underlying cellular and molecular mechanisms can differ between the HNF1A-MODY variants. Previous studies using human induced pluripotent stem cells (hiPSCs) reported that null *HNF1A* mutations as well as mutations in the transactivation (such as *P291fsinC*) and DNA binding (such as *R200Q*, *H126D*) leading to haploinsufficiency result in impaired pancreatic islet cell fate acquisition, altered islet architecture, a reduced number of pancreatic progenitors, loose cell identity confinement, or decreased glucose sensing and insulin secretion defects (Cardenas-Diaz et al. 2019; Cujba et al. 2022; González et al. 2022). In contrast, mutations that exert exclusively a dominant negative effect (such as *R272C*) lead to insulin hypersecretion without changes in the ratio of two main islet cell populations (Hermann et al. 2023). Furthermore, mutations in the dimerization domain (Narayana et al. 2001) impact β -cell development also by preventing the necessary dimerization with other factors such as HNF1B (Cujba et al. 2022).

One of the key events in early pancreas development is the compulsory inhibition of the Hedgehog signaling pathway; its ectopic activation leads to increased duodenal cell fate specification at the expense of the pancreatic lineage (Apelqvist et al. 1997; Kawahira et al. 2005) in an apparently dose-dependent manner (Nakayama et al. 2008; Lau and Hebrok 2010). Conversely, the inhibition of Hedgehog signaling leads to the expansion of the pancreatic expression domain (Kim and Melton 1998; Hebrok et al. 2000) during regeneration while also being involved in cell fate specification in the adult pancreas (Cigliola et al. 2018).

In most tissues, the Hedgehog signaling receptors are based in the primary cilia (Rohatgi et al. 2007; Sigafos et al. 2021), an evolutionarily conserved organelle involved in mediating mechano-chemical signaling (Goetz et al. 2009), with disrupted Hedgehog signaling being responsible for many defects observed in ciliopathies (Niewiadomski et al. 2019). Hedgehog signaling is activated by the binding of the Hedgehog ligands to the Patched1 (Ptch1) receptor, leading to Smoothened (Smo) translocation into the primary cilia. This blocks the proteolytic

cleavage of Gli2/Gli3 and the activation of Hedgehog target gene transcription. In the absence of the Hedgehog ligands, the Ptch receptors repress Smo activity, and Gli2/3 are sequestered by Suppressor of Fused (Sufu) and marked for proteolytic cleavage, which generates the repressor forms (Gli2R and Gli3R) (Sigafos et al. 2021) responsible for Hedgehog target gene repression. The proteolytic cleavage of Gli2 and Gli3, a process also associated with the primary cilia, is not equally efficient (Pan et al. 2006; Infante et al. 2018), with Gli2 being present mainly as Gli2FL (transcriptional activator), while Gli3 mostly acts as a transcriptional repressor (Gli3R) (Ruiz i Altaba 1999; Kasper et al. 2006). Thus, Gli3R is the main repressor of Hedgehog target genes, with its loss leading to severe developmental defects. Despite its proven importance, the molecular mechanisms by which Hedgehog signaling controls lineage segregation in the posterior foregut require further investigation. Our recent pilot study using human induced pluripotent stem cells (hiPSCs) suggested that Hedgehog signaling inhibition and the Gli3R repressor are important for HNF1A expression at this developmental stage (Unger et al. 2024). However, the precise regulatory relationship between these components as well as the consequences of their altered interaction remain unknown.

Here, by combining CRISPR-edited cells bearing the *P291fsinC* hotspot dominant negative *HNF1A* mutation and mouse transgenic models, we comprehensively investigate the regulatory interplay between Hedgehog signaling and HNF1A, the effect of the mutation on this loop, and its potential role in the broad spectrum of *Hnf1a*-deficient phenotypes.

Results

HNF1A mutation impacts *Gli3R* functionality via cilium proteins

First, to determine the impact of Hedgehog signaling and HNF1A in islet cell identity acquisition, we generated stem cell (SC) islets (Fig. 1A) from CRISPR-edited hiPSCs bearing the heterozygous “hotspot” *HNF1A* mutation (*HNF1A*^{*P291fsinC*}) (Supplemental Fig. S1A,B) and their isogenic control (*HNF1A*^{WT}) by employing a highly efficient differentiation protocol developed by the Otonkoski group (Balboa et al. 2022; Barsby et al. 2022). Similar to HNF1A-MODY patients, these cells are heterozygous for the *P291fsinC* mutation, which occurs within the HNF1A transactivation domain, leading to a truncated yet stable version of the HNF1A protein (Yamagata et al. 1998). Of note, as in all efficient *in vitro* islet cell differentiation protocols (Pagliuca et al. 2014; Reznika et al. 2014; Nostro et al. 2015), the required Hedgehog signaling inhibition was induced at the posterior foregut stage (e.g., first day of stage 3 [S3], corresponding to day 7 of the differentiation protocol; referred to here as S3_[D7]) and actively maintained until the formation of the endocrine progenitors (stage 5 [S5]) (Fig. 1A, yellow line).

The RNA-seq analysis of the early differentiation stages revealed a significant downregulation of *HNF1A*

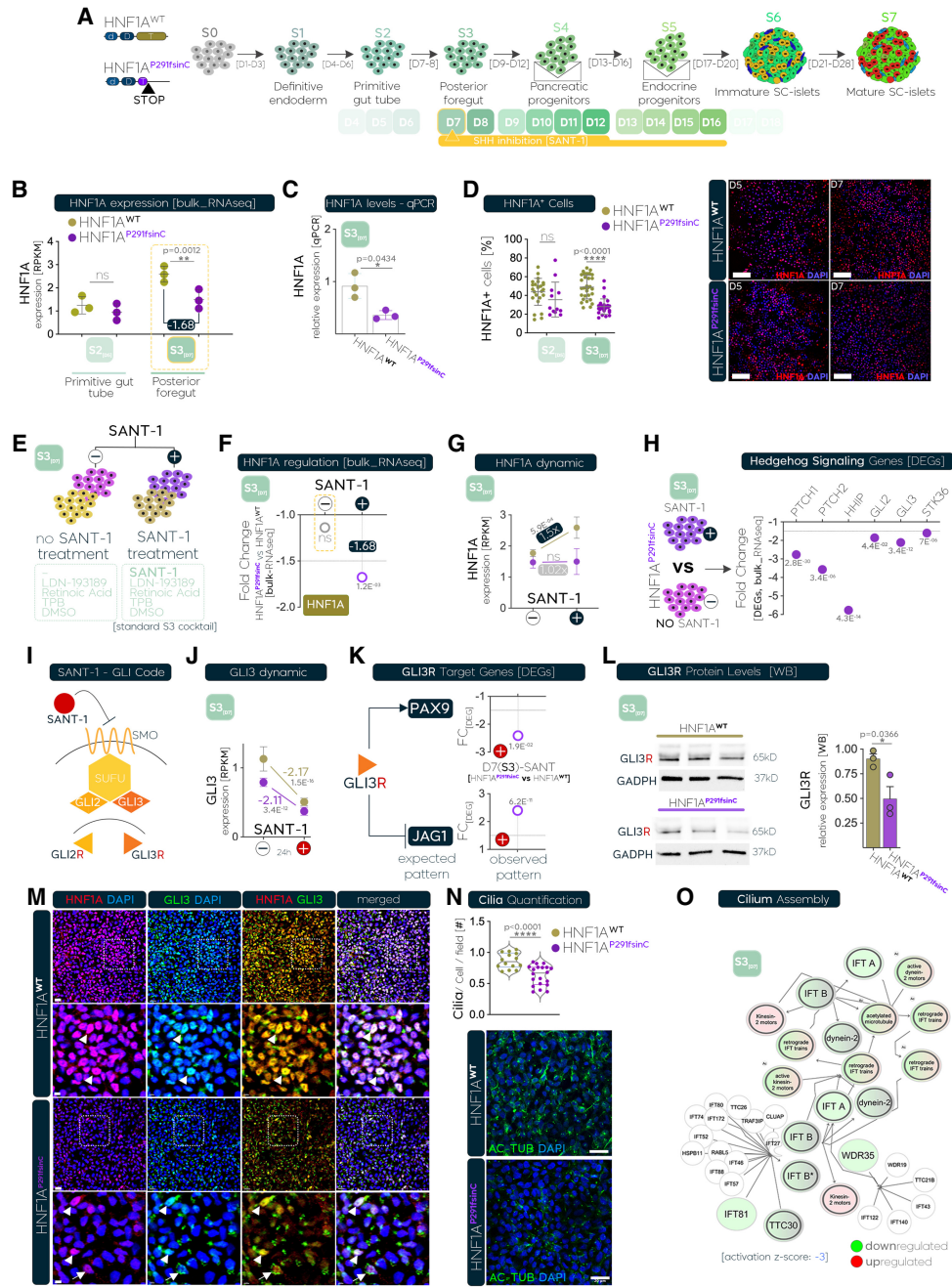


Figure 1. Defective cilium assembly and *GLI3* processing in *HNF1A*^{*P291fsinC*} cells at the posterior foregut stage. (A) Schematic representation of the WT and *P291fsinC* mutated *HNF1A* gene as well as the layout of the islet cell differentiation protocol staging, including the initiation and period of Hedgehog signaling inhibition by SANT-1. (B) Graph displaying the *HNF1A* levels in mutated *HNF1A*^{*P291fsinC*} and control *HNF1A*^{WT} at the primitive gut tube (S2_[D5]) and posterior foregut (S3_[D7]) stages based on the bulk RNA-seq RPKM values of each relevant condition (Welch's *t*-test). (C) Graph depicting the *HNF1A* relative expression levels by qPCR (Welch's *t*-test). (D) Quantification of the *HNF1A*⁺ cell percentages in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} conditions (one-way ANOVA) at D5 (S2_[D5]) and D7 (S3_[D7]) and representative immunofluorescence images of *HNF1A* staining in these conditions. (Red) *HNF1A*, (blue) DAPI. Scale bar, 50 μ m. (E) Scheme depicting the SANT-1 treatment conditions (with or without) and the respective differentiation cocktail compositions. (F) Graph indicating the regulation (fold change [FC]) of *HNF1A* expression in *HNF1A*^{*P291fsinC*} cells in the absence and presence of SANT-1, as revealed by RNA-seq. (G) Graph displaying *HNF1A* expression dynamic (RPKM) in the absence and presence of SANT-1 in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells (Welch's *t*-test). (H) Graph depicting the differential expression (RNA-seq) of Hedgehog signaling genes in the presence of SANT-1 as compared with no SANT-1 administration in *HNF1A*^{*P291fsinC*} cells. (I) Schematic representation of the expected Gli code in response to SANT-1. (J) Graph displaying the *GLI3* expression dynamic (RPKM) in the absence and presence of SANT-1 in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells. (K) Scheme depicting the expected outcome of *GLI3* regulation of *PAX9* and *JAG1* and graph displaying their observed differential expression in *HNF1A*^{*P291fsinC*} cells as revealed by RNA-seq. (L) Western blots and the respective quantifications (over GADPH) for *GLI3R* protein in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells (*t*-test). (M) Representative immunofluorescence images of *HNF1A* (red) and *GLI3* (green) staining patterns in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells. (Arrowheads) *GLI3* nuclear staining, (arrow) *GLI3* cytoplasmic staining. (Blue) DAPI. Scale bar, 20 μ m; magnification scale bar, 5 μ m. (N) Quantification and representative immunofluorescence images of acetylated tubulin (green) cilium staining in *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells (Welch's *t*-test). (Blue) DAPI. Scale bar, 20 μ m. (O) Cilium assembly pathway activity pattern (Z-score = -3) in *HNF1A*^{*P291fsinC*} based on the differential landscape (RNA-seq, S3_[D7]) between *HNF1A*^{*P291fsinC*} and *HNF1A*^{WT} cells (generated by Ingenuity pathway analysis).

expression in $\text{HNF1A}^{\text{P291fsinC}}$ as compared with HNF1A^{WT} conditions (Fig. 1B, $P = 1.2 \times 10^{-03}$; Supplemental Data S0, S1) at the initiation of the posterior gut stage (S3_[D7]) (Fig. 1B). This result was validated by qPCR (Fig. 1C, $P = 0.0434$) and at the protein level (Fig. 1D, $P < 0.0001$). Of note, similar results were obtained when differentiating $\text{HNF1A-MODY}^{\text{P291fsinC}}$ patient-derived hiPSCs, which also exhibited fewer HNF1A^+ cells ($P < 0.0001$) (Supplemental Fig. S1C; Supplemental Data S2) and reduced *HNF1A* expression ($P = 1.6 \times 10^{-05}$) (Supplemental Fig. S1D) at S3_[D7]. Furthermore, an independent CRISPR-edited mutant clone ($\text{HNF1A}^{\text{P291fsinC_D9}}$) differentiated using an alternative protocol (Rezania et al. 2014; Ghila et al. 2022) confirmed the statistically significant reduction in HNF1A^+ cells at the posterior foregut stage (Supplemental Fig. S1E, $P < 0.0001$, $P = 0.0131$).

To assess whether the observed mutation-related discrepancy in *HNF1A* levels was connected with the Hedgehog pathway, the Hedgehog signaling inhibitor SANT-1 was removed from the typical S3 differentiation cocktail (SANT-1^{minus}), while the rest of the components were maintained at their standard concentrations (Fig. 1E). A comparison of S3_[D7] HNF1A^{WT} and $\text{HNF1A}^{\text{P291fsinC}}$ cells via RNA-seq revealed similar baseline *HNF1A* expression levels in the absence of SANT-1 (Fig. 1F, yellow inset; Supplemental Data S1). However, a significant difference emerged after the treatment, with an increase in *HNF1A* expression being exclusively observed in the S3_[D7] HNF1A^{WT} cells (Fig. 1G, mustard, $1.5\times$, $P = 5.9 \times 10^{-04}$). The $\text{HNF1A}^{\text{P291fsinC}}$ cells failed to mount an equivalent response (Fig. 1G, purple) even though SANT-1 successfully downregulated the primary Hedgehog signaling components in these cells (Fig. 1H). Of note, $\text{HNF1A-MODY}^{\text{P291fsinC}}$ patient-derived S3_[D7] cells exhibited higher expression of Hedgehog signaling molecules than their respective controls after SANT-1 exposure (Supplemental Fig. S2A).

Overall, these data indicate that Hedgehog signaling inhibition is necessary for an *HNF1A* expression surge at the posterior foregut stage. However, its failure to upregulate the truncated form suggests a potential regulatory loop where *HNF1A* is required for proper Hedgehog signaling regulation.

To test this scenario, we investigated the effects of Hedgehog signaling inhibition in $\text{HNF1A}^{\text{P291fsinC}}$ mutant cells. The binding of the SANT-1 antagonist to Smoothed (SMO) leads to Hedgehog signaling inhibition and the generation (by proteolytic cleavage) of the repressor GLI forms GLI3R and, to a much lesser extent due to inefficient processing, GLI2R (Fig. 1I; Pan and Wang 2007). These repressor forms of GLIs, especially GLI3R, are not transcriptionally inert and actively repress Hedgehog target gene expression, with GLI3R deletions in many cases leading to gain-of-function phenotypes of Hedgehog signaling (Niewiadómski et al. 2019).

We thus focused on GLI3R for testing the actual efficiency of Hedgehog inhibition in $\text{HNF1A}^{\text{P291fsinC}}$ mutant cells. As expected, overall *GLI3* expression was significantly downregulated by SANT-1 exposure regardless of the mutation status (Fig. 1J). Despite this rather similar

dynamic in response to SANT-1 exposure, key GLI3R target genes in $\text{HNF1A}^{\text{P291fsinC}}$ cells exhibited a regulatory pattern opposite to the one normally associated with GLI3R activity as well as opposite to the one observed in control HNF1A^{WT} cells (Fig. 1K; Supplemental Fig. S2B). For example, *PAX9*, a GLI3R-activated target gene (McGlinn et al. 2005; Bastida et al. 2020), was upregulated only in HNF1A^{WT} (Supplemental Fig. S2B) but not in the mutant cells, consequently presenting significantly lower levels in the $\text{HNF1A}^{\text{P291fsinC}}$ cells (Fig. 1K). Similarly, GLI3R-repressed target genes, such as *JAG1* (McGlinn et al. 2005; Bastida et al. 2020) and *OTX1* (Theil et al. 1999) among others, were upregulated in mutant $\text{HNF1A}^{\text{P291fsinC}}$ cells in the presence of SANT-1 (Fig. 1K; Supplemental Fig. S2B,C). Moreover, important GLI3R regulators and partners (Engelen et al. 2011; Gao et al. 2024), like *CDH7* and *SOX2*, presented significantly decreased expression in $\text{HNF1A}^{\text{P291fsinC}}$ cells (Supplemental Fig. S2D). Taken together, these data suggest a disrupted GLI3R activity in the mutant $\text{HNF1A}^{\text{P291fsinC}}$ cells despite SANT-1 action.

In line with these observations, GLI3R protein levels were reduced in $\text{HNF1A}^{\text{P291fsinC}}$ cells (Fig. 1L), while GLI3 staining displayed distinct patterns between the mutated and WT cells (Fig. 1M). A clear GLI3 nuclear localization (Fig. 1M, arrowheads) was observed in the overwhelming majority of cells with high *HNF1A* expression (abundant in WT S3_[D7] cells) (see also Fig. 1D), while a speckled-like cytoplasmic pattern characterized the cells with low or absent *HNF1A* protein expression. Furthermore, cells with high *HNF1A* protein expression but cytoplasmic GLI3 were observed in the mutant cells (Fig. 1M, arrow).

Given the established critical role of cilia in regulating GLI3 protein levels and its processing to the repressor GLI3R form (Wen et al. 2010; Besse et al. 2011), we investigated whether the observed differences in GLI3 output and cellular localization pattern observed in $\text{HNF1A}^{\text{P291fsinC}}$ mutant cells stem from underlying changes in cilium structure or function. Acetylated tubulin (AC-TUB) staining revealed a statistically significant cilium number reduction in S3_[D7] $\text{HNF1A}^{\text{P291fsinC}}$ mutant cells as compared with controls, consistent with a cilium formation defect (Fig. 1N). Moreover, the pathway analysis of the differential transcriptional landscape inferred the inactivation of the cilium assembly signaling (negative Z-score = -3) in S3_[D7] $\text{HNF1A}^{\text{P291fsinC}}$ cells as compared with the control counterparts (Fig. 1O), with key genes required for both anterograde and retrograde cilium transport being observed significantly downregulated (Fig. 1O; Supplemental Fig. S2E). This observation was also reproduced in $\text{HNF1A-MODY}^{\text{P291fsinC}}$ patient-derived S3_[D7] cells where cilium gene deregulation was observed, including *ITF70A* or *ITF70B* downregulation (Supplemental Fig. S2F) among others. Interestingly, mutations in many of these downregulated cilium proteins were shown to impede GLI3FL processing to GLI3R and to lead to defective Hedgehog signaling, thus further pointing toward a GLI3 processing error in the *HNF1A* mutant cells.

To further study this possibility, similar to our previous pilot study (Unger et al. 2024), we modified the expected Gli code by replacing SANT-1 with GANT61, another potent Hedgehog signaling inhibitor (Zubčić et al. 2020). GANT61 acts by directly inhibiting GLI1 and GLI2 but not GLI3 (Agyeman et al. 2014; Matissek and Elswa 2020), and consequently, in contrast to SANT-1, it does not induce the processing of GLI3FL to GLI3R (Supplemental Fig. S2G). GANT61 treatment significantly decreased the proportion of HNF1A⁺ cells in the S3_[D7] HNF1A^{WT} cells to levels observed in the mutant HNF1A^{P291fsinC} counterparts (Supplemental Fig. S2H, mustard). In contrast, the proportion of HNF1A⁺ cells in S3_[D7] HNF1A^{P291fsinC} was not significantly impacted by GANT61 treatment (Supplemental Fig. S2H, purple), suggesting that a GLI3FL-like code was already present in the mutant cells. Of note, while the *HNF1A*-relative expression was slightly yet significantly downregulated after 4 h of GANT61 treatment in the HNF1A^{WT}, this was recovered by 24 h, indicating the transient effect of GANT61 inhibition of Hedgehog signaling (Supplemental Fig. S2I).

Taken together, these data suggest a scenario in which GLI3R is required for increasing *HNF1A* expression at the initiation of the posterior foregut stage, while the fully functional HNF1A is essential for GLI3R processing, with the mutated HNF1A^{P291fsinC} impeding this process by deregulating cilium proteins.

The tethered HNF1A–Hedgehog signaling regulatory loop leads to early shifts in cell identity acquisition

To differentiate the impact of the deficient regulatory loop between HNF1A and Hedgehog signaling, we mined the data for critical transcriptional regulators, which follow a regulatory pattern similar to the one exhibited by HNF1A in response to SANT-1 treatment (i.e., upregulated in HNF1A^{WT} but not in HNF1A^{P291fsinC} cells) (Fig. 2A). The analysis identified the significant upregulation of *HHEX* ($P = 1.2 \times 10^{-09}$) exclusively in control HNF1A^{WT} cells (Fig. 2B; Supplemental Fig. S3A). In contrast, the same gene was downregulated in HNF1A-MODY patient-derived S3_[D7] cells (Supplemental Fig. S3B). *HHEX* is a known type 2 diabetes risk gene (Sladek et al. 2007), a coactivator of HNF1A (Tanaka et al. 2005), and a key developmental cell fate selector in the gut (Yang et al. 2022). Moreover, previous studies demonstrated that *HHEX* is negatively regulated by Hedgehog signaling during pancreatic endoderm formation (Dumasia et al. 2021) as well as the critical role of *HHEX* in promoting pancreatic progenitors' fate at the expense of the duodenal and liver progenitor alternative programs, with its loss leading to a switch toward liver and duodenum lineage identity (Fig. 2C; Yang et al. 2022).

Indeed, we observed that in the absence of *HHEX* upregulation, the key intestinal marker *CDX2* was significantly upregulated in both HNF1A^{P291fsinC} (Fig. 2D) and HNF1A-MODY^{P291fsinC} (Supplemental Fig. S3C) patient-derived cells. Of note, previous research demonstrated that *CDX2* is a direct target gene of HNF1A (Verzi et al. 2010; Song et al. 2023) as well as a direct regulatory inter-

action partner (Mitchelmore et al. 2000; Boudreau et al. 2002). This increase in *CDX2* was also confirmed at the protein level in the HNF1A^{P291fsinC} condition, which demonstrated an elevated number of *CDX2*⁺ cells (Supplemental Fig. S3D, $P = 0.0201$). In contrast, *CDX2* expression was significantly downregulated in the control cells (Supplemental Fig. S3E). Of note, a similar transcriptional regulatory pattern was shared also by the critical liver marker AFP (Fig. 2D), another direct target gene of HNF1A (McVey et al. 1993; Nagata-Tsubouchi et al. 2005; Yu et al. 2008; Li et al. 2019).

In line with these findings, a wide range of duodenal- and liver-specific markers were observed upregulated in the HNF1A^{P291fsinC} (Fig. 2E) and HNF1A-MODY (Supplemental Fig. S3F) patient-derived cells. This upregulation was also confirmed by running single-cell RNA sequencing (sc-RNA-seq) on the posterior foregut S3_[D7] cells (Supplemental Fig. S3G,H). Cluster annotation (Supplemental Fig. S3G,H) and module scores (Supplemental Fig. S3I–K) indicated the presence of pancreas (cluster 2), liver (cluster 7), duodenum (cluster 8), and mixed hepato-intestinal (cluster 10) progenitor clusters. As anticipated, the analysis revealed a higher representation in the mutant (Fig. 2F, bottom pie) of the liver (Fig. 2G, top; Supplemental Data S3), duodenal (Fig. 2G, cluster 8, bottom; Supplemental Data S3), and early hepato-intestinal (Supplemental Fig. S4A) progenitors. Moreover, certain duodenal and liver markers were observed significantly upregulated in the HNF1A^{P291fsinC} mutant cells of these clusters (Fig. 2H; Supplemental Fig. S4B).

CDX2 is an essential factor for intestine development, its ectopic expression being sufficient to promote intestinal differentiation in the murine gastric mucosa in vivo (Silberg et al. 2002). Conversely, its loss leads to early anteriorization events (i.e., foregut transcriptional program expansion) due to the expansion of the *SOX2* domain (Beck et al. 1999; Gao et al. 2009), thus further highlighting the importance of *CDX2* inhibition observed exclusively in the control HNF1A^{WT} cells (Supplemental Fig. S3E). Of note, *SOX2*, the key partner of *CDX2* for establishing the gastric duodenal border, is severely downregulated in HNF1A^{P291fsinC} mutant cells ($\approx -70\times$, Fig. 2I, top graph) in response to SANT-1, thus leading to much lower levels compared with its control counterpart (Fig. 2I, bottom graph). This, combined with the increased *CDX2* levels, suggests a posteriorization of cell identity acquisition in this context. The sc-RNA-seq of the D7 cells (Supplemental Fig. S3G) confirmed this observation, indicating an underrepresentation of the *SOX2* population and an overrepresentation of the *CDX2* population in mutant HNF1A^{P291fsinC} cells (Fig. 2J).

In line with the observed shift toward a duodenal/hepatic progenitor program, a wide range of pancreatic fate markers was significantly downregulated in both the HNF1A^{P291fsinC} (Fig. 2K) and HNF1A-MODY^{P291fsinC} (Supplemental Fig. S4C) patient-derived S3_[D7] cells. Moreover, key markers controlling the fusing of the dorsal and ventral pancreatic buds (such as *BARX1* [Patzek et al. 2023] among others) were upregulated only in the control HNF1A^{WT} cells (Supplemental Fig. S4D). In addition, the

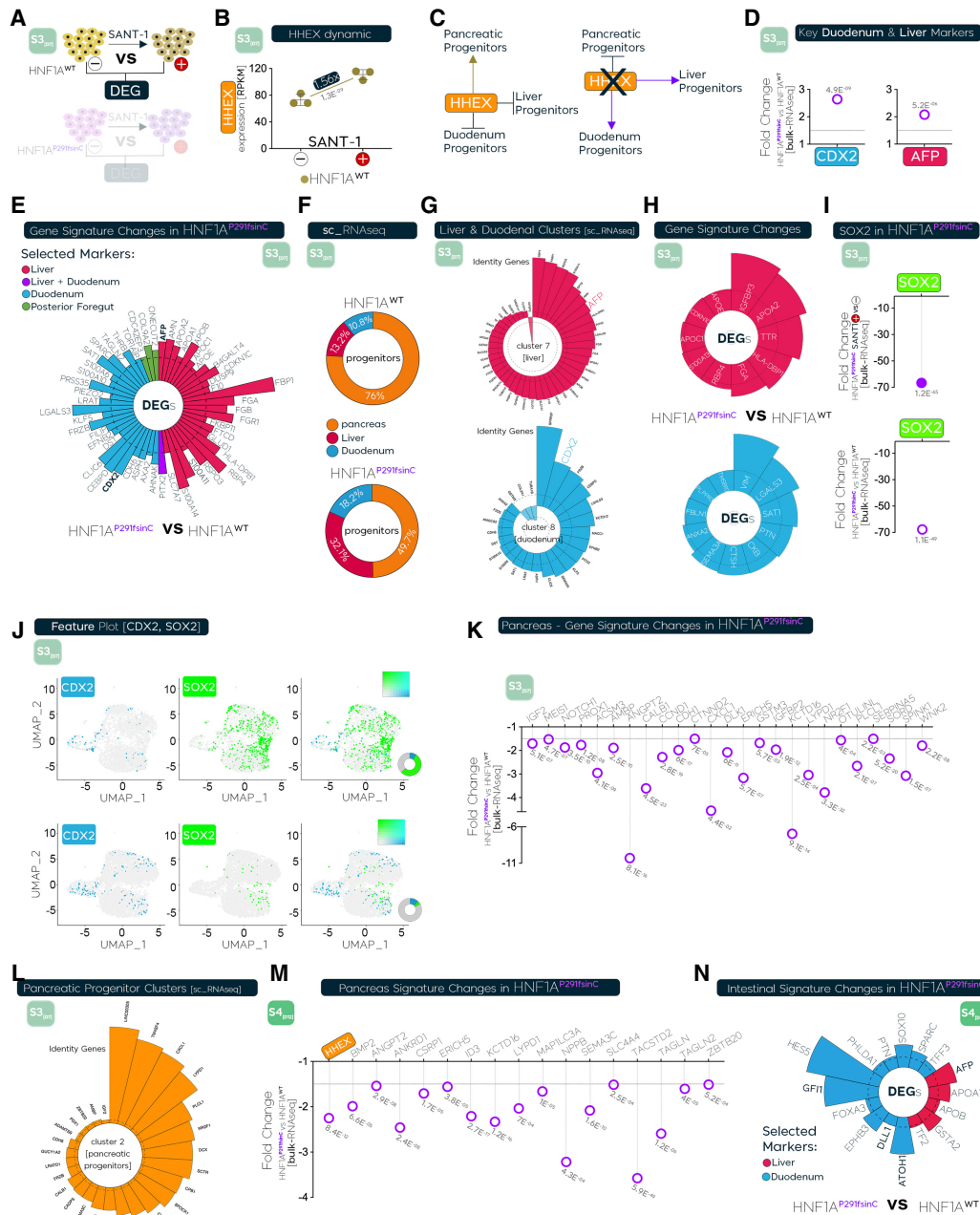


Figure 2. Specification program signature changes in HNF1A^{P291fsinC} cells at the posterior foregut and pancreatic progenitor stages. (A) Scheme depicting the conditions analyzed focusing on the Hedgehog signaling inhibition effects exclusively on HNF1A^{WT} cells (S3_{D7}). (B) Graph displaying *HHEX* expression dynamic (RPKM, S3_{D7}) in the absence and presence of SANT-1 in HNF1A^{WT} cells. (C) Scheme depicting the reported role of *HHEX* in controlling lineage choices in the foregut. (D) Graphs depicting the differential expression (RNA-seq, S3_{D7}) of *CDX2* and *AFP* in the presence of SANT-1 in HNF1A^{P291fsinC} as compared with HNF1A^{WT}. (E) Circle plot exhibiting the upregulation of duodenal and liver markers in HNF1A^{P291fsinC} at the posterior foregut stage (S3_{D7}), based on RNA-seq DEG data sets ($P < 0.05$, FC > 1.5). (F) Pie diagrams illustrating the percentage representation of pancreatic (orange; cluster 2), liver (pink; cluster 7), and duodenum (blue; cluster 8) clusters in HNF1A^{WT} and HNF1A^{P291fsinC} conditions based on sc-RNA-seq data. (G) Circle plots depicting the expression of liver (pink) and duodenum (blue) progenitor markers in cluster 7 (liver progenitors) and cluster 8 (duodenum progenitors), respectively, as compared with the expression of all other clusters (DEGs; sc-RNA-seq at S3_{D7}). (H) Circle plots depicting the upregulated liver (pink) and duodenum (blue) progenitor markers in HNF1A^{P291fsinC} at the posterior foregut stage (S3_{D7}), based on sc-RNA-seq. (I) Graphs displaying *SOX2* differential expression (FC) in HNF1A^{P291fsinC} in response to SANT-1 in HNF1A^{P291fsinC} cells (up) and as compared with HNF1A^{WT} cells (down) (bulk RNA-seq). (J) Feature plots for *CDX2* (blue) and *SOX2* (green) genes in HNF1A^{WT} and HNF1A^{P291fsinC} cells. (K) Graph depicting the differential expression of selected pancreatic signature markers in HNF1A^{P291fsinC} (RNA-seq) at the posterior foregut stage (S3_{D7}). (L) Circle plots depicting the upregulation of pancreatic progenitor markers in cluster 2 as compared with all other clusters (DEGs; sc-RNA-seq at S3_{D7}). (M) Graph depicting the differential expression of selected pancreatic signature markers in HNF1A^{P291fsinC} (RNA-seq) at the pancreatic progenitor stage (S4_{D12}). (N) Circle plot exhibiting the upregulation of duodenal and liver markers in HNF1A^{P291fsinC} at the pancreatic progenitor stage (S4_{D12}).

single-cell analysis revealed that there are fewer cells (Fig. 2F) bearing a pancreatic progenitor profile in the HNF1A^{P291fsinC} condition (cluster 2) (characterized in Fig. 2L; Supplemental Fig. S4E; see also Supplemental Fig. S3G–K; Supplemental Data S3). Taken together, these data further confirm a shift toward duodenal specification in the mutant cells. Of note, the significant decrease in *HHEX* and other pancreatic progenitors' marker levels was maintained at S4_[D12] (pancreas progenitor stage) in HNF1A^{P291fsinC} mutant cells (Fig. 2M; Supplemental Data S1), alongside key islet cell identity genes (like *GCG*, *INS*, *MAFB*, and *PCSK1*) (Supplemental Fig. S4F).

In contrast, due to the daily administration of LDN-193189, a standard component of the differentiation cocktail (administered between D7 and D20) (Fig. 1E), we observed a significant decrease of the liver progenitors' signature between S3_[D7] and S4_[D12] in both HNF1A^{WT} and HNF1A^{P291fsinC} cells (Supplemental Fig. S4G; Supplemental Data S1), which was more pronounced in the mutant (Supplemental Fig. S4H). LDN-193189 is a BMP inhibitor demonstrated to block the liver-inducing BMP signaling (Rossi et al. 2001; Gouon-Evans et al. 2006; Shin et al. 2007; Ang et al. 2018; Yang et al. 2022) and promote intestinal cell fate in the absence of *HHEX* (Supplemental Fig. S4I; Yang et al. 2022). In contrast, the duodenal signature still progressed at S4_[D12], exhibiting the upregulation of specific intestinal markers essential for the development of the intestinal lineage such as *DLL1*, *ATOH1*, and *GFI1* (Fig. 2N; Supplemental Fig. S4J).

Overall, these data suggest that the absence of *HHEX* upregulation in HNF1A^{P291fsinC} mutant cells leads to misexpression of duodenal and liver signatures, with the first being further promoted by the presence of BMP inhibitors in the differentiation cocktail composition.

The generation of endocrine progenitors (S5) is boosted by aggregating cells into three-dimensional structures (see also Fig. 1A), a process that was demonstrated to also exclude the undifferentiated or off-target cells (Barsby et al. 2022). Expectedly, we observed a robust increase in islet cell markers in both HNF1A^{P291fsinC} and HNF1A^{WT} cells between the pancreatic progenitor and endocrine progenitor stages. However, certain key α -cell markers, such as *GCG*, *ARX*, and *MAFB*, displayed higher upregulation in the mutant cells as compared with HNF1A^{WT} (Supplemental Fig. S4K; Supplemental Data S4). In contrast, the increase in the endocrine progenitor expression was similar or slightly more elevated in the HNF1A^{WT} cells at S5 compared with S4. The difference between the regulatory dynamic was maintained during the differentiation of S5 cells toward SC islets (S7) with a robust upregulation of α -cell markers (such as *GCG* and *ARX*) in mutant HNF1A^{P291fsinC} cells, which was not matched by the control (Supplemental Fig. S4L; Supplemental Data S4). Conversely, key β -cell markers (such as *INS* and *SLC2A2*) presented a stronger induction in control HNF1A^{WT} than in HNF1A^{P291fsinC} mutant cells between S7 and S5. Taken together, these results suggest a decreased β -cell fate acquisition coupled with an increased α -cell fate acquisition during the last stages of the differentiation sequence of HNF1A^{P291fsinC} cells.

Enriched *GLI3* expression and a shift toward α -cell identity in differentiated SC islet cells with HNF1A mutation

Despite the signature shifts observed at earlier stages, HNF1A^{P291fsinC} mutant cells are still able to form SC islets in response to the imposed differentiation sequence (Cardenas-Diaz et al. 2019; Legøy et al. 2020; Low et al. 2021; Cujba et al. 2022; González et al. 2022), yet the total number of secretory CHGA⁺ was lower than in the HNF1A^{WT} (Fig. 3A, $P=0.0338$). The resulting mutant SC islets present glucose regulation apparently similar to their control HNF1A^{WT} counterparts (Fig. 3B); however, both CRISPR-edited HNF1A^{P291fsinC} (Fig. 3C, $P<0.0001$; Supplemental Fig. S5A [$P=0.0041$], B) and HNF1A-MODY^{P291fsinC} (Supplemental Fig. S5A, $P<0.0001$) patient-derived conditions were characterized by significantly fewer insulin⁺ (INS⁺) cells and decreased β -cell markers (such as *INS*, *MAFA*, *SLC2A2*, and *PAX4*) (Fig. 3D) by RNA-seq (Supplemental Data S1). In contrast, the HNF1A^{P291fsinC} SC islets displayed significantly increased glucagon secretion (Fig. 3E, $P=0.0011$) and number of glucagon⁺ (GCG⁺) cells (Fig. 3F, $P<0.0001$; Supplemental Fig. S5B,C, $P=0.0315$), as well as significantly elevated expression of α -cell markers (such as *GCG*, *ARX*, and *IRX2*) (Fig. 3G; Supplemental Data S1). Moreover, they presented a slight yet significant increase in the number of GCG⁺/INS⁺-expressing cells (Fig. 3H, $P<0.0001$). Of note, although slightly increased in the mutant, the nuclear CDX2 was not detected by IF in any of the GCG⁺ cells of the SC islet cells regardless of the mutation status (Supplemental Fig. S3D).

To further explore the observed shift in cell identity selection, we performed single-cell RNA-seq of S7 (mature SC islet stage) on HNF1A^{WT} and HNF1A^{P291fsinC} SC islets. This confirmed the presence of the three main islet endocrine populations (insulin, glucagon, and somatostatin) in HNF1A^{P291fsinC} SC islets (Fig. 3I; Supplemental Fig. S5E,F; Supplemental Data S3). Of note, very few cells expressed ghrelin (GHR) across all clusters (Supplemental Fig. S5G), and we identified a small population of enterochromaffin cells (cluster 6) (Supplemental Fig. S5H), a known in vitro differentiation artifact.

The RNA-seq analysis confirmed the significant increase of glucagon-expressing (Fig. 3J; Supplemental Fig. S5I,J) and bihormonal (GCG/INS) (Fig. 3J; Supplemental Fig. S5I,J) cell populations observed in the bulk approach. Of note, the single-cell approach further revealed a significantly lower expression of key β -cell markers such as *INS* and *MAFA* in the mutant HNF1A^{P291fsinC} mature (cluster 0) insulin-expressing cells (Fig. 3J, top graph; Supplemental Data S1), while the critical α -cell determinant *ARX* was significantly upregulated in these cells (Fig. 3K, top graph), suggesting a less stringent β -cell identity regulation. Correspondingly, the comparison of the mature α -like cells (cluster 1) (Supplemental Data S1; Supplemental Fig. S5E,F) between HNF1A^{P291fsinC} and HNF1A^{WT} identified a significantly increased *GCG* expression in HNF1A^{P291fsinC} (Fig. 3K, bottom graph), further

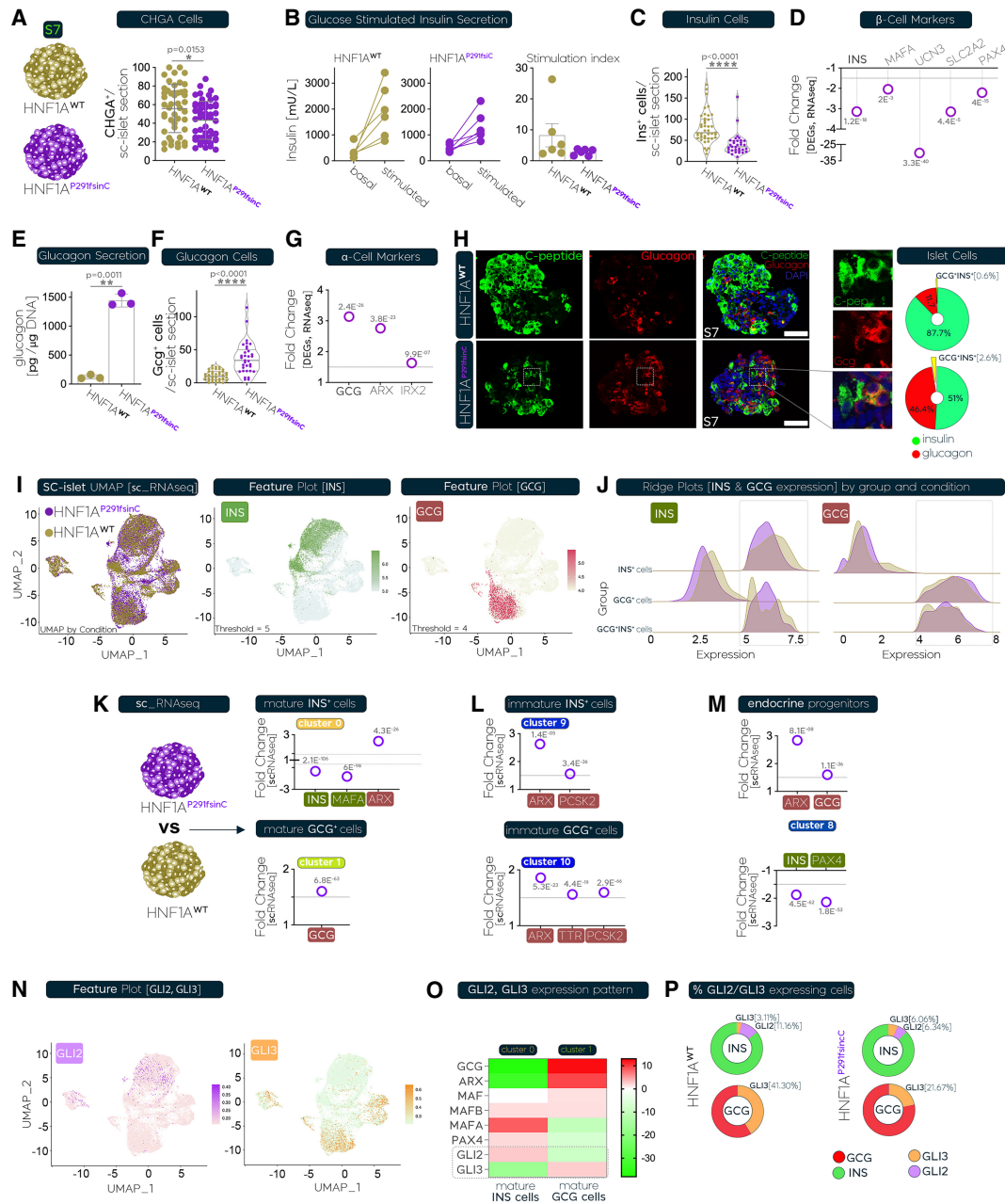


Figure 3. Islet signature changes in HNF1A^{P291fsinC} SC islets. (A) Quantification of secretory (CHGA⁺) cells per SC islet section in HNF1A^{P291fsinC} and HNF1A^{WT} conditions (Welch's *t*-test). (B) Glucose-stimulated insulin secretion (GSIS) of HNF1A^{P291fsinC} and HNF1A^{WT} cells. (C) Quantification of insulin-producing (C-peptide⁺) cells per SC islet section in HNF1A^{P291fsinC} and HNF1A^{WT} conditions (Mann-Whitney). (D) Graph illustrating the significant downregulation of key β -cell markers in HNF1A^{P291fsinC} (RNA-seq). (E) Glucagon secretion in HNF1A^{P291fsinC} and HNF1A^{WT} cells (Welch's *t*-test). (F) Quantification of GCG⁺ cells per SC islet section in HNF1A^{P291fsinC} and HNF1A^{WT} conditions (Mann-Whitney). (G) Graph illustrating the significant upregulation of key α -cell markers in HNF1A^{P291fsinC} (RNA-seq). (H) Representative immunofluorescence images of HNF1A^{WT} and HNF1A^{P291fsinC} cells stained for C-peptide (green), glucagon (red), and DAPI (blue) illustrating the occurrence of bihormonal GCG⁺INS⁺ cells, and pie diagrams illustrating the percentage representation of glucagon, insulin, and bihormonal cell types. Scale bar, 50 μ m. (I) UMAP of HNF1A^{P291fsinC} and HNF1A^{WT} conditions and feature plots for *INS* and *GCG* genes (sc-RNA-seq). (J) Ridge plots for *INS* and *GCG* genes in insulin, glucagon, and bihormonal cells (sc-RNA-seq) in HNF1A^{P291fsinC} and HNF1A^{WT}. (K) Graphs depicting the upregulation of key α -cell markers in mature β -like (up; cluster 0) and α -like (down; cluster 1) clusters in HNF1A^{P291fsinC} cells (sc-RNA-seq). (L) Graphs depicting the upregulation of key α -cell markers in immature β -like (up; cluster 9) and α -like (down; cluster 10) clusters in HNF1A^{P291fsinC} cells (sc-RNA-seq). (M) Graphs depicting the upregulation of key α -cell markers and downregulation of key β -cell markers in the endocrine progenitor cluster (cluster 8; sc-RNA-seq). (N) Feature plots for *INS* and *GCG* genes. (O) Heat map of *GLI2*, *GLI3*, and selected α -cell and β -cell markers in mature insulin and glucagon cells based on sc-RNA-seq data. (P) Pie diagrams illustrating the percentage representation of *GLI2*- and *GLI3*-expressing insulin and glucagon cells in HNF1A^{P291fsinC} and HNF1A^{WT} conditions based on sc-RNA-seq data.

supporting an overall shift toward an α -cell identity with the *HNF1A* mutation. This was also true in immature β -like (cluster 9) and α -like (cluster 10) cells, both of which exhibit significantly increased α -cell markers (Fig. 3L). Furthermore, the upregulation of key α -cell markers (*ARX* and *GCG*) and downregulation of β -cell markers (*INS* and *PAX4*) were also significant in the endocrine progenitor cluster (cluster 8) (Fig. 3M), which was also underrepresented in the mutant (4.93% vs. 6.24%)

Of note, the analysis revealed the different GLI usage between the two main hormone cell types, with *GLI2* being expressed mostly by insulin-expressing cells, while *GLI3* was expressed mainly in glucagon-expressing cells (Fig. 3N) including the respective mature population clusters (Fig. 3O). However, this segregation was skewed in the *HNF1A*^{P291fsinC} mutant context, with an overall decrease in *GLI2*-expressing insulin cells and *GLI3*-expressing glucagon cells (Fig. 3P).

The persistent progenitor population is underrepresented and exhibits a residual intestinal signature in cells with the HNF1A mutation

The single-cell analysis of S7 cells revealed a cell population (cluster 7) that was strongly enriched in pancreatic progenitor markers with significantly higher levels of expression compared with all the other clusters (Fig. 4A; Supplemental Fig. S6A–C; Supplemental Data S3). Correspondingly, the expression of critical islet cell determinants such as *PDX1*, *NKX6.1*, and *ARX* was reduced compared with islet α -cell and β -cell clusters (Supplemental Fig. S6D). Similarly, markers for nonislet enterochromaffin cells, a cell type known to artifactually occur during in vitro islet-like cell differentiation (Zhu et al. 2023), displayed downregulation in this cluster (Supplemental Fig. S6E). Of note, in the actual enterochromaffin cluster (cluster 6), the only statistically significant

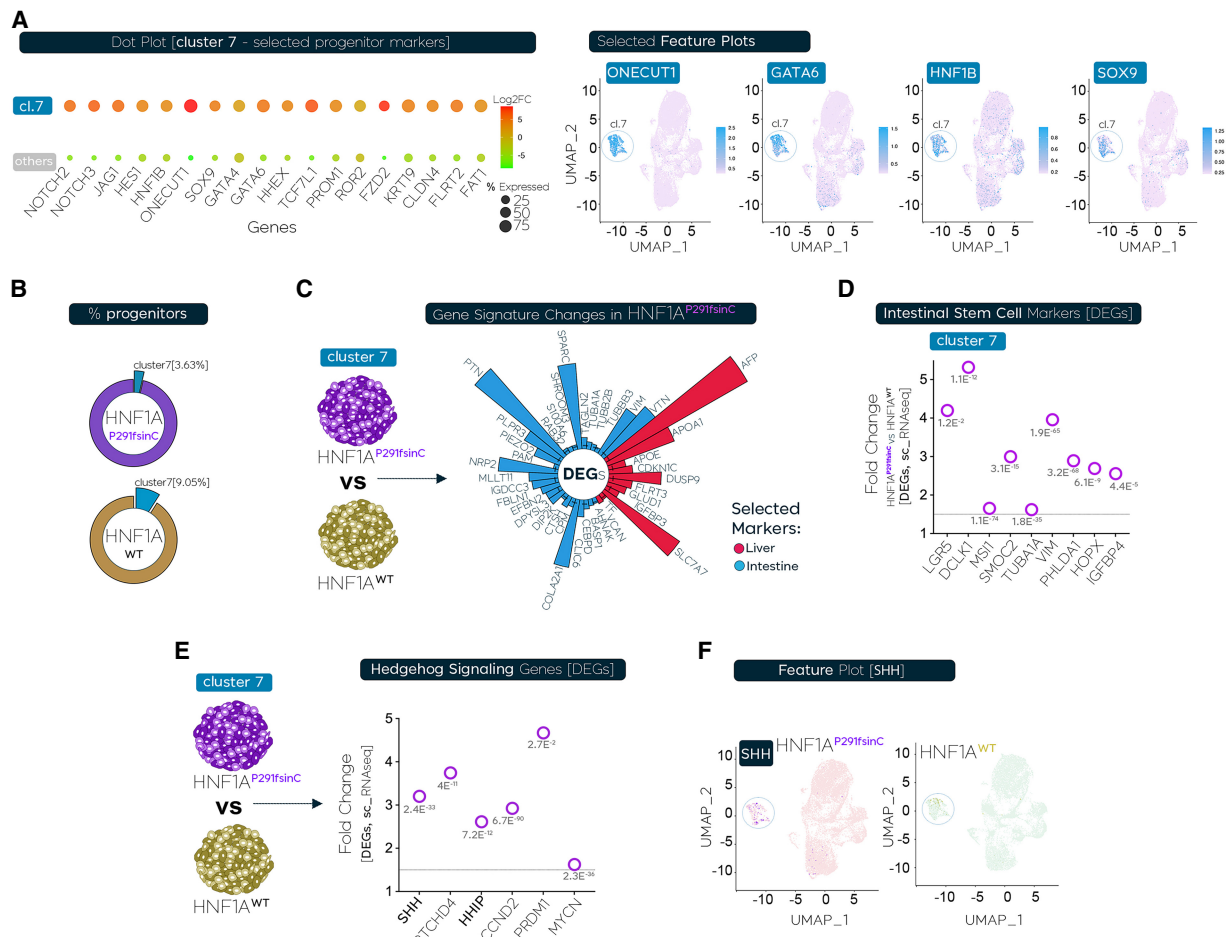


Figure 4. Decreased residual progenitors with an increased intestinal signature in *HNF1A*^{P291fsinC} SC islets. (A) Dot plot displaying selected progenitor marker expression in cluster 7 and feature plots for ONECUT1, GATA6, HNF1B, and SOX9 genes. (B) Cluster 7 representation (in percentages) in *HNF1A*^{P291fsinC} and *HNF1A*^{WT} cells. (C) Circle plot exhibiting the upregulation of intestinal and liver progenitor signatures in *HNF1A*^{P291fsinC} cluster 7 cells based on sc-RNA-seq DEG data sets ($P < 0.05$, logFC > 0.585). (D) Graph illustrating the significant upregulation of key intestinal markers in *HNF1A*^{P291fsinC} (sc-RNA-seq). (E) Graph illustrating the significant upregulation of Hedgehog signaling genes in *HNF1A*^{P291fsinC} (cluster 7; sc-RNA-seq). (F) Feature plot of SHH (in percentages) in *HNF1A*^{P291fsinC} and *HNF1A*^{WT} cells.

upregulated enterochromaffin marker between HNF1A^{P291fsinC} and the control HNF1A^{WT} was *CXCL14* (Supplemental Fig. S6F). Overall, these data suggest the preservation of a progenitor-like population in S7.

This population (cluster 7) is underrepresented in the HNF1A^{P291fsinC} condition (~2.5× less cells in the mutant) (Fig. 4B), and the comparison with the HNF1A^{WT} control revealed the upregulation of known intestinal and liver markers in the mutant HNF1A^{P291fsinC} cells (Fig. 4C), including major intestinal stem cell players such as Musashi-1 (*MSI1*) and *LGR5* among others (Fig. 4D).

Furthermore, a fraction of the cluster 7 cells expressed Hedgehog signaling pathway components (Supplemental Fig. S6G). In line with the observed intestinal signature, the HNF1A^{P291fsinC} cluster 7 cells displayed a regulatory pattern compatible with Hedgehog signaling activation (Fig. 4E), exhibiting significantly higher levels of Hedgehog-interacting protein (*HHIP*; a benchmark marker for Hedgehog signaling activity) and Sonic Hedgehog (*SHH*) (Fig. 4F) among others.

Hnf1a mutation in the glucagon lineage leads to increased intestinal length and villus area in vivo in mice

In vitro differentiation protocols rely on the progressive and constant reinforcement of the differentiation program, thus persistently imposing the selection of the desired cell fate. This approach rescues cells with low developmental robustness, which otherwise will adopt a different cell fate in vivo. Consequently, to further differentiate the impact of *HNF1A* mutation, we investigated its effects in a previously generated HNF1A^{fl/exon4} mouse model (floxed exon 4 spanning the human P291fsinC mutation site) (Mathisen et al. 2024), where the *Cre*-mediated mutation is expected to generate a truncated but stable *Hnf1a* form, similar to its human counterpart.

We thus employed a *Pdx1Cre*, *Hnf1a^{fl/exon4}* (*Pdx1-Hnf1a*) transgenic setup where the *Hnf1a* mutation is introduced using a *Pdx1Cre* driver. Pancreas duodenal homeobox 1 (*Pdx1*) is a key transcription factor expressed in the posterior foregut endoderm, which is involved in the specification of both the duodenum (Offield et al. 1996) and pancreas (Jonsson et al. 1994). It is essential for repressing *Cdx2* in the pancreatic domain, preventing intestinal cell fate (Shih et al. 2015). In the intestine, *Pdx1* is most abundant in the proximal duodenum, with its levels decreasing toward the distal part of the intestine. Moreover, *Pdx1* is expressed by both the enterocytes and enteroendocrine cells, with its loss in enteroendocrine cells leading to significant reduction of key hormone-expressing populations, including GLP-1-secreting cells (L cells) (Chen et al. 2009). The heterozygous *Pdx1-Hnf1a* mice (*Pdx1*-driven *Cre*-induced *Hnf1a* mutation) were normoglycemic (Fig. 5A) but had a significantly increased intestine length compared with their control counterparts (Fig. 5B, $P=0.0055$), suggesting that the *Hnf1a* mutation in the murine pancreas/gut prompts intestinal elongation. Moreover, the morphology of the duodenum was altered with decreased villus area (Fig. 5C, $P=0.0007$), while vil-

lus length was unaffected (Fig. 5D). In contrast, in the *Ptf1aCre*, *Hnf1a^{fl/exon4}* (*Ptf1-Hnf1a*) transgenic mice (Fig. 5E), no intestinal elongation was observed (Fig. 5F). *Ptf1a* is a master regulator of pancreas development, directing progenitors toward a pancreas instead of an intestinal cell fate (Kawaguchi et al. 2002), suggesting that the observed intestinal elongation in the *Pdx1-Hnf1a* model is driven by the mutation within the intestinal tissue itself, independent of systemic factors from the pancreas.

To further identify the cell population responsible for the observed elongation, we subsequently introduced the *Hnf1a* mutation in the enteroendocrine/neuroendocrine lineage by employing a *Ngn3Cre*, *Hnf1a^{fl/exon4}* (*N-Hnf1a*) transgenic setup. In these mice, all *Ngn3*-expressing cells and their progeny express the mutated *Hnf1a* from the first moment of *Ngn3* expression during development. *Ngn3* is critical for adopting a secretory neuroendocrine cell fate in both the pancreas (Gradwohl et al. 2000) and intestine (Jenny et al. 2002). *Ngn3*-driven *Cre* activity was reported in all pancreatic endocrine cells (α [glucagon], β [insulin], δ [somatostatin], ϵ [ghrelin], and γ [PP] cells), endocrine portions of the stomach, small intestine enteroendocrine cells ($I_{[cholecystokinin]}$, $K_{[GIP]}$, $L_{[glucagon-like\ peptide\ 1,\ GLP-1]}$, $N_{[neurotensin]}$, and $S_{[secretin]}$ cells), and some nonendocrine intestinal cells (Schonhoff et al. 2004). While the heterozygous *Ngn3Cre*, *Hnf1a^{fl/exon4}* mice are normoglycemic, the homozygous mice are hyperglycemic (Fig. 5G, $P<0.0001$) and display a significant reduction in islet cell numbers (Fig. 5H, $P<0.0001$). Of note, similar to the human SC islet HNF1A^{P291fsinC} counterpart (see Fig. 3), we observed a significant β -cell decrease (Fig. 5I, left, $P<0.0001$) coupled with a significant increase in α -cell population (Fig. 5I, right, $P=0.0019$). Interestingly, like *Pdx1-Hnf1a*, *Ngn3Cre*, *Hnf1a^{fl/exon4}* also presented a significant increment in the intestine length (Fig. 5J, $P=0.0004$, $P<0.0001$). This was coupled with larger villus length (Fig. 5K, $P=0.0035$) and area (Fig. 5L, $P<0.0001$) as well as increased proliferation, as indicated by KI67⁺ staining (Fig. 5M, $P=0.0001$). Both heterozygous and homozygous mice exhibited similar intestine elongation, further indicating that glycemia does not play a role in the elongation process.

Based on the increased numbers of glucagon-expressing α cells observed in the islets of these mice and differentiated human SC islets, we further investigated whether the downstream glucagon/GLP-expressing lineage is driving intestine elongation. For this, we employed a *GluCre*, *Hnf1a^{fl/exon4}* (*Hnf1a* mutation restricted to the glucagon/GLP-expressing cells [*G-Hnf1a*]) transgenic setup. In this model, both heterozygous and homozygous mice are normoglycemic (Fig. 6A) and exhibit significant intestinal elongation (Fig. 6B, $P=0.0002$, $P=0.0005$), which is similar to what was observed in the *Ngn3Cre*, *Hnf1a^{fl/exon4}* mice (Fig. 5F), suggesting that glucagon/GLP cell-restricted mutation is sufficient to elicit a phenotype. Further confirming this hypothesis, no increase in intestine size was observed in a *RIPCre*, *Hnf1a^{fl/exon4}* model (*Hnf1a* mutation restricted to the insulin-expressing cells) (Supplemental Fig. S7A) even in older hyperglycemic animals (Supplemental Fig. S7B).

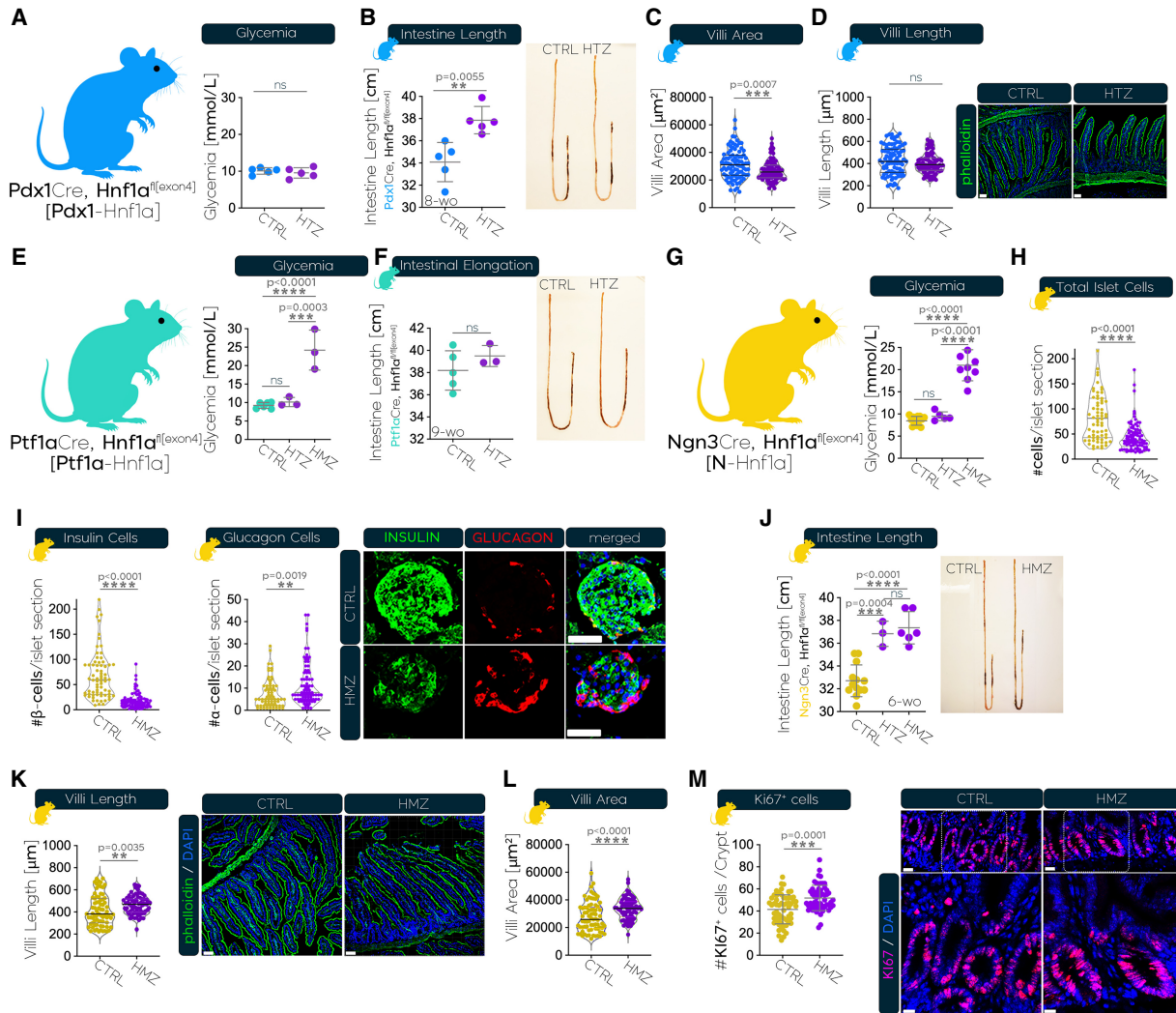


Figure 5. Significantly elongated intestines in *Pdx1-Hnf1a*, *Ptf1a-Hnf1a*, and *N-Hnf1a* mice. (A) Glycemia measurement in control (CTRL; *Pdx1Cre, Hnf1a*^{wt/wt}) and heterozygous (HTZ; *Pdx1Cre, Hnf1a*^{fl/wt}) *Pdx1-Hnf1a* mice (Welch's *t*-test). (B) Intestine length of CTRL and HTZ *Pdx1-Hnf1a* mice and representative pictures of intestines (Welch's *t*-test). (C) Graph displaying the changes in villus area between CTRL and HTZ *Pdx1-Hnf1a* mice (Mann-Whitney). (D) Graph displaying the changes in villus length between CTRL and HTZ *Pdx1-Hnf1a* mice (Mann-Whitney) and representative images of villi. (Green) Phalloidin, (blue) DAPI. Scale bar, 50 μm . (E) Glycemia measurement in control HTZ and HMZ *Ptf1a-Hnf1a* mice (one-way ANOVA). (F) Intestine length of CTRL (*Ptf1aCre, Hnf1a*^{wt/wt}) and HTZ *Ptf1a-Hnf1a* (*Ptf1aCre, Hnf1a*^{fl/wt}) mice and representative pictures of intestines (Welch's *t*-test). (G) Glycemia measurement in control (CTRL; *Ngn3Cre, Hnf1a*^{wt/wt}), heterozygous (HTZ—*Ngn3Cre, Hnf1a*^{fl/wt}), and homozygous (HMZ; *Ngn3Cre, Hnf1a*^{fl/fl}) *N-Hnf1a* mice (one-way ANOVA). (H) Graph illustrating the number of cells per islet section in CTRL and HMZ *N-Hnf1a* mice (Mann-Whitney). (I) Quantification of INS⁺ (left graph) and GCG⁺ (right graph) cells per islet section in CTRL and HMZ *N-Hnf1a* mice and representative islets stained for C-peptide (green), glucagon (red), and DAPI (blue; Mann-Whitney). Scale bar, 50 μm . (J) Graph displaying the intestine length measurements of CTRL, HTZ, and HMZ *N-Hnf1a* mice and representative pictures of intestines (one-way ANOVA). (K) Graph displaying the changes in villus length between CTRL and HMZ *N-Hnf1a* mice (Mann-Whitney) and representative images of villi. (Green) Phalloidin, (blue) DAPI. Scale bar, 50 μm . (L) Graph displaying the changes in villus area between CTRL and HMZ *N-Hnf1a* mice (Mann-Whitney). (M) Quantification of Ki67⁺ cells in the crypt of CTRL and HMZ *N-Hnf1a* mice (Mann-Whitney) and representative images of Ki67 staining. (Red) Ki67, (blue) DAPI. Scale bar, 20 μm ; inset scale bar, 10 μm .

In addition, as in the *N-Hnf1a*, the immunofluorescence (IF) of the *GluCre, Hnf1a*^{fl/exon4} small intestine revealed an expansion of Ki67⁺ cells in the crypt (Fig. 6C, $P < 0.0001$, $P < 0.0001$) in both heterozygous and homozygous mice, suggestive of increased proliferation. This was coupled with an enlarged villus area (Fig. 6D, $P < 0.0001$, $P < 0.0001$) and a steep increase of the irreversible traced

YFP⁺ cells in the *G-Hnf1a* mouse crypts (Fig. 6E; Supplemental Fig. S7C). In the genetically traced *G-Hnf1a* mice (*GluCre, Hnf1a*^{fl/exon4}, R26YFP), cells expressing glucagon (e.g., the Gcg/Glp lineage) are irreversibly labeled with YFP due to the Cre-mediated removal of the stop codon in the R26YFP transgene prompting YFP expression (see scheme in Fig. 6E). The specificity of the

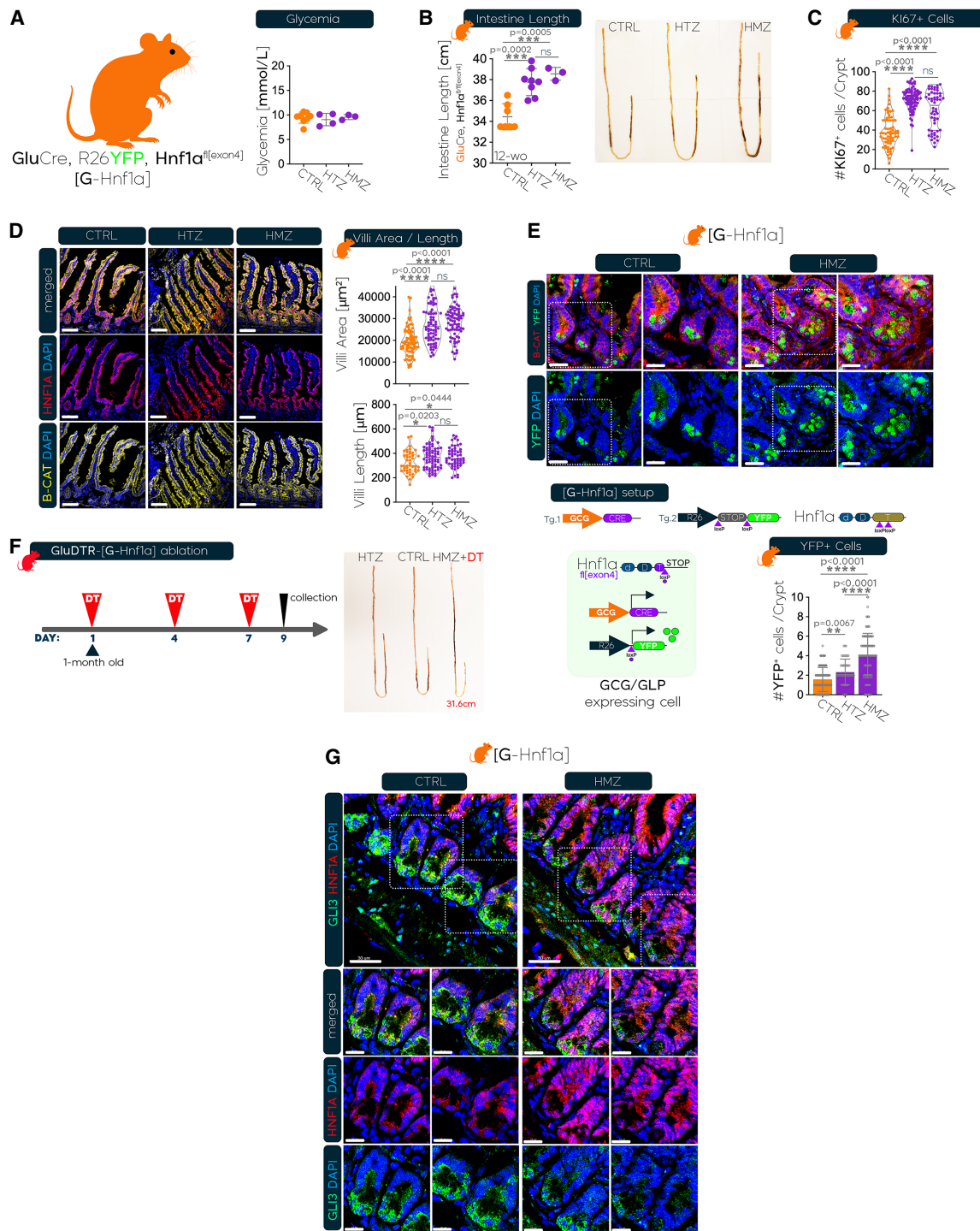


Figure 6. Significantly elongated intestine and altered villus and crypt architecture in *G-Hnf1a* mice. (A) Glycemia measurement in control (CTRL; *GluCre*, *Hnf1a*^{wt/wt}), heterozygous (HTZ; *GluCre*, *Hnf1a*^{fl/wt}), and homozygous (HMZ; *GluCre*, *Hnf1a*^{fl/fl}) *G-Hnf1a* mice (one-way ANOVA). (B) Intestine length of CTRL, HTZ, and HMZ *G-Hnf1a* mice and representative pictures of intestines (one-way ANOVA). (C) Quantification of Ki67⁺ cells in the crypts of CTRL and HMZ *G-Hnf1a* mice (one-way ANOVA). (D) Graph displaying the changes in villus area and length between CTRL, HTZ, and HMZ *G-Hnf1a* mice (one-way ANOVA) and representative immunofluorescence pictures of villi and crypts stained for HNF1A (red), β-catenin (yellow), and DAPI (blue). Scale bar, 75 μm. (E) Representative YFP (green) and β-catenin (red) staining indicating increased YFP-traced cells in HMZ *G-Hnf1a* mice compared with controls and schematic transgenic setup of the lineage tracing strategy and quantification of YFP⁺ cells per villus (one-way ANOVA). (Blue) DAPI. Scale bar, 30 μm; magnification scale bar, 20 μm. (F) Experimental design used for GCG/GLP cell ablation using DT in the *GcgDTR-G-Hnf1a* mice and representative pictures of intestines. (G) Representative *Hnf1a* (red) and *Gli3* (green) staining indicating changes in their expression domains in HMZ *G-Hnf1a* mice as compared with control. (Blue) DAPI. Scale bar, 30 μm; magnification scale bar, 15 μm.

YFP labeling for the GCG/GLP cells was controlled using the GluDTR ablation system (Supplemental Fig. S7D, transgenes scheme), which allows the specific ablation of all glucagon-expressing cells, including the ones in the intestine (Thorel et al. 2011; Perez-Frances et al. 2024). Briefly, in this setup, the diphtheria toxin receptor (DTR) is expressed on the surface of the glucagon/GLP-expressing cells, thus rendering them sensitive to the diphtheria toxin (DT) action. Following DT administration, the DTR-expressing cells die rapidly by apoptosis (Supplemental Fig. S7D, cartoon). DT exposure in *G-Hnf1a* mice prompted the nearly total disappearance of all YFP⁺ cells in the gut (a drop to 0.025 YFP cells; ~98% ablation efficiency) (Supplemental Fig. S7D), validating the YFP staining specificity for the GCG/GLP line. Of note, unspecific fluorescently labeled structures were observed in the villus laminae but not in the villus lining or the crypt and therefore were not considered in the counting. Interestingly, repetitive (every 3 days) DT administration (Fig. 6F) led to unexpectedly high mortality in the heterozygous and homozygous *G-Hnf1a* mice but not in their control counterparts (*GluCre*, *R26YFP*). In this setup, four mice died before the end of the experiment, while the surviving one presented a drastic shortening of the intestine (Fig. 6F), reiterating the role of *Hnf1a*-deficient GCG/GLP-expressing cells in regulating gut expansion.

Taken together, these data advocate for a proliferation-driven population expansion. Moreover, a striking difference in pattern was observed for *Hnf1a*⁺ cells, with control animals presenting few to no *Hnf1a*⁺ nuclei in crypts, while the crypts of mutant animals were characterized by a broad *Hnf1a*⁺ nuclear expression excluded in only a few cells at the base (Fig. 6G; Supplemental Fig. S7E). Interestingly, the *Gli3* staining in the crypts displayed an opposite expression pattern, with the broad cytoplasmic staining observed in the control crypts being restricted to only a few basal cells in the mutants (Fig. 6G). These data point toward a complex cross-talk between *Hnf1a* and *Gli3* in the crypts, leading to changes in proliferation rate, villus area, and intestine length in *Hnf1a* mutant mice. Furthermore, the mutation of *Hnf1a* in the glucagon/GLP lineage is sufficient to induce these phenotypes.

Hnf1a mutation in the GLP/GCG lineage prompts molecular changes suggestive of conversion of L cells toward an enterocyte fate

To further characterize the relation between the GCG/GLP lineage *Hnf1a* deficiency and intestine length, we performed RNA-seq on a crypt- and villus-enriched intestinal fraction from HTZ *G-Hnf1a* mice and their control counterparts. The pathway analysis revealed signaling involved in transport, extracellular matrix, digestion, and absorption in the top pathways characterizing the differential transcriptional landscape (Fig. 7A; Supplemental Data S5). Of note, the top second signaling was the intestinal absorption pathway being inferred with an activated regulatory pattern (Z-score = 2.000). Moreover, in the top predicted upstream regulators, *Ets1* was demonstrating statistically significant upregulation in the HTZ *G-*

Hnf1a (Fig. 7B). Previous studies indicated that *Ets1* is a proto-oncogene (Dittmer 2003) promoting cell proliferation (Cao et al. 2019) and a direct target of *Cdx2* (Boyd et al. 2010). ETS is strongly associated with malignancy in intestinal tumors (Wai et al. 2006; Peng et al. 2009), inducing epithelial-to-mesenchymal transition (Bellocin et al. 2006). Accordingly, processes related to proliferation, such as proliferation of progenitor cells, were predicted as activated in the top biological functions (Fig. 7C).

Interestingly, the top deregulated gut cell type signature was defined by the upregulation of key enterocyte markers, including *Si*, *Maf*, *Fabp1*, and *Krt20* among others (Fig. 7D). Along with the upregulation of the enterocyte genes, we identified the robust upregulation of *Bmp4* (8.9×) (Fig. 7E), a key signal restricting intestinal stemness (Qi et al. 2017) and epithelium hyperproliferation (Clevers 2013) that is also essential for enterocyte differentiation (Beumer et al. 2022; Berková et al. 2023). Of note, previous studies showed that following BMP treatment, L cells (GLP1-secreting cells in the intestine) reduced their *Glp-1* (*Gcg*) expression and presented secretory and molecular signature characteristics very similar to those of K cells (GIP-secreting cells) (Beumer et al. 2018).

Based on these previous findings, we investigated whether a fraction of the *Hnf1a*-deficient L cells in the *G-Hnf1a* mice attempted to adopt an enterocyte identity. To test this hypothesis, we assessed the YFP⁺ cells for the presence of the enterocyte marker sucrose-isomaltase (*Si*), which was observed to be upregulated in the RNA-seq assay (Fig. 7D). As the YFP labeling in the *G-Hnf1a* model is an effect of glucagon expression during development, the induction of enterocyte markers will indicate an acquisition of enterocyte features. The immunofluorescence indicated the presence of scattered but frequent YFP⁺*Si*⁺ cells (Fig. 7F), further suggesting a potential destabilization of L-cell identity in the HMZ *G-Hnf1a* model.

Discussion

A seminal body of work demonstrated that proper Hedgehog signaling inhibition is essential for pancreatic specification during development in vertebrates (Kim and Hebrok 2001; Hebrok 2003) from fish (Roy et al. 2001) to chicks (Hebrok et al. 1998), mice (Apelqvist et al. 1997; Hebrok et al. 2000; Kim et al. 2000; Ramalho-Santos et al. 2000), and humans (Klieser et al. 2016). Alterations in the normal activity pattern significantly interfere with pancreas development (Kim and Melton 1998), with ectopic *Shh* expression in mice leading to intestinal mesenchyme instead of pancreatic mesoderm (Apelqvist et al. 1997). To the best of our knowledge, we show here for the first time that impeded GLI3 processing due to primary cilium protein deregulation impacts pancreatic cell fate specification. The essential role of cilium proteins in GLI3 processing and Hedgehog pathway functionality was recently highlighted in other major organ systems by a series of studies examining mutations of key genes involved in cilium assembly (Rana et al. 2004; Badano et al. 2006; He et al. 2014; Caparrós-Martin et al. 2015; Taylor et al.

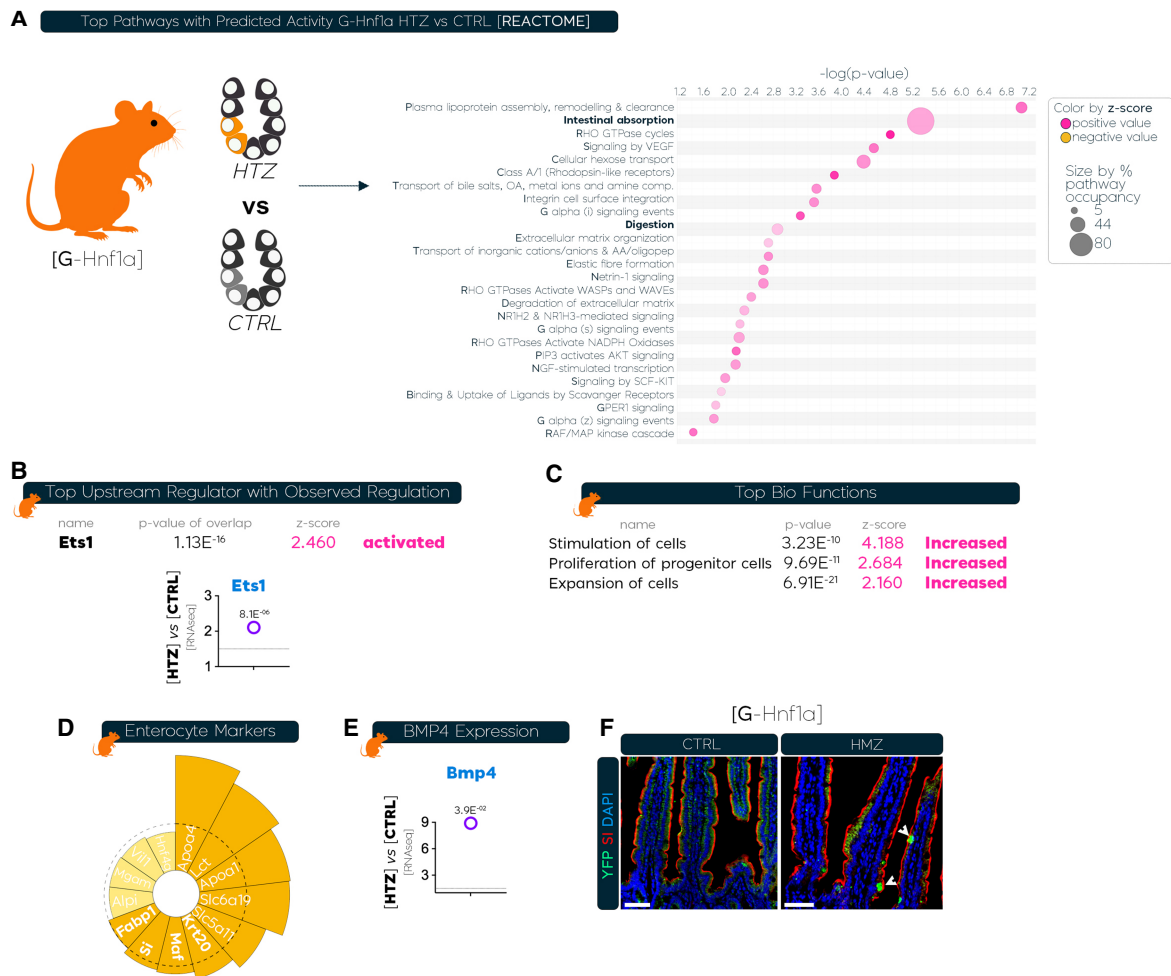


Figure 7. The transcriptional landscape and cell fate changes in *G-Hnf1a* mice. (A) The top non-immunology-related signaling pathways (reactome) with predicted activity characterizing the differential transcriptional landscape between heterozygous (HTZ; *GluCre*, *Hnf1a*^{fl/wt}) and control (CTRL; *GluCre*, *Hnf1a*^{wt/wt}) mice. (B) The top predicted upstream regulators for the analyzed transcriptional landscape and the observed upregulation of *ETS1* in HTZ *G-Hnf1a*. (C) The top developmental processes characterizing the analyzed transcriptional landscape. (D) Circle plots of key enterocyte markers deregulated in HTZ *G-Hnf1a*. (E) Graph depicting the observed upregulation of *BMP4* in HTZ *G-Hnf1a*. (F) Representative YFP (green) and Si (red) staining in HMZ *G-Hnf1a* mice. (Blue) DAPI. Scale bar, 50 μ m. (Arrowheads) YFP*Si⁺ cells in the villi.

2015). Besides their role in activating the GLI proteins, the cilia are also essential for GLI3FL processing to its GLI3R repressor form (Dorn et al. 2012; Caparrós-Martin et al. 2013; Schembs et al. 2022). The primary cilium is a microtubule-based structure that functions as a signaling hub for several signaling pathways (Berbari et al. 2009), with its formation and function requiring the intraflagellar transport (IFT). The IFT, through its IFT-A and IFT-B complexes, provides bidirectional traffic in the cilium. Previous studies disrupting the anterograde cilium transport in the pancreas by *Cre*-mediated mutation of the *Kif3a* gene elegantly demonstrated that cilia regulate Hedgehog signaling in part by curbing Gli3 protein levels (Cervantes et al. 2010). In humans, patients carrying pathogenic *HNF1A* mutations exhibit smaller pancreas volumes than nondiabetic controls, with the degrees of pancreatic atrophy being correlated with the duration of diabetes and

exocrine dysfunction (Vesterhus et al. 2008a). Thus, the pancreas volume reduction may be a result of the altered activity pattern in the pancreas, though we cannot rule out that reduced insulinotropic effects can also play a role in the pathogenesis.

Here we newly identified the deregulation of components belonging to both the retrograde and anterograde transport in the *HNF1A*^{P291fsinC} cells, such as downregulation of *DYNC2LI1* (IFT-A) or *WDR35* (IFT-A). Human autosomal recessive mutations in these two genes were demonstrated to cause defects in cilium assembly, retrograde transport, and Hedgehog signaling through compromised GLI3 processing (Caparrós-Martin et al. 2015; Taylor et al. 2015) during skeletogenesis. *DYNC2LI1* is one of the main components of the IFT-A dynein 2 complex, and its decreased activity by mutations or RNAi leads to increased cilium length variations and

accumulation of the IFT-B complex proteins (Taylor et al. 2015). Of note, in these contexts, SMO displayed localization errors consistent with ectopic Hedgehog signaling activation in the absence of Hedgehog ligand stimulation. Moreover, similar to our observation at the posterior foregut stage in HNF1A^{P291fsinC} cells, GLI3 processing was affected, exhibiting a decreased GLI3FL to GLI3R ratio, suggesting impaired proteolytic activity. Similarly, the WDR35 mutation leads to inhibition of GLI3FL processing, with a decline in GLI3R levels (Caparrós-Martin et al. 2015). Thus, based on these observations, the decrease of IFT-A components in HNF1A^{P291fsinC} cells coupled with the mislocalization of GLI3 protein and an ~2× decrease in GLI3R protein levels points toward an incomplete inhibition of the Hedgehog signaling caused by the HNF1A mutation.

We further show that the lack of stringent Hedgehog signaling inhibition and decreased GLI3R levels in HNF1A^{P291fsinC} cells prompt the loss of HNF1A upregulation, which normally occurs in the HNF1A^{WT} cells (Fig. 8A). This unexpectedly leads to a similar dysregulation pattern of the *HHEX* gene, an essential selector gene controlling lineage commitment in the gut (Keng et al. 2000; Martinez Barbera et al. 2000) and a type 2 diabetes risk gene (Sladek et al. 2007). *HHEX*-null mouse embryos fail to specify the ventral pancreas while retaining liver specification (Bort et al. 2004). Although *HHEX* and HNF1A are direct interaction partners (Tanaka et al. 2005), the binding of HNF1A to the *HHEX* promoter was not demonstrated. However, *HHEX* was shown to be negatively regulated by Hedgehog signaling during pancreatic endoderm formation (Dumasia et al. 2021), suggesting a scenario in which HNF1A impacts its levels indirectly by preventing proper Hedgehog signaling inhibition through defective GLI3 processing to GLI3R. This model can explain the upregulation observed exclusively in the HNF1A^{WT} control line, characterized by a robust Hedgehog signaling inhibition. It should also be stated that previous studies indicated that the HNF1A^{P291fsinC} mutation impaired HNF1B-dependent transcription, and thus it is possible that some of the observed defects could be elicited via

this mechanism at the stages where the two factors' expressions coincide (Cujba et al. 2022).

More recently, a comprehensive study demonstrated that *HHEX* acts as a gatekeeper in differentiating hiPSCs, being intrinsically required for both pancreatic progenitor specification and the prevention of the acquisition of liver and duodenum cell fates (Yang et al. 2022). The *HHEX*-null cells presented decreased pancreatic progenitor markers while exhibiting *CDX2* expression, a key regulator of duodenal/intestinal fate. In line with these previous observations, we report here for the first time the failure to upregulate *HHEX* in HNF1A^{P291fsinC} cells, which leads to the undesired diversification of the specification programs, as illustrated by the increase in both the liver and duodenum/intestine signatures. In parallel, the HNF1A^{P291fsinC} cells also fail to downregulate *CDX2*, exhibiting a significantly higher expression level than control cells at this stage. Of note, a regulatory loop between *CDX2* and HNF1A was reported previously in the gut epithelium (Silberg et al. 2002), with HNF1A direct binding to the *CDX2* promoter being reported previously (D'Angelo et al. 2010; Verzi et al. 2010; Song et al. 2023).

The presence of a liver signature in HNF1A^{P291fsinC} was reported previously (Low et al. 2021), yet the use of the BMP inhibitor LDN-193189, a key component of the differentiation cocktail (between D7 and D20; i.e., S3–S5), was demonstrated to potentially inhibit the liver program (Yang et al. 2022). Indeed, the liver signature in HNF1A^{P291fsinC} cells was drastically reduced by the end of S4, though *AFP* expression was still higher in the mutant cells, suggesting the maintenance of a residual liver signature. As with *CDX2*, previous studies demonstrated direct HNF1A binding to the *AFP* promoter (McVey et al. 1993; Nagata-Tsubouchi et al. 2005; Yu et al. 2008; Li et al. 2019). It is interesting to note that germline *HNF1A* mutations in humans confer an increased risk of developing hepatocellular neoplasms in the liver ranging from solitary lesions to adenomatosis by a classic tumor suppressor two-hit mechanism (Jeannot et al. 2010; Mingo Alemany et al. 2014; Fu et al. 2020).

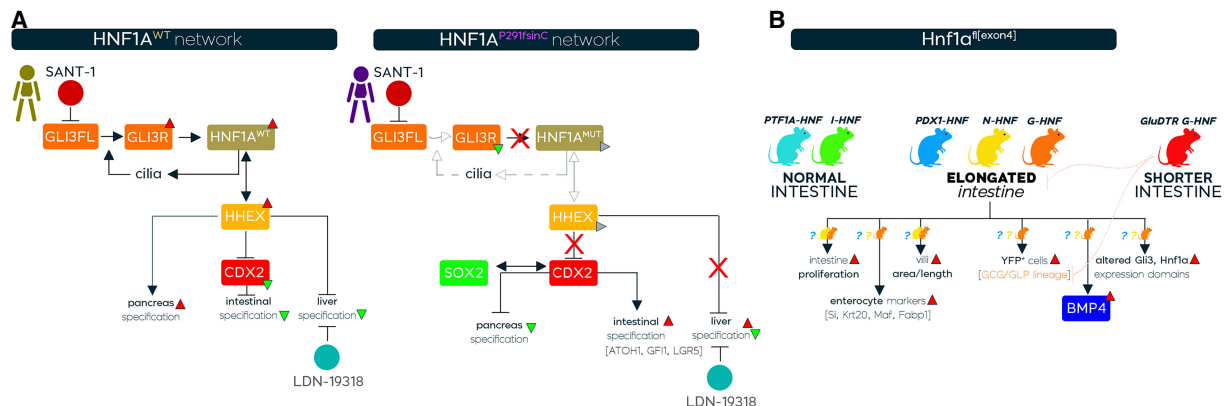


Figure 8. Proposed molecular model. (A) Schematic representation of the HNF1A^{P291fsinC} and HNF1A^{WT} putative regulatory networks. (B) Schematic representation of Hnf1a deficiency effects in the intestine according to the different murine setups analyzed.

Along with the increased *CDX2* levels, we identified a striking, previously unreported, downregulation of *SOX2* (~70×) in the *HNF1A*^{P291fsinC} cells compared with the control counterpart. Previous studies demonstrated the essential role of the *SOX2* and *CDX2* domains in establishing the gastrointestinal junction boundary (Silberg et al. 2002; Gao et al. 2009; Coskun et al. 2011). The loss of *CDX2* will expand the *SOX2* distal boundary, causing an early anteriorization event with the activation of the foregut program in the anterior intestine (Gao et al. 2009), while the ectopic activation of *CDX2* leads to intestinal differentiation of the gastric mucosa (Silberg et al. 2002). Thus, the increased *CDX2* levels combined with decreased *SOX2* suggest the emergence of an intestinal rather than gastric signature in the *HNF1A*^{P291fsinC} cells. To our knowledge, we are the first to report a posteriorization effect of the *P291fsinC* mutation with potential clinical implications for *HNF1A*-MODY patients.

Despite the relaxation of the specification program selections, the persistent reinforcement of the guided differentiation sequence led to the formation of SC islets in the *HNF1A*^{P291fsinC} cells. The capacity of the *HNF1A*^{P291fsinC} cells to give rise to all islet endocrine cell types was also elegantly demonstrated by others (Cardenas-Diaz et al. 2019; Legøy et al. 2020; Low et al. 2021; Cuijba et al. 2022; González et al. 2022), indicating their high robustness in vitro. Moreover, mouse models of *HNF1A* loss/mutation also indicated the formation of the pancreas and islets in vivo (Lee et al. 1998; Boj et al. 2001; Párizas et al. 2001; Ferrer 2002; Servitja et al. 2009; Mathisen et al. 2024). However, the islet architecture is changed, with significantly lower numbers of insulin-expressing cells being reported both in vitro (Cardenas-Diaz et al. 2019; González et al. 2022) and in vivo (Lee et al. 1998; Pontoglio et al. 1998). Moreover, we report here an increase in glucagon-expressing α -cell numbers and glucagon expression in vitro in SC islets and in vivo in mouse islets bearing the *HNF1A* mutation. Of note, the in vitro increase in glucagon expression was also reported in recent studies using different null *HNF1A* cell lines (Cardenas-Diaz et al. 2019; González et al. 2022). In contrast, a recent study on a *HNF1A* mutation located in the DNA-binding domain (*HNF1A*^{+R272C}) did not uncover a change in the α -cell or β -cell fraction (Hermann et al. 2023). These apparently conflicting findings can be explained by distinct mechanisms of action between the mutations and provide precious insights into *HNF1A* regulation. *R272C* is a missense mutation in the DNA-binding domain leading to a full-length nonfunctional protein acting exclusively in a dominant negative fashion. In contrast, *P291fsinC* is a frameshift mutation in the transactivation domain leading to a truncated protein, which can exert a dominant negative effect but also can cause haploinsufficiency due to nonsense-mediated decay. The fact that the bias toward α -cell fate was also reported in the null *HNF1A* mutations suggests that haploinsufficiency, rather than a dominant negative mechanism, is responsible for the changes in the islet population specification. This hypothesis is also supported by the observations by Bjørkhaug et al. (2003), which did not find evidence for a dominant nega-

tive effect in the variants investigated, including the hot-spot *P291fsinC* mutation.

However, the exact mechanism behind the robust α -cell increase was not identified. Previous research into glucagon gene expression indicated that the *CDX2* gene is involved in its transcriptional regulation, with its loss leading to decreased glucagon expression (Zhao et al. 2005). *CDX2* promotes glucagon (*GCG*) gene expression by directly binding to the G1 element of the *GCG* promoter (Jin and Drucker 1996; Laser et al. 1996) and by forming a transcriptionally active complex with *PAX6*, which significantly increases its activity (Zhao et al. 2005). Although *PAX6* levels were similar in *HNF1A*^{P291fsinC} and *HNF1A*^{WT} cells, the increased *CDX2* expression in the *HNF1A*^{P291fsinC} suggests a likely involvement of *CDX2* in the observed increase in overall glucagon expression, thus providing for the first time a mechanistic explanation for this phenomenon.

To our knowledge, we are the first to report an increase of glucagon cells in vivo in *Hnf1a* transgenic mice, indicating that the similar in vitro observations are not an artifact of the differentiation protocol. Of importance, the enrichment in glucagon cells also occurred in mice where the *Hnf1a* mutation was restricted to the neuroendocrine lineage (*N-Hnf1a*), indicating that the tilt toward the glucagon cell fate can also occur later; that is, after pancreatic progenitor specification. Although there are no data indicating chronically elevated glucagon in patients with a pathogenic *HNF1A* mutation, it is important to note that increased glucagon could augment the effect of reduced insulin secretion. This is particularly important, as early chronic hyperglycemia and pronounced, slowly declining postprandial glucose spikes are recognized hallmarks of *HNF1A*-MODY (Byrne et al. 1996). However, the translatability to human condition should be carefully assessed, and it should be stated that the mouse model used here is not a true disease model of *HNF1A*-MODY but a model of cell-specific *Hnf1* deficiency.

Moreover, we report a significant elongation of the small intestine in the *Pdx1-Hnf1a*, *N-Hnf1a*, and *G-Hnf1a* transgenic lines (Fig. 8B). Of note, the intestinal elongation and changes in villus architecture were also observed previously in a full-body *Hnf1a*-null allele mouse model (Lussier et al. 2010); however, the developmental mechanism was unclear. Interestingly, in the *Pdx-Hnf1a* model, we observed a decreased villus area, while the enteroendocrine-specific lines (*N-Hnf1a* and *G-Hnf1a*) exhibited an increased villus area. This difference might be explained by the different cell populations expressing the factors, with *Pdx1* being expressed in both enterocyte and enteroendocrine cells (Chen et al. 2009), whereas *Ngn3* and *Gcg* are typically expressed just by enteroendocrine cells. Thus, the effect of *Hnf1a* deficiency on enterocytes could contribute to the decrease in villus size in the *Pdx-Hnf1a* model.

Here, by using diverse lineage tracings and Cre lines, we were the first to pinpoint the *Gcg*/*Glp1* cell population (*G-Hnf1a*) as a culprit for the observed phenotypes caused by *Hnf1a* mutation, with the mice exhibiting intestinal elongation similar to that of *Pdx1-Hnf1a* and *N-Hnf1a* mice.

A large number of *Hnf1a*⁺ cells ectopically emerged at the base of the villi and inside crypts in the *G-Hnf1a*, changing the localization and expression domain of *Gli3*, which was suggestive of a disruption of the *Hnf1a*/*Gli3* cross-talk. Interestingly, a recent study demonstrated ciliary Hedgehog signaling involvement in intestine elongation, with its attenuation or activity disruption leading to shortened small intestines (Yang et al. 2021). Therefore, it is tempting to speculate that the incomplete inhibition of the Hedgehog signaling observed in the posterior gut HNF1A^{P291fsinC} cells (due to decreased GLI3 processing by IFT-A proteins) also occurs at ectopic locations in the *G-Hnf1a* intestinal crypts, causing changes in the signaling landscape that lead to intestinal elongation. Supporting this hypothesis, we observed a robust increase in BMP4 signaling, a factor known to promote and stabilize enterocyte fate, coupled with an increase in enterocyte markers. Furthermore, healthy L-cell identity is destabilized by BMP treatment, with the cells adopting a K-cell profile, a phenomenon reminiscent of transdifferentiation. This, coupled with an increase in proliferation and YFP-labeled cells in crypts, suggests a model in which supernumerary *Hnf1a*-deficient L cells migrate apically and adopt an enterocyte identity when exposed to BMP4 in the villi, thus leading to increased enterocyte numbers, which in time contribute to elongated villus and gut size. However, properly proving such a scenario will require extensive in-depth analysis.

Patients carrying mutations in HNF1A exhibit increased excretion of fat in the feces (Vesterhus et al. 2008b). This exocrine dysfunction is likely not due to reduced pancreatic volume, which these patients also have. Thus, a link to the gut finding that we report here cannot be excluded. So far, there are no published imaging studies or biopsies investigating intestinal length or histopathology (crypt depth and villus height) in HNF1A-MODY patients. Our findings indicate that a more careful investigation of patients, including MRI and biopsies, may be warranted to further explore the nature of the gut phenotype. Along the same lines, a recent study demonstrated that a specific subgroup of neuroendocrine carcinoma (e.g., the gastric carcinoma) is defined by a HNF1A–HNF4A network (Wang et al. 2024). Interestingly, these tumors are also characterized by increased *GCG* expression. In light of these results and ours, it will be important to study the occurrence of gut carcinoids and gastroenteropancreatic (GEP) carcinoids in HNF1A-MODY patients.

Materials and methods

Mice

Animal work was approved and performed according to the regulations of the Norwegian Animal Research Authority (authorization nos. FOTS 10785, 12105, 19800, 20547, 25526, 25531, 30348, 30350, and 30353) and in accordance with European Union (EU) Directive 2010/63/EU. All mice were fed ad libitum with standard diet

RM1A (Special Diets Services) or V1534-703 mouse maintenance feed (ssniff-Spezialdiäten GmbH), with free access to water. Mice were housed in groups of two to five in individual ventilated cages (IVC systems) in a 12 h light/dark cycle at 22°C.

Multiple transgenic mouse strains were used with C57BL6/J mice (mixed background) and carrying the following transgenes: *Pdx1-cre* [B6.FVB-Tg(Pdx1-cre)6Tuv/J; RRID: IMSR_JAX:014647], *Ptf1a-cre* [Ptf1atm1(cre)Hnak/Rsch]; RRID: IMSR_JAX:023329], *Ngn3-cre* [Tg(Neurog3-cre)C1Able/J; RRID: IMSR_JAX:005667], *Glu-DTR*; *Glu-cre* [Tg(Gcg-cre)1Herr], *RIP-cre* [Tg(Ins2-cre)-23Herr], *R26-YFP* [B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP)Cos}/J; RRID: IMSR_JAX:006148], and *Hnf1a*^{fllox/fllox} (exon 4 KO; generated by us [Mathisen et al. 2024]). Upon Cre-recombination, exon4 of *Hnf1a* was deleted, removing the IPR001356 homeobox domain and producing a nonfunctional downstream sequence (Mathisen et al. 2024). Experimental mice were identified by genotyping using the following primers for the *Hnf1a*^{fllox} allele: 5'-AACCACCC TCTCTCCAGTAAG-3' (forward) and 5'-GTGTGTGT AACCGGAGTAGAAG-3' (reverse).

Cell source and maintenance

Commercially available human induced pluripotent stem cells (hiPSCs) generated from PGP1 donor skin fibroblasts (Coriell GM23338) by retroviral reprogramming were used to generate the HNF1A^{P291fsinC} heterozygous knock-in cell clones by Synthego by introducing a cytosine (CCA → CCCA) in the polycytidine tract of codon 291 (p291fsinsC) of HNF1A, which led to a frameshift mutation and premature stop codon. Clone H3 was used for most of the experiments, while replication experiments were performed using clone D9 (use of D9 clone is specifically mentioned in the figures and legends). Additionally, patient-derived iPSC line 5-B (described by Bjorlykke et al. 2019) that carries a HNF1A P291fsinsC mutation was used for replication experiments (HNF1A-MODY patient-derived cells). This cell line was obtained by Sendai reprogramming of skin fibroblasts by Takara Bio, Inc., using a CytoTune-iPS 2.0 Sendai reprogramming kit (Life Technologies A16517). The patient-derived iPSC work was approved by the Regional Committee of Medical and Health Research Ethics (REK 2010/2295). The patient gave written informed consent.

All iPSC lines were maintained in mTeSR Plus cGMP-stabilized, feeder-free maintenance medium (Stem Cell Technologies 100-0276), as described previously (Unger et al. 2024). For passaging, hiPSC colonies were detached after treatment with Gentle cell dissociation reagent (Stem Cell Technologies 100-0485). Prior to differentiation induction, the hiPSC cultures were confirmed to be mycoplasma-free using the MycoAlert mycoplasma detection kit (Lonza LT07-418).

In vitro differentiation

For all the differentiation sequences described for this study, with the exception of Supplemental Figure S1E,

the hiPSCs were differentiated toward stem cell islets following a previously published stepwise protocol (Barsby et al. 2022), starting with 2 million cells/well in 6 well plates or 15 million cells/10 cm cell culture dish precoated with Geltrex LDEV-free reduced growth factor (Gibco A1413202). The protocol was followed until day 44 of the differentiation. Cells were collected along the differentiation time line at specified time points (days).

For Supplemental Figure S1E, the hiPSCs were differentiated using a different differentiation protocol developed by Rezanian et al. (2014) and performed as described by Ghila et al. (2020).

GLI modulation

To modulate the GLI code, SANT-1 (Sigma-Aldrich S4572) was replaced with GANT-61 (Stem Cell Technologies 73692) at a consistent concentration of 10 μ M in all protocol steps where SANT-1 was added (differentiation stages 3–5 and days 7–16).

RT-qPCR

RNA was extracted from lysed cells using the RNeasy mini kit (Qiagen 74104) following the manufacturer's protocol. The RNA was eluted with 30 μ L of nuclease-free water. Gene expressions were determined using QuantiNova SYBR Green RT-PCR kit (Qiagen 208352) and QuantiTect primer assays (Supplemental Table S1).

Glucose-stimulated insulin release assay

All incubation steps were conducted in a humidified incubator at 37°C and 5% CO₂.

Twenty-four hours prior to the glucose-stimulated insulin secretion (GSIS) test, approximately one-third of the SC islets were transferred to new ultralow-attachment (ULA) 6 well plates containing treatment media devoid of ITS-X to eliminate exogenous insulin. Next, 1 mL of media was collected, and the SC islets were transferred to a 12 well plate pretreated with antiadhesion solution (Stem Cell Technologies) and washed twice with KREBS buffer (129 mM NaCl, 5 mM NaHCO₃, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 0.3% BSA at pH 7.4). Initially, the SC islets were primed in KREBS buffer containing 1.67 mM glucose for 30 min. Following priming, the SC islets were incubated in 1.67 mM fresh glucose KREBS buffer for 90 min, after which the media was collected for analysis. Next, the SC islets were exposed to high-glucose conditions using 20 mM glucose KREBS buffer for 90 min, and afterward, the media was collected. The SC islets were then washed once with 1.67 mM glucose solution before being returned to basal conditions with 1.67 mM glucose KREBS buffer for 60 min followed by media collection. In the final stimulation step, the SC islets were treated with 30 mM KCl KREBS buffer for 60 min to induce maximum insulin release, and the media was collected. To determine total insulin content, the SC islets were lysed using 75% ethanol containing 1.5% HCl, followed by sonication at 30% pow-

er for two 20 sec intervals. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher) and used for normalization. Insulin secretion was measured by insulin ELISA (Mercodia) of the media following the manufacturer's protocol.

Glucagon secretion

A total of 300 μ L of culture medium was collected from each sample of stem cell-derived islets following a 72 h incubation period. Glucagon secretion was quantified using a glucagon ELISA kit (Mercodia 10-1271-01). The protocol adhered to the standard procedures outlined, with the exception that 200 μ L of 1 $\times$ enzyme conjugate solution was added to each well, followed by overnight incubation.

Protein extracts and Western blotting

To collect samples for Western blot analysis, the culture media was removed, and cells were washed with 1 $\times$ Dulbecco's phosphate-buffered saline (DPBS). The cells were then resuspended in 300 μ L of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and a 1 $\times$ protease inhibitor cocktail. The samples were stored at –80°C. Upon thawing on ice, the samples were sonicated at 30% amplitude for 30 sec, followed by centrifugation at 14,000g for 15 min at 4°C. The protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific 23225). From each sample, 10 μ g of protein was then used for Western blot analysis. The appropriate amount of lysate sample was diluted 3:1 with a mixture of 4 $\times$ Laemmli buffer (Bio-Rad 1610747) and β -mercaptoethanol (Sigma-Aldrich M3148) and incubated for 5 min at 95°C. Ten microliters of each sample was then loaded and run for 40 min at 200 V along with the ladder using Mini-Protean TGX gels (Bio-Rad 4561094) in a chamber filled with SDS running buffer (Bio-Rad 1610772). The samples were then blotted from the gel onto a 0.2 μ m PVDF membrane using Trans-Blot Turbo transfer pack (Bio-Rad 1704156) following the manufacturer's protocol. The next step was to block the membrane using 5% BSA in PBST (PBS + 0.05% Tween) for 1 h with shaking at room temperature. The membrane was then incubated in 10 mL of 5% BSA in PBST + Gli-3 antibody (1:5000) or HNF1A antibody overnight at 4°C while shaking. The next day, the membrane was washed five times for 5 min each with PBST before adding 10 mL of secondary antibody in 5% BSA in PBST (1:5000). After 1 h of incubation at room temperature while shaking, the membrane was washed five times for 5 min each with PBST. Finally, the membrane was incubated for 5 min in a drawer using Clarity Max Western ECL substrates (1:1; Bio-Rad 1705062) to analyze the membrane in a ChemiDoc imaging system (Bio-Rad). To validate the Western blot, the membrane was cleaned using Restore Western blot stripping buffer (Thermo Scientific 21059), and GAPDH or actin was used as a loading control following a staining technique similar to that mentioned above.

Tissue and SC islet preparation for imaging

Harvested SC islets were fixed in 4% PFA in PBS overnight at 4°C, followed by dehydration in a sucrose gradient (up to 30%). The sucrose was then replaced with Tissue Tek O.C.T. (Sakura) and kept overnight at 4°C. The SC islets were moved to embedding molds, covered with Tissue Tek O.C.T., and flash-frozen in beakers using isopentane surrounded by liquid nitrogen. Frozen tissue samples were stored at –80°C until ready for sectioning. Frozen tissue blocks used for staining were sectioned at 5 µm on Superfrost Plus microscope slides (Eprelia) using a cryostat (Leica CM1950).

The intestine was dissected between the duodenum/stomach and ileum/cecum, and the full length was measured using a ruler on wet blotting paper to prevent drying. It was then opened longitudinally, washed with ice-cold PBS, dried minimally using Kimwipes (Kimtech Science), and rolled from distal to proximal end with the mucosal layer facing inward using tweezers. The roll was pinned using a 27 gauge needle and fixed using 4% PFA in PBS overnight at 4°C, followed by 30 min washes with 5× PBS.

The pancreas was dissected, fixed in 4% PFA in PBS for 2 h at room temperature, and subsequently washed for 30 min with 5× PBS. Both tissues were then processed similarly to SC islets, except the pancreas was sectioned at 10 µm onto Superfrost Plus microscope slides, and the intestine was sectioned at 10 µm onto gelatin-coated microscope slides.

Immunofluorescence staining

For cell differentiation, 12 mm glass coverslips (VWR 631-1577) were cleaned and placed in 6 well plates prior to Geltrex coating, and then hiPSCs were seeded, differentiated, and treated with Gli modulators as described above. At specified stages, the coverslips covered by differentiating cells were transferred using tweezers into a clean 12 well plate prefilled with PBS and further fixed for 20 min in 2% PFA at room temperature. Coverslips were washed multiple times with PBS before permeabilization. Similarly, sections of SC islets, pancreas, and intestine were rehydrated in PBS for 10 min before permeabilization. Cells, SC islet sections, and tissue sections were then permeabilized with 2% Triton X-100 in PBS for 10 min, followed by blocking with 2% BSA in PBS for 30 min. The coverslips or sections were incubated with primary antibody (Supplemental Table S2) diluted in 2% BSA in PBS overnight at 4°C. For antigen retrieval of the nuclear target antibody staining on tissue sections, heat-induced retrieval was carried out in 10 mM citrate buffer (pH 6.0) for 2 min using a pressure cooker (Ninja) at the high-pressure setting. Following primary antibody incubation, samples were washed three times with PBS and then incubated with secondary antibody and DAPI nuclear stain (Molecular Probes D1306) for 1 h at room temperature. After incubation, the slides were washed three times for 10 min each with PBS followed by a final 10 min wash using Mili-Q. Finally, coverslips were mounted on microscope slides, while glass slides were mounted on the SC islet and tissue

sections using aqueous mounting medium (Abcam ab128982).

Microscopy

Images of stained cells were acquired with a confocal Leica TCS SP8 STED 3× microscope and a Leica Stellaris (Leica Microsystems) in the same imaging session for each experiment using the exact same parameters for every experimental set. Stained pancreas and intestine images were acquired with 20× and 40× objective lenses on a Leica TCS SP8 STED 3× confocal microscope and a Leica Stellaris (Leica Microsystems). Intestinal morphology was imaged using an Olympus VS120 S6 slide scanner, starting with a 4× objective lens for an overview scan. Regions of interest were then selected for tile scanning with a 20× objective lens.

Automated quantification

Per-cell immunofluorescence (IF) expression was quantified using a custom FIJI macro combined with an R script generated and described previously by us (Unger et al. 2025). The source code and all documentation have been deposited in GitHub and are freely accessible at <https://www.github.com/LucasUnger/SAM>.

Images were processed to assess expression in up to four channels, with nuclear segmentation based on DAPI. Using the StarDist 2D plugin, regions of interest (ROIs) were created for each nucleus detected based on the DAPI staining. For each additional IF channel, the background threshold was set individually by measuring the background signal and multiplying by a factor of 1.5–2. The threshold was applied to exclude high background and isolate the relevant IF signal within each nuclear ROI. The FIJI macro measured signal intensity within the ROIs across all IF channels and saved these measurements to separate CSV files according to channel and sample. The accompanying R script then aggregated these measurements, calculating the total number of cells, the number of cells with expression above the background threshold, and the percentage of cells exhibiting significant nuclear IF expression per channel.

Blood glucose measurement

Conscious animals were restrained using modified 50 mL Falcon tubes. A blood drop was obtained by nicking the lateral tail vein using a sterile scalpel blade or by pricking the vein using a sterile needle. One clean droplet of blood was placed on a glucose test strip, which was read using a commercial glucometer (Contour XT glucometer, Bayer).

Crypt isolation

Crypt isolation was performed following the protocol described by Díez-Sánchez et al. (2024). A 5 cm segment of mouse duodenum was isolated, rinsed with ice-cold PBS, opened longitudinally, and cleared of mucus and villi using a coverslip. The duodenum was cut into 2–4 mm

fragments, transferred to a 50 mL tube, and washed repeatedly with ice-cold PBS using a BSA-coated pipette until the supernatant was clear (a minimum of 15 times). Tissue fragments were then incubated with gentle rotation at 30 rpm for 30 min at 4°C in 2 mM EDTA/PBS (Sigma-Aldrich 60-00-4). After discarding the supernatant, fragments were vigorously shaken in 10 mL of ice-cold PBS to release crypts. This step was repeated up to three times until most crypts were released. The suspension was filtered through a 70 µm strainer into a BSA-coated 50 mL tube, centrifuged at 300g for 5 min, and washed using ice-cold PBS. To remove single cells, the fraction was washed again with shaking at 200g for 2 min. After washing, the pellets were centrifuged at 300g for 3 min, and 350 µL of RLT buffer was added (Qiagen 79216) with 0.1% β-mercaptoethanol (Sigma-Aldrich M6250) for downstream RNA extraction.

RNA extraction

RNA was extracted from lysed cells using the RNeasy mini kit (Qiagen 74104) following the manufacturer's protocol. The RNA was eluted with 30 µL of nuclease-free water, and its concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Bulk sequencing

RNA samples were processed by either Qiagen or Novogene. All RNA samples except for stage 7 were processed by Novogene, where a second quality control and library preparation [poly(A) enrichment] were performed. The sequencing was performed on NovaSeq X Plus series (PE150) with 30 million reads for each end.

The library preparation for RNA samples from stage 7 was processed by Qiagen by using the QIAseq stranded mRNA kit (Qiagen). The mRNA was enriched from 100 ng of starting material. The RNA was heat-fragmented. After first and second strand synthesis, the cDNA was end-repaired and 3'-adenylated. Sequencing adapters were ligated to the overhangs. Adapted molecules were enriched by using 16 cycles of PCR and purified by a bead-based cleanup. Library preparation was quality-controlled using capillary electrophoresis (Tape D1000). High-quality libraries are pooled based on equimolar concentrations. The library pools were quantified using qPCR, and an optimal concentration of the library pool was used to generate the clusters on the surface of a flow cell before sequencing on a NextSeq 2000 (Illumina, Inc.) instrument (P3 cartridge and 1 × 75 or 2 × 10 bp) according to the manufacturer's instructions (Illumina, Inc.). Raw data were demultiplexed, and FASTQ files for each sample were generated using bcl2fastq v2.20.0.422 (Illumina, Inc.).

Data and pathway analysis

FASTQ files from sequencing were handled in CLC Genomics Workbench 24.0 (Qiagen). Preprocessing included

adapter and quality score-based trimming using the default setting provided by the trimming tool in the CLC software. Alignment and quantification were carried out using the RNA-seq analysis tool following the default settings provided by CLC Workbench. To generate the DEG lists, groups were compared using the "empirical analysis of DGE" algorithm of the CLC software. For S5 versus S4 and S7 versus S5, a cutoff of 15 was employed on the maximum group mean. The DEG lists were subsequently uploaded to Ingenuity pathway analysis for further analysis (Krämer et al. 2014) as described previously (Petrenko et al. 2020; Mathisen et al. 2023). Only DEGs with at least a fold change (FC) ≥ 1.5 and *P*-value of < 0.05 were included for pathway prediction; further network settings were kept at the default.

Single-cell sequencing

Single-cell RNA sequencing was performed using Chromium single-cell 3' reagent kit v3.1 (10x Genomics) following the manufacturer's protocol. Briefly, a single-cell suspension was prepared in 1% BSA-PBS, ensuring $> 90\%$ cell viability. Cell concentration was adjusted to 700–1000 cells/µL, targeting the capture of ~10,000 cells per sample.

Cells were loaded into Chromium chip B, and gel beads in emulsion (GEMs) were generated using the chromium controller to encapsulate single cells with barcoded gel beads. Reverse transcription was performed within GEMs to generate barcoded cDNA, followed by cleanup using silane magnetic beads.

The barcoded cDNA was amplified by PCR, and the product was purified using SPRIselect magnetic beads. Sequencing libraries were constructed by fragmenting the cDNA, performing end repair, A-tailing, and ligating sequencing adapters. After another round of PCR amplification, the libraries were purified using SPRIselect beads and quantified using TapeStation 4150 (Agilent G2992AA).

The 10x Genomics gene expression libraries, each containing ~10,000 cells and targeting 68,000 reads per cell, were pooled together and sequenced on an Illumina NovaSeq 6000 system using an S2-100 flow cell. Paired-end sequencing was performed with a configuration of read 1 (28 bp), read 2 (90 bp), and dual-index reads (10 bp each). A 1% ΦX control library was spiked into the flow cell to serve as a sequencing control.

Data analysis using single-cell RNA sequencing

Single-cell RNA sequencing data were processed using 10x Genomics Cell Ranger v7.0.4 for demultiplexing, barcode processing, gene counting, and sample aggregation. The filtered feature–barcode matrices generated by Cell Ranger were used for downstream analysis. Data processing was conducted in R using the Seurat package. Quality control (QC) metrics were applied to retain high-quality single-cell data; cells with < 500 detected genes (nFeature_RNA) or < 3000 total RNA counts (nCount_RNA) were removed. Doublet identification was performed

using scDblFinder, and only singlet cells were retained for downstream analyses. For signatures, we used the top highly expressed duodenal/liver/pancreas markers in the posterior foregut single-cell data set published by Yang et al. (2022). For enterochromaffin cells, we used the gene signature described by Veres et al. (2019).

Statistical analyses

Statistical analyses were performed using GraphPad Prism v10.4.1 (GraphPad Software, Inc.). To assess the statistical differences between groups for the immunofluorescence data quantification and physiological parameters, we used one-way ANOVA tests unless specified otherwise in the corresponding figure legends. In the figures, data are represented as mean $\pm$ SEM (standard error of the mean) unless specified otherwise. Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****).

Data availability

The data sets have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO series accession numbers GSE288431, GSE288932, GSE285253, GSE284213, GSE291570, GSE300388, GSE311839, and GSE312607. Mice and iPSC lines are available for sharing upon sending material transfer agreements (MTAs) to the corresponding author.

Competing interest statement

The authors declare no competing interests.

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Author contributions: L.U. performed the in vitro differentiations, treatments, RNA preparations, and omics data analyses. U.L. generated the intestine data (including crypt isolation, immunostaining, confocal imaging, processing, and counting) and collected the physiological data from *Ptf1a-cre*, *Pdx1-cre*, *Ngn3-cre*, *Glu-cre*, and *RIP-cre* mice. T.A.L. performed differentiations and insulin secretion measurements and prepared RNA samples, staining, imaging, and quantifications. M.K.H. performed the Western blots, tissue processing, staining, imaging, and quantifications. J.H.G. performed staining, imaging, and quantifications. A.F. generated SC islets and performed glucagon secretion measurements. S.S. measured glycemia of diabetic mice. L.U. and S.C. performed the pathway analysis for the single-cell and bulk transcriptomics data sets. P.R.N. supervised the work and edited the manuscript. H.R. generated the patient-derived iPSC line. P.L.H. generated the *Glu-DTR*, *Glu-cre*, and *RIP-cre* mice and edited the manuscript. L.G. and S.C. acquired funding, conceived the experiments, supervised the work, interpreted the observations, and wrote the manuscript. All authors edited and approved the final version of the manuscript.

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