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THE TRIDENT OF THE PANCREATIC DUCT: STEMNESS, MORPHOGENESIS AND ENDOCRINOGENESIS

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Cover illustration: The Z-projection of the principal islet in 45 dpf Tg(neurod1:EGFP) juvenile fish. The anti-Vasnb staining (white) is used to visualize the ductal tree while the neurod1:EGFP is used to display the pancreatic islet.

The Trident of the pancreatic DUCT: Stemness, Morphogenesis and Endocrinogenesis

Thesis for Doctoral Degree (Ph.D.)

By

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Popular science summary of the thesis

Mammalian animals, such as mouse and human, can generate different cell types during embryonic development. Yet, such capability dramatically declines soon after birth, which lead to inefficient tissue recovery or cell type restoration in a disease state. We are particularly interested in pancreatic β-cells as in either type 1 or type 2 diabetes, both diseases are featured by a massive loss of functional β-cells. Despite the emerging pharmaceutics that can alleviate diabetes symptoms, they cannot help cure the diseases and the β-cell functions would undergo irreversible deterioration along the disease progression. One promising strategy to replenish β-cells is from the endogenous stem cell population. Harnessing the stem cell to give rise to the lost cells can avoid adverse drug reaction and risks of cell therapies. Zebrafish, the most widely used animal models in regeneration research, is recognized as the superstar in the field of tissue regeneration. Even in the adult stage, the zebrafish can replenish the extreme loss of β-cells and recover the blood glucose levels very rapidly. However, the underlying mechanisms remains the mystery, in particular, we have limited knowledge about stem cell properties in zebrafish pancreas and how the different cell types orchestrate together to restore the normal tissue architecture. The work presented in the thesis aims to address these questions by making new tools to identify the real stem cell populations that can continuously give rise to endocrine cells during development and introducing a drug discovery story which we identified a chemical in inducing β-cell differentiation. Leveraging these tools, we characterized the new pattern and the mode of action of β-cell development and regeneration and identify the molecular pathways that are crucial in β-cell differentiation or de-differentiation. The finding represents a valuable resource, not only it defines a refined principle in tissue remodeling and endocrinogenesis which for decades puzzle the researchers, but also it shed new light on the utility and advantage of using zebrafish to study human diseases.

Abstract

Different subtypes of diabetes are all featured by the deterioration and loss of functional β-cell. Currently, there is still lack of therapeutic strategies to cure diabetes. However, harnessing the innate stem cell population to become functional β-cell could be a potential way for future treatment. Mammalian animals, including mouse and human, can barely replenish the lost β-cell in the disease state; however, early vertebrates, especially zebrafish, has astonishing tissue regeneration capacity. In particular, zebrafish can quickly recover from the extreme β-cell loss and recovery body glucose level very quickly. Understanding the underlying cellular and molecular events is pivotal for the drug discovery in translational studies to treat diabetes.

In **Paper I**, we introduced a compound, named CID661578, which we identified from a large-scale chemical screening experiment. Combining yeast hybrid assay, biochemistry experiments, polysome sequencing, single-cell RNA-seq and genetic zebrafish mutant, we confirmed that *mknk2b* in zebrafish and MNK2 in human are the major functional target. Functional testing indicated that CID661578 can induce duct-to-β-cell neogenesis in both zebrafish and neonatal pig islet.

In **Paper II**, we introduced a novel CRISPR/Cas9 knock-in method to generate zebrafish lines for multiple utility (i.e. cell labeling and lineage tracing). We creatively used double-stranded DNA with 5' modification as the donor. Such clone-free, one-step knock-in strategy allow researchers to target different loci in a quick and scalable fashion. Using the newly generated lines, we are able to delineate the developmental paths of zebrafish pancreas and liver and explore the origins of the regenerative hepatocytes under different injury conditions.

In **Paper III**, the newly developed knock-in tools allow us to decipher the developmental paths of zebrafish endocrinogenesis in normal and β-cell ablation conditions. Combining single-cell RNA-seq, lineage tracing, cell targeted ablation, immunofluorescence, in situ hybridization, we identified a previously unrecognized ductal heterogeneity in zebrafish pancreas as well as the endocrine precursor cells during β-cell development. We delineate the key cellular and molecular events in β-cell differentiation and de-differentiation and pinpoint the distinct origins of β-cells during the development and regenerative conditions. This study offers a good resource and provides novel mechanistic insights into β-cell development and regeneration in zebrafish.

List of scientific papers

I. Karampelias C, Watt K, Mattsson CL, Ruiz ÁF, Rezanejad H, **MI J**, Liu X, Chu L, Locasale JW, Korbutt GS, Rovira M, Larsson O, Andersson O. Nature Chemical Biology, 2022, Sep;18(9):942-953.

II. **Mi J**, Andersson O. Efficient knock-in method enabling lineage tracing in zebrafish. Life Sci Alliance. 2023 Mar 6;6(5):e202301944.

III. **Mi J**, Liu KC, Andersson O. Decoding pancreatic endocrine cell differentiation and beta-cell regeneration in zebrafish. (Under revision)

Contents

List of abbreviations

GIP	Glucose-dependent insulinotropic polypeptide
DPF	days post-fertilization
HPF	hours post-fertilization
PPCs	pancreatic progenitor cells
Peri-PCs	Peri-pancreatic cells
BMP	Bone morphogenesis protein
FUCCI	fluorescence ubiquitination cell cycle indicator
RA	Retinoic Acid
RAR	Retinoic Acid Receptors
RXR	Retinoid X Receptors
RAREs	Retinoic Acid Response Elements
HAT	Histone acetyltransferase
Hh	Hedgehog
FGF	Fibroblast growth factor
ALK8	Activin A receptor, type 1 like
PGE2	Prostaglandin E2
HPD	Hepatopancreatic duct
BAC	Bacterial artificial chromosome
EPD	Extra-pancreatic duct
IPD	Intra-pancreatic duct
MTOR	Mechanistic target of rapamycin
ROS	Reactive oxygen species
H2O2	Hydrogen peroxide
ER	Endoplasmic reticulum
NTR	Nitroreductase
MTZ	Metronidazole
hHB-EGF	human heparin-binding epidermal growth factor-like growth factor
STZ	Streptozotocin
BID	BH3 Interacting Domain Death Agonist
IKKs	IκB kinases
TBK1	TANK-binding kinase 1
ARQiv	Automated reporter quantification in vivo

FDA	Federal Drug Administration
NECA	5’-N-ethylcarboxamidoadenosine
LUCCI	luminescence ubiquitination-based cell cycle indicator
SIKs	Salt-inducible kinases
UPR	Unfolded protein response
SOX	SRY-like HMG-box
CRISPR	Clustered regularly interspaced short palindromic repeats
HDAC1	Histone deacetylase 1
DNMT1	DNA methyltransferase 1
ZFNs	Zinc finger nucleases
TALENs	TAL effector nucleases
HDR	Homology directed repair
NHEJ	Non-homologous end joining
HAs	Homologous arms
HMEJ	Homology mediated end joining
UTR	Untranslated region

1 Introduction

Similar to mammalian pancreas, zebrafish pancreas is primarily composed of exocrine and endocrine cells. In exocrine pancreas, ductal cells gradually form luminal structure to facilitate the transportation of digestive enzymes, which are secreted by acinar cells, to the intestine. The endocrine cells cluster together and construct the delicate tissue structure named pancreatic islets. Within the islets, there are several endocrine cell types, including insulin-secreting β-cells, glucagon-secreting α-cells, somatostatin-secreting δ-cells, ghrelin-positive ε-cells, and gastric inhibitory polypeptide (GIP)-positive cells. In addition, zebrafish pancreas is a highly vascularized organ with a substantial number of vascular endothelial cells, smooth muscle cells, and pericytes. The islet vasculature system is essential in regard with maintaining the whole-body glucose homeostasis, as it can help the islet cells sense the blood glucose level. Consequently, it involves in regulating the paracrine/autocrine effects of islet cells and fine-tune the balance of insulin and glucagon secretion. Zebrafish pancreas is also innervated by peripheral nervous cells. Although the major function of zebrafish pancreas innervation is largely unknow, it could be similar to that of mammalian pancreas in regulating vessel constriction and hormone secretion. The lymphatic system is also present in zebrafish pancreas, while its function is yet to be very well determined. Interestingly, researchers have found that the innate immune cells including macrophages and neutrophils serve as security guards and constantly pass in and out of islet to remove apoptotic cells during physiological conditions. Adaptive immune cells, such as T cells, NK cells, B cells begin to develop at 21 dpf; however, their function in the pancreas is still elusive. Mesothelial cells form out-layer sheath surrounding the whole pancreata. Various types of stromal cells intermingle with parenchymal cells and produce extracellular matrix as the scaffold. Such complex composition of many distinct cell types, all together, forms a delicate interconnected microenvironment, which are critical for both the normal physiological function (i.e. body glucose level control, food digestive enzyme production) and proper injury response for the tissue recovery.

Here, I mainly summarize the major discoveries of zebrafish endocrine and exocrine pancreas development and regeneration; in the chapter 4, I will briefly discuss the cell-cell interactions within the pancreas from recent findings based on the state-of-the-art developmental genetic tools and single-cell RNA-seq.

2 Zebrafish pancreas development

2.1 Gastrulation, foregut endoderm development and tissue patterning

The primordium of zebrafish pancreas starts to develop at the 4-somite stage (12 hours post-fertilization [hpf]). The pancreatic progenitor cells (PPCs) appear as a monolayer cells in the endodermal sheath, which is located at the ventral side of the embryo. These PPCs, which will eventually differentiate into endocrine pancreatic, lie closest to the midline (M) in the endodermal sheet; whereas Peri-pancreatic cells (Peri-PCs), which are destined to become exocrine pancreatic cells, as well as the progenitors of liver, intestine, swim bladder and hepatopancreatic duct, are located in the medium or lateral region (Figure 1) (1).

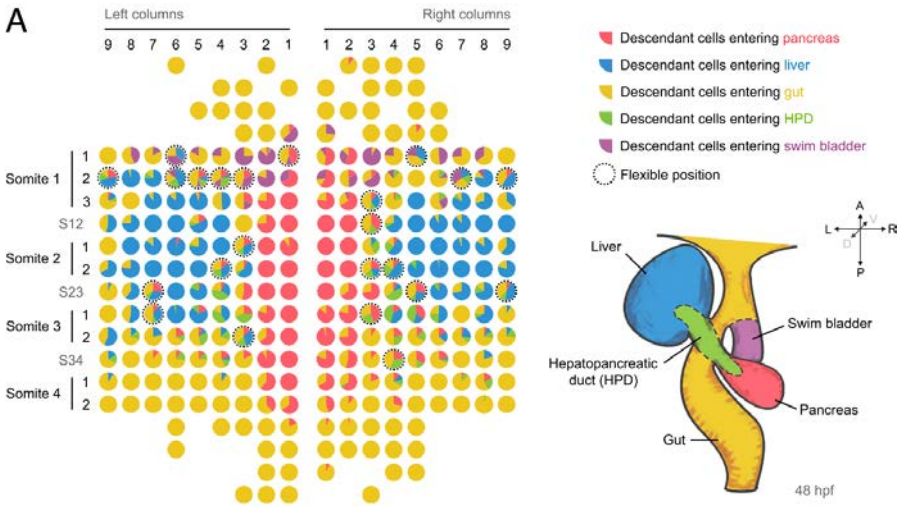

Figure 1. The fate map of single-cells in the endodermal sheath at 4-somite stage zebrafish embryos. (Yang et al. 2021, PNAS)

Multiple factors, including but might not restricted to secreted signals and cell cycle states, place combinatorial effects on the early pancreas fate decision. Evidence from *swirl* (*bmp2b*) and *chordino* (*chordin*) mutants indicated that bone morphogenesis protein (BMP) signaling is important for the expansion of pancreatic primordium during anteroposterior patterning period (2). Using photoactivatable lineage tracer DMNB-caged fluorescein dextran conjugate in transplanted zebrafish embryos, Chung et al discovered that *Bmp2b*, which is mainly produced in the lateral plate mesoderm, can promote liver versus pancreas/intestine fate determination (3). Furthermore, Chung et al also proposed that Hedgehog/Smoothened signals derived from the lateral part of endodermal sheath, can induce pancreatic β-cells formation in a cell non-autonomous manner (4). Recently, with the help of dual fluorescence ubiquitination cell cycle indicator (FUCCI) system, Yang et al showed that PPCs are featured by a relatively longer G1 phase compared with peri-PCs; intriguingly, tuning up the duration of G1 phase by morpholino

knock-down Geminin, a DNA replication inhibitor, can drive the peri-PCs towards PPCs fate (1).

Retinoid acid (RA) signaling has been confirmed to be crucial in endoderm patterning and pancreas regionalization. RA, which is synthesized by *Aldh1a2* (also known as *Raldh2*) from β-carotene, is present in the anterior paraxial mesoderm adjacent to the foregut. Upon binding to RA, the Retinoic Acid Receptors (RAR) would form dimer with Retinoid X Receptors (RXR), and such heterodimers can bind to DNA regulatory sequences, called "Retinoic Acid Response Elements" (RAREs), residing in the promoters or enhancers. Subsequently, they will recruit transcriptional co-activator and histone acetyltransferase (HAT) to activate gene expression (5). In early zebrafish embryos, the RA forms a two-tailed gradient with the highest levels in the mid-trunk region and relative lower levels towards the anterior and posterior regions, and such gradient pattern is crucial in regionalization of different organs (6), (7), (8). Disruption of RA signaling shown by *neckless* (*nls*) mutant (the disruption of aldh1a2) and embryos treated with RA chemical antagonist at the end of gastrulation stage can reduce the numbers of pancreatic precursors and eventually inhibit β-cell formation (9), (10). Conversely, exogenous RA administration or the disruption of *cyp26a* genes, which code for a RA-degrading enzyme, can lead to a dramatic expansion of pancreatic endocrine cells in the anterior foregut, indicating that RA can induce early endodermal cells, which are normally destined to become other endodermal organs, towards the pancreatic endocrine cells at late gastrulation stage (11). Recent ChIP-seq data in zebrafish confirmed the auto-regulatory feedback loop of RA in the control of RA metabolizing gene (*cyp26a*, *dhrs3*) expression. Furthermore, it also identified the direct binding site of RAR, especially the cis-regulatory elements of pancreatic genes, including *hnf1ba*/*b*, *gata6*, *insm1b*, *jag2b*, and *mnx1* (12). These studies, all together, suggested that RA is a mesoderm-to-endoderm instructive signal mediating the induction of endocrine precursors.

Also, during this time period, the dominance of different Notch ligands play divergent roles in α- or β-cell determination (13). For example, the deltaA knock-down embryos lack α-cells, while the jagged1b (*jag1b*) mutant embryos develop excessive number of α-cells (13). The mechanism of limiting pancreatic field in the posterior region will be discussed in the following chapter.

2.2 Hepatopancreatic regionalization and liver versus pancreas development

The Wnt, Hedgehog (Hh), RA, BMP, and fibroblast growth factor (FGF) signaling generate an intricate regulatory network and all together play interrelated and sequential roles in the endodermal organ induction and specification. Liver and ventral pancreas are descendants of common multipotent progenitor cells. A series of studies from Ober et al. reported that two Wnt ligands, *Wnt2* and *Wnt2bb*, which originate from the lateral plate mesoderm, can activate frizzled homolog 5 (*fzd5*) receptor, and subsequently mediate liver versus pancreas fate

specification (14), (15). Additionally, Lu et al. showed that the membrane protein *EpCAM* is a Wnt de-repressor and can promote the endodermal cells to respond to Wnt2bb activation for the liver induction in a cell autonomous manner (16). Furthermore, Lancman et al showed a bidirectional role of Wnt signaling and emphasized an essential time window for the synergistic effects between Wnt signaling and hnf1ba-driven transcriptional network in the ventral pancreas specification. Lastly, the Wnt signaling is crucial for hepatopancreatic progenitor cells specification prior to liver bud formation and can also support liver expansion at a later developmental stage (17).

The Hh signaling, however, play an opposite role in pancreatic progenitor and β-cells differentiation during gastrulation stage. Axial mesoderm derived Sonic Hedgehog (*Shh*) can specifically induce β-cells formation by restricting the effects of BMP signaling (18), (19). The proper localization of pancreatic tissue also relies on Shh, as zebrafish embryos with *smoothen* mutation or treated with Hh antagonist showed duplicated pancreatic primordium surrounding the gut (20). Further lines of evidence from betaine homocysteine S-methyltransferase (*bhmt*) morpholino knock-down and *Shh*-deficient mutant suggested that Shh functions downstream of *Bhmt* to promote β-cells development (21). Additionally, the overexpression of Shh ligand, rather than Bmp ligand, can rescue the reduction of endocrine cells led by accelerated cell turnover in PPCs (1). Nevertheless, at the end of gastrulation, Hh switches to a repressive mode for the β-cells differentiation, and facilitates exocrine pancreas development. On the contrary, during this time period, RA can induce β-cell differentiation by antagonizing Hh signaling (18).

As described in the above chapter, RA confers its impact on pancreas specification starting from the end of gastrulation (9). RA signaling is crucial in establishing the anterior and midline β-cells convergence pattern as well as increasing β-cell numbers (22). Contrarily, transcription factor *cdx4* expressed in the endoderm can prevent the RA signaling from the induction of β-cells. The exogenous RA treatment in *cdx4* mutant can induce the development of a large number of β-cells scattered throughout the posterior endoderm (22).

The BMP signaling demonstrates multiple roles in several steps of pancreas development and hepatopancreatic regionalization. *Bmp2b*, which is secreted from the lateral plate mesoderm, acts through *Alk8* (activin A receptor, type 1 like, also known as *Acvr1l* or *Alk2*) to promote hepatogenesis and to allow the foregut endoderm to retain differentiation competence into dorsal bud derived endocrine cells (3). This effect was, at least partially, mediated by Four and a Half LIM Domains 1b (*Fhl1b*), as the augmentation of *Fhl1b* in pancreatic and intestinal progenitor cells can cell-autonomously downregulate *Pdx1*, the master regulator in pancreas development, and subsequently divert the cells to liver lineages (23). Intriguingly, prostaglandin E2 (PGE2) was also shown to interact with BMP signaling to regulate liver versus pancreas cell fate (24). Genetically blocking BMP and FGF signaling by temporally-controlled overexpression of dominant-negative BMP and FGF receptor can cause a severe reduction of the liver anlage without affecting the neighbouring endodermal and mesodermal tissues (25). *Noggin2*, an endogenous BMP inhibitor secreted from the notochord, can confine the inhibitory effect of BMP in pancreatic progenitor expansion and β-cells differentiation (26).

The inhibitory roles of BMP signaling in the induction of the ventral pancreatic bud, hepatopancreatic duct (HPD) formation and ductal-derived endocrinogenesis will be discussed in the following chapters.

Lastly, *slow-muscle-omitted* (*smu*) mutants with Hedgehog signaling defects showed a universally decreased expression of endocrine markers without affecting *ptf1a* expression, indicating its differential roles in dorsal and ventral pancreatic anlagen specification (27).

2.3 Two pancreatic anlagen formation, bud fusion and multipotent pancreatic progenitor cell formation

Zebrafish pancreas originates from two buds. The first bud (also named first anlage or dorsal bud), is located at the dorsal region of the developing gut, and starts to develop at around 24 hpf. The second bud (also named second anlage or ventral bud), is residing on the ventral side of the developing gut and located anterior to the dorsal bud, and it starts to develop at around 40 hpf (28). These two buds fuse by 52 hpf. The dorsal bud only gives rise to endocrine cells; whereas the ventral bud can contribute to both exocrine and endocrine cells (28). Immunostaining of bacterial artificial chromosome (BAC) reporter line *Tg(nkx6.1:EGFP)* demonstrated that both the dorsal bud pancreas and ventral bud multipotent progenitor cells are *nkx6.1* positive before 48 hpf; however, shortly afterwards, *nkx6.1* only presents specifically in intra-pancreatic ductal cells and is absent in mature endocrine cells and acinar cells (29). The GFP signal can be detected in both Notch irresponsive extra-pancreatic duct (EPD) and Notch responsive intra-pancreatic duct (IPD) at 65 hpf (29).

The BMP signaling is critical during bud fusion period. This was shown by the *alk8* mutant and alk8 splice-blocking morpholino knock-down embryos, that displayed remarkable ventral bud pancreas developmental deficiency, as indicated by the inability to engulf the dorsal bud at 72 hpf (30). Moreover, lateral plate mesoderm derived Bmp2a is required in ventral bud development (31). The analysis of myosin phosphatase targeting subunit 1 mutant (*mypt1*^{sq181}) showed a disruption of lateral plate mesoderm migration and hence impaired the *Bmp2a*-producing cell position. Such disorganized structure can eventually compromise the proper distribution of BMP ligand sending to the adjacent exocrine pancreas, which ultimately leads to exocrine pancreas dysplasia (32), (33). Overexpression of *Bmp2a* can rescue such exocrine developmental defects led by mypt1 loss of function (32). One recent study showed that 12-Lipoxygenase (12-LOX)-Gpr31 axis is critical in pancreatic bud fusion and pancreatic duct morphogenesis; however, whether this effect is mediated through BMP signaling is still inconclusive (34). Aside from BMP signaling, reciprocal endoderm and lateral plate mesoderm communications by *Fgf24* and *Fgf10* are crucial in the specification and growth of ventral bud pancreas (35). *Fgf10*$^{-/-}$; *fgf24*$^{-/-}$ double homozygous mutant displays ventral bud agenesis and ectopic hepatocytes (31).

Yap/*Tead* signaling is critical in the expansion of multipotent progenitor cells in the endodermal organ (36). Genetically knock-down of Yap1 or overexpression of dominant negative *Tead1* (protein fused to a transcriptional repressor domain Engrailed) showed a dramatic reduction of sox9b-expressing multipotent progenitor cells (36). Analysis of $yap^{1-/-}$; $taz^{+/-}$ embryos demonstrated multiple endodermal organ developmental defects, such as smaller liver and pancreas as well as non-looped intestine (37).

2.4 Hepatopancreatic duct development

The HPD is a tissue structure connecting pancreas, liver, and the gut. This structure can be clearly observed at 48 hpf but its specification is pre-determined as early as 1-4 somite stage. Based on the high-resolution microscopy for single-cell fate mapping in 216 foregut endoderm cells, the progenitors of HPD are mainly located at two to three cells away from the midline (column 2 and 3) (1). The specification of HPD is determined by Fgf10 secreted from the adjacent mesenchyme, as *fgf10* mutant showed severely dysmorphic HPD epithelium (38). Transcription factor *sox9b* is essential in the HPD identity maintenance. Homozygous *sox9b* mutant displays significant HPD dysmorphism, accompanied by cells dysregulated differentiation towards hepatic and pancreatic cell fates (39). Furthermore, sox9b expression in EPD requires various Fgf signals (*Fgf10*, *Fgf24*) from the surrounding tissue (40). Multiple Bmp ligands (*Bmp2b*, *Bmp4*, *Bmp6*) are produced from the mesenchymal cells surrounding the ventral bud pancreas and HPD; while the endogenous BMP inhibitor, including Gremlin1a and Noggin1, were also present in this region to restraint the effects of Bmp ligands. Although BMP signaling can inhibit dorsal and ventral bud derived β-cells formation, activation of *Alk8* is indispensable at an earlier stage for the HPD formation and ventral bud expansion, indicating a delicate spatiotemporal regulation of BMP signaling on HPD morphogenesis (30). Lastly, the *EphB*/*EphrinB* signaling can drive the de novo HPD remodeling, which starts from several nascent lumina and ultimately clump together and merge into a single-lumen structure, implicating that cell-cell contact might also play a role in HPD development (41).

2.5 EPD and IPD specification, ventral bud derived β-cells in the principal islet

The pancreatic ductal system can be subdivided into EPD and IPD based on their location and these two types of ducts are separately specified during development. Based on a *nkx2.2a* reporter line, the Argenton group showed that the common duct precursor cells can split into one group giving rise to EPD, with another group differentiating into IPD (42). Morpholino knock-down of ptf1a can dramatically reduce the number of *nkx2.2a* positive IPD cells without interfering with EPD, indicating distinct transcriptional regulatory networks in EPD and IPD development (42).

After ventral and dorsal bud pancreas fusion, the ventral bud can continuously contribute to endocrine cells in the principal islet. The most straightforward evidence came from time-lapse imaging done by Kimmel et al. This was accomplished since the whole ductal system can be visualized by pan-epithelial and endocrine marker *cldnb* transgenic reporter, *Tg(-8.0cldnb:lynEGFP)* (43). This study showed that *neurod1* positive endocrine cells can continuous bud out from the EPD and migrate towards the principal islet during normal development (43). BMP signaling inhibition by dorsomorphin (a selective inhibitor of the BMP type I receptors *Alk3*, *Alk6*, and *Alk8*) treatment can lead to a substantial increased ectopic β-cells formation from the EPD, implicating a latent competence of EPD as endocrine progenitors and such differentiation process is BMP dependent (30) (Figure 2). Overexpression of *Fhl1b* can repress the induction of ventral bud derived endocrine cells by inhibiting *pdx1* expression; whereas inhibition of Fhl1b promotes β-cells formation by upregulating *pdx1* and *neurod1* expression in HPD in both developmental and regenerative conditions (23). Of note, the Peers group showed that upon bud fusion, all ventral bud derived cells are *nkx6.1* positive; during development, *nkx6.1* positive cells maintained in both EPD and IPD. Together with previous findings, the *nkx6.1* positive cells were multipotent at early stages and gradually restricted in bipotent progenitors which are capable of continuously differentiating into endocrine cells (29).

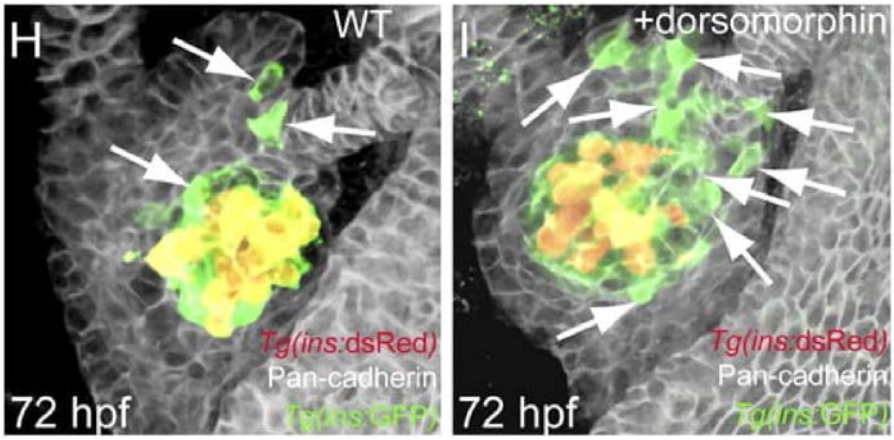

Figure 2. Dorsomorphin treatment can greatly induce β-cell formation from EPD (Chung et al, 2008, PNAS)

2.6 IPD elongation and pancreas expansion

IPD, which is composed by Notch-responsive cells, is surrounding the principal islet and form delicate ductal network throughout the whole pancreas. The fully developed IPD is crucial for the expansion of the pancreatic mass. This is supported by several loss-of-function experiments. Zebrafish larvae with morpholino knock-down of *jag2* displayed a remarkable agenesis of exocrine tissue and a decrease of exocrine-to-endocrine ratio (13). The *jag1b*/*jag2b* double homozygous mutant showed hypomorphic IPD and a dramatic reduction of pancreatic size (44).

Early inhibition (30-72 hpf) of Notch signaling using a gamma secretase inhibitor, LY-411575, phenocopied the above ductal and pancreas development deficiency (44). Another evidence is from the *donuts*^{908}, a mutant contains a missense mutation disrupting the proteolytic maturation of hepatocyte growth factor (HGF) tyrosine kinase receptor, Met. It showed that apart from Notch signaling, PI3K/Akt and *Stat3* signaling pathways are also essential in proper IPD morphogenesis (45). Lack of Met signaling can cell-autonomously block "tip cell" elongation in pancreatic tail region. Cell transplantation chimeric studies suggested the wild-type IPD can partially rescue the mutant phenotypes (44), (45). *Sox9b*, the transcription factor important in ductal cell development, is maintained by Notch signaling. *Sox9b* homozygous mutant demonstrated a deficit of ductal morphogenesis, which subsequently leads to the impairments of endocrinogenesis in response to Notch inhibition in the pancreatic tail, and β-cell regeneration in the principal islet (40).

2.7 Secondary islet formation

Notch-responsive ductal cells have been considered as ductal progenitors giving rise to all endocrine cells upon Notch inhibition at the larval stage. In physiological condition, higher calorie intake can induce β-cell replication as well as duct-to-endocrine differentiation (also named neogenesis). Short- and long-term lineage tracing experiment using the *tp1* promoter (contains six concatemerized Notch-responsive elements including in total 12 RbpJ binding sites from the Epstein Barr Virus terminal protein 1 [TP1] gene) driving inducible Cre recombinase showed that Notch-responsive IPD (in larvae) or *tp1* positive centroacinar cells (in adult), lying in between the large duct and acinar cells, can be mosaically labelled (46). These cells can differentiate into β-cells in the peripheral region of the principal islet as well as β-cells in the secondary islets in development, β-cell chemical ablation, and partial pancreatectomy models (47), (48), (49), (50). Mechanistically, nutrient-dependent ductal progenitor activation involved a key step involving downregulation of Notch signaling. More importantly, the levels of Notch activity (from high-Notch, intermediate-Notch and sustained Notch inhibition) have been proposed to define the phenotypic switch of IPD between quiescent, cell proliferation and initiation of cell differentiation (51). Upon calorie intake, mechanistic target of rapamycin (mTOR) is indispensable for β-cell neogenesis in response to glucose specifically, whereas insulin/IGF1 pathway mediates lipid-rich diet induced β-cell development (47), (52). Furthermore, genetic manipulation of fgf1 demonstrated that secreted Fgf1 can profoundly induce β-cell neogenesis with or without overnutrition condition (53). Moreover, an increased demand of insulin production by overnutrition can itself induce neogenesis (54). Modulating key genes in β-cell metabolism can also affect β-cell neogenesis. Genetically blocking or activating ATP-sensitive potassium (K ATP) channels can either potentiate or suppress β-cell neogenesis, respectively (54). Overexpression of hyperactive glucokinase gene can also induce β-cell neogenesis independent of nutritional states (54).

As mentioned earlier, *sox9b* is crucial in hepatopancreatic biliary system development, however, contradictory results remain in terms of the functionality of *sox9b* in β-cells neogenesis in both physiological and β-cell ablation conditions (39), (40), (55). This might be

due to the facts that different studies used different *sox9b* mutant zebrafish lines. To avoid the innate limitations of different missense mutation and the early disruption of IPD development in such mutants, Huang et al. generated a spatiotemporal-controlled *sox9b* gain-or-loss of function model in IPD by overexpressing wild type or truncated version of *sox9b* (lack of transactivation domain) (55). This study showed that *sox9b* performs as a downstream mediator in both Notch and RA signaling pathway for the maintenance of IPD cell identity. The loss of functional *sox9b* showed synergistic effects upon Notch and RA inhibition. Unexpectedly, heterozygous *sox9b* mutant showed an accelerated β-cell regeneration in adult fish indicated by increased number of single β-cell per section (55). Other genetic defects, such as mutations *pdx1*, *jag1b*/*jab2b*, can all lead to developmental defects of IPD and ultimately have a negative impact on neogenesis in response to Notch inhibition. Pharmacological manipulation of RA also suggested that although RA is initially required for specification of the dorsal bud derived endocrine cells, it later functions as a gatekeeper to maintain ductal progenitors in an undifferentiated state and prohibit β-cell differentiation (56). Kimmel et al. put forward a two-wave endocrinogenesis model, indicating that the first wave originates from pancreatic progenitor in the dorsal bud followed by a second wave from Notch-responsive latent progenitors located in the EPD and pancreatic tail (43).

During the larvae stage, nearly all *tp1* positive cells co-express *nkx6.1* in pancreatic tail region (44), suggesting that *nkx6.1* is a marker of IPD. In homeostatic condition, adult zebrafish ductal cells are mainly quiescent; however, upon β-cell ablation, *nkx6.1* ductal progenitors will re-enter the cell cycle and begin to differentiation (more discussion in the following chapter). One recent study done by Rovira et al. reported that the transcriptional repressor, REST, is a major suppressor in endocrine differentiation across different species (57). Chemical inhibition by a selective REST inhibitor can promote a marked increase of α-cell differentiation in zebrafish, indicating a conserved role of REST in regulating ductal-derived β-cell neogenesis.

2.8 β-cell proliferation and β-cell heterogeneity

β-cell proliferation is a continuous process for the expansion of β-cell mass. In zebrafish larvae, β-cells rarely self-replicate due to *Cdk4* mediated inhibition. Overexpression of mutant *cdk4*, which is insensitive to inhibition by cyclin-dependent kinase inhibitors, can enhance β-cell proliferation in zebrafish larvae and improved glucose tolerance at the expense of dampened β-cell neogenesis (58). Intriguingly, preceding studies also showed that the turn-over rate of β-cells varies and it accounts for the β-cell heterogeneity. Most β-cells stay quiescence, whereas very few β-cells can keep dividing in a slow pace. Using hot-cre temporal labeling system, Hesselson et al. successfully distinguished the dorsal bud derived β-cells from the ventral bud derived β-cells (59). The dorsal bud derived β-cell normally sustain in a quiescent state indicated by limited EDU incorporation. Ventral bud derived β-cells are not fluorescently labelled but maintain proliferative. These findings were further extended to juvenile stage, by using the *Tg(ins:CreERT2)* transgenic line for temporal labeling β-cells, β-cell specific

multicolor betabow and β-cell specific Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) system. Based on the *Tg(ins:CreERT2)* temporally-labeling system to separate old and newly-formed β-cells, Singh et al. proposed an "Hourglass model" demonstrating that the newly formed β-cells are located at the anterior region of principal islets showing elevated turn-over rate as compared with pre-existing β-cells located at the posterior region (49). However, these proliferative β-cells are less functional mature as manifested by less glucose responsive calcium influx. The maturity of these newly-formed β-cells will catch up when the fish grow up to 35 dpf. This is indicated by the fact that the β-cells from both the anterior and the posterior regions of principal islet showed similar percentage of glucose responsive β-cells (49).

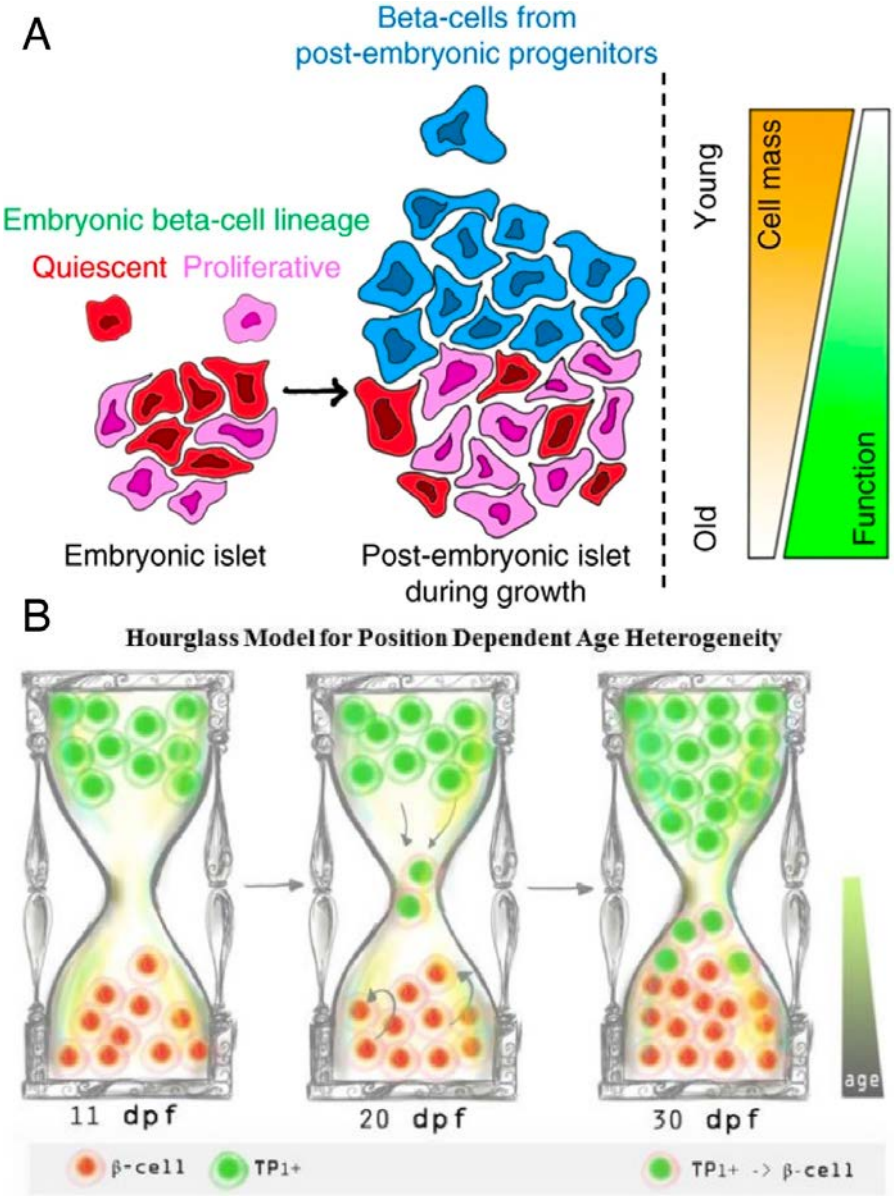

Figure 3A and B, The schematics of the "Hourglass" modeling indicating differential origin and features of pancreatic islet cells in zebrafish.

There are several factors contributing to β-cell proliferation in zebrafish. One pilot study showed that high-calorie intake can accelerate β-cell proliferation (47). Mechanistically, higher nutrient metabolism can elevate the level of reactive oxygen species (ROS) production. A modest increase of intracellular hydrogen peroxide (H2O2) levels is sufficient enough to boost β-cell proliferation (60). Conversely, reducing H2O2 level can block β-cell proliferation induced by nutrients consumption (60). However, a marked increase of H2O2 above a certain threshold can also play inhibitory roles in β-cell proliferation, yet in the meantime, promote duct-to-β neogenesis (60).

Aging and local immunological status can also affect the cell cycle state in β-cells (61). Old fish islets contain a higher proportion of *nfkb*^{high} β-cells and can recruit more macrophages and neutrophils. Transcriptomics analysis showed that islets from older fish upregulate genes coding for protein machinery in cytokine release, ER (Endoplasmic reticulum) stress, and inflammation. Among them, *Socs2* exerts a major inhibitory effect on β-cell proliferation (61).

Lastly, β-cells can be further characterized according to their glucose responsiveness. In both mouse and zebrafish, leader cells were identified, which constitute a portion of β-cells that responding quickly to an increase of glucose levels in the first place, and subsequently coordinate other β-cell calcium influx within the whole islet. Photoablation of leader cells can disrupt the whole islet responsiveness to glucose (62). Single-cell RNA-seq analysis suggested that these leader cells highly express glucose kinase gene with less insulin expression (62).

2.9 β-cell maturation

The final step of β-cell development is β-cell functional maturation. In zebrafish, thyroid hormone can activate *pax6b* and *mnx1* expression while downregulate *arxa* expression (63). α-cell and β-cell maturation during the larvae-to-juvenile transition is shown as a thyroid hormone-dependent process by enhancing glucose sensitivity (63), (64). As described above, the old β-cells are functionally mature based on glucose stimulated calcium influx, and the newly formed β-cells are more prone to be proliferative and immature. However, such subcategories of mature and immature β-cell become blurry when the fish grows up (49).

3 Zebrafish pancreas regeneration

3.1 Tools used in zebrafish pancreas injury models

3.1.1 Nitroreductase-based system

Nitroreductase (NTR) mediated cell ablation is a widely used chemo-genetic methodology in zebrafish research to deplete different cell types (such as hepatocytes, β-cells, cardiomyocytes) in a temporally-controlled fashion (65), (66). NTR is a bacterial enzyme which can convert the prodrug, MTZ (metronidazole), into a DNA cross-linking agent and cause cell death in a dose-dependent manner. Once it is washed away, the cell toxicity effect can be discontinued. Such feature is crucial in zebrafish regenerative research as it allows tissue with high regenerative capacity (such as heart, liver, and β-cells) to recover right after ablation (65). Mechanistically, Anderson et al. reported that NTR/MTZ mediated cell ablation is a macrophage-dependent process as MTZ treatment can lead to a remarkable production of reactive oxygen species (ROS) and subsequently recruit immune cells (mainly macrophages) with peak infiltration observed 6 hours after drug treatment (67), (68). Intriguingly, antioxidant agents can protect β-cells from MTZ induced cell death. In addition, blocking 12-lipoxygenase (12-LOX)-CXCR3 axis, which is responsible in myeloid cell migration, can efficiently reduce islet macrophage infiltration and preserve β-cell mass (67).

The first-generation of NTR only exerts its effect with high concentration of prodrugs. Meanwhile, several cell types, for example macrophages, are proven to be resistant to such system. The next generation of NTR (NTR 2.0) was recently introduced based on rational engineering and cross-species validation demonstrated that the ablation efficiency is much improved (~100-fold) even with lower concentration of prodrug (69).

Furthermore, to overcome the variability of cell ablation efficacy across different batches, Nifurpirinol, another nitroaromatic antibiotic, was introduced as a reliable alternative reagent with a working concentration ~ 2000 lower than MTZ. Nifurpirinol has been proved to be efficient across various cell types (β-cells, osteoblast, dopaminergic neurons) (70). More importantly, the working concentration of Nifurpirinol is three times below its toxic threshold. Thus, nifurpirinol provides another option in NTR based cell ablation model, particularly useful in MTZ/NTR resistant cell types (70).

In summary, the NTR-based cell ablation system provides an easy and amenable way to deplete cell types in a reversible manner. Such method is of great value in regenerative research and has also been used as a genetic tool for mechanistic studies of cellular function and cell-cell communications.

3.1.2 DTR and DTA system

Diphtheria toxin (DTA) is a cytotoxin, which inhibits protein synthesis in both proliferating and non-proliferating cells. It was firstly described in a transgenic mouse model with overexpression of human heparin-binding epidermal growth factor-like growth factor (hHB-

EGF), which is an endogenous receptor of DTA, in hepatocytes. In zebrafish, overexpression of diphtheria toxin receptor (DTR) driven by acinar cell specific promoter (*ela3l* promoter) has been proven to kill acinar cells efficiently (71). However, the apoptosis process is slower than that shown in NTR/MTZ model. Alternatively, DTA has also been applied to deplete specific cell types in either constitutive or tissue specific manner. Transgenic lines with a DTA cassette downstream of a floxed terminator, and under the control of ubiquitous promoter (such as *bactin2* promoter, heat-shock promoter) or tissue specific promoter have been generated. These lines can be crossed with tissue specific Cre lines for temporally-controlled cell specific ablation in cardiomyocytes, V1 neurons, β-cell, and enteroendocrine cells (72), (73), (74), (75).

3.1.3 Caspase 8 system

Direct activation of the apoptosis pathway is another useful approach to induce cell death. This is accomplished by the overexpression of membrane bound Caspase8 together with treatment of the dimerization agent AP20187 (Dim) (76). Schmitner et al characterized this system by generating an ela3l-expressing acinar cell depletion model. They found that Caspase8 induced-apoptosis can be detected after 8 hours treatment in zebrafish larvae (71). This method can achieve 95%-99% ablation efficiency with 8 μM Dim treatment for 48-96 hours. Moreover, single dose of intraperitoneal injection of Dim can also induce massive acinar cell loss (71).

3.1.4 Streptozotocin (STZ) treatment

STZ is a commonly used reagent in ablating β-cells in rodents. In zebrafish, instead of adding the compound directly in water, intraperitoneal injection of STZ with very high concentration is needed to kill β-cells in adult zebrafish, which limits its usage (77). The utility of STZ in zebrafish larvae and juvenile fish has not yet been reported.

3.1.5 Overexpression of truncated human BH3 Interacting Domain Death Agonist (BID) gene

BID is a “BH3 domain only” pro-apoptotic subgroup of BCL-2 family member, which can induce cell apoptosis (78). Li et al. generated a zebrafish β-cells specific ablation model by overexpressing truncated human BID in β-cells with the design of tetracycline and ecdysone-inducible system. The β-cells can be ablated with doxycycline hyclate (100 mmol/l in ethanol) and tebufenozide (50 mmol/l in DMSO) treatment for 48 hours (54), (79). However, compared with NTR-based system, this method is less potent in ablation efficiency, with on average 7.5 cells remaining after the treatment.

3.2 Zebrafish islet inflammation model

Insulitis, cytokine production and immune cell infiltration are key features of type I diabetes. Notably, RIPK3-dependent interleukin-(IL)1β induction in β-cells is a conserved instigator of islet inflammation (80). Overnutrition in a muscle insulin resistant zebrafish model (by overexpression of a dominant-negative insulin growth factor 1 receptor in the muscle) can cause islet inflammation, β-cell dysfunction, and β-cell loss (80). Nevertheless, a more straightforward way to stimulate islet inflammation is to overexpress proinflammatory cytokines in the islet. Delgadillo-Silva et al generated transgenic zebrafish lines with IL1β constitutively overexpressed in β-cells (81). Combining live imaging and calcium imaging, researchers observed the substantial macrophage infiltration and prolonged macrophage retention in the islet, which also leads to α-cell expansion and the deficiency of glucose stimulated calcium influx. In order to mimic islet inflammation in a reversible, spatiotemporally-controlled manner, Anderson et al. carried out a further optimization by generating a novel Cre-based tetracycline-inducible transgenic zebrafish line, named CETI-PIC3. This system can switch-on and -off three main proinflammatory cytokines (TNFα, Interferon gamma and IL1β) specifically in β-cells and it showed major features of type I diabetes in the islet, including ROS-mediated oxidative stress, endoplasmic reticulum stress, and various immune cells infiltration (82).

3.3 Partial pancreatectomy model and pancreatic ductal ligation

Surgical removal of 90% pancreatic tissue has been commonly used in rodents as a model to study β-cell regeneration, and researches have reported a profound compensatory β-cell proliferation in the remaining pancreata (83). In zebrafish, partial pancreatectomy can dramatically induce β-cell and ductal cells proliferation (77). Additionally, lineage tracing experiments showed that centroacinar cells can contribute to regenerative β-cells to a small extent in adult zebrafish under this condition (50). Pancreatic ductal ligation, a severe injury model triggering an immune response, demonstrated that *neurog3* positive endocrine progenitor cells may present in mouse pancreatic duct, indicating duct-to-β cell neogenesis as an important source for β-cell regeneration (84). However, this model has never been exploited in adult zebrafish model.

3.4 β-cell regeneration

Studies have shown that zebrafish keep several ways to replenish the loss of β-cells. Currently, zebrafish is considered as an excellent model to study cellular and molecular mechanism of β-cell regeneration. Additionally, zebrafish larva is widely used as an in vivo drug screening platform to identify potent small molecules inducing β-cell regeneration. These advantages have paved the new way for further translational studies in higher

organisms for drug discovery. Here, I summarize the preceding findings based on the different sources of β-cell regeneration and followed with the associated translational works.

3.4.1 β-cell proliferation

β-cell proliferation is considered as the major approach to replenish β-cell loss in mouse and human. In zebrafish, there was one systematic analysis examining cell proliferative state in three β-cell injury models (STZ, MTZ and pancreatectomy) in adult zebrafish (77). It showed that in STZ model, β-cell proliferation is rarely found within 7 days after ablation; however, the ins:GFP positive cells, indicating regenerative β-cells, can be identified in the ductal epithelium (77). Two weeks after STZ treatment, a number of scattered Pcna positive cells were present in the islets, in contrast to vehicle controls. In MTZ model, a large number of pdx1 positive dividing cells can be detected in both the islet and ductal epithelium. However, in two weeks after ablation, the Pcna positive cells are mainly restricted outside of islets; whereas the proliferative state within the islets is comparable to vehicle controls. In partial pancreatectomy model, we can see more prominent β-cell proliferation (77). These results suggested that distinct regenerative cellular responses can be stimulated by different injury models.

In zebrafish, chemical screening is an unbiased method to identify novel drivers of β-cell proliferation. Several low-medium-throughput chemical screening studies reported that chemical inhibitors of non-canonical IκB kinases (IKKs), TANK-binding kinase 1 (TBK1), IκB kinase ε (IKKε), and serotonin modulator are potential inducers for β-cell proliferation (85), (86). Wang et al. developed a zebrafish high-throughput screening platform, named automated reporter quantification in vivo (ARQiv), for targeted chemical screening involving all FDA (Federal Drug Administration) approved drugs. They found that two chemicals targeting serotonin signaling, paroxetine and amitriptyline, which belong to norepinephrine/serotonin reuptake inhibitor, can increase the number of proliferating β-cells (87). More importantly, two large-scale chemical screening studies done by Andersson et al. and Charbord et al. identified 2 potent small molecules in zebrafish and demonstrated translational potential in mouse or human cells. In the first study, by screening more than 7000 small molecules in the β-cell NTR/MTZ ablation model, Andersson et al identified several hits that converge on the adenosine pathway (88). In particular, 5'-N-ethylcarboxamidoadenosine (NECA), an adenosine receptor A2aa agonist, displays highest potency. Interestingly, the pro-proliferative effect from NECA is very mild during normal development. More importantly, NECA can also promote β-cell proliferation in isolated mouse islet as well as in vivo mouse STZ model (88). In the follow-up study, using β-cell-specific adenosine receptor A2a mutant mice, Schulz et al. demonstrated a reduction of β-cell proliferation at the basal level and a deficiency of compensatory β-cell proliferation during pregnancy (89). Another study, which was conducted independently during the same time in the Melton lab, employed a cell-based platform and also identified that a class of adenosine kinase inhibitors can selectively enhance β-cell proliferation in three species (mouse, rat and pig) without affecting the proliferative state

in other cell types (90). The second study done by Charbord et al. creatively applied a novel luminescence cell cycle indicator, luminescence ubiquitination-based cell cycle indicator (LUCCI) system, under the control of ins promoter to indicate β-cell proliferative states using luminescence as the readout for in vivo drug screening. Using this system, the researchers screened more than 3,000 small molecules and identified that HG-9-91-01 is a potent driver of β-cell proliferation by inhibiting salt-inducible kinases (SIKs), which would induce activating transcription factor (ATF)6-dependent unfolded protein response (UPR) prior to cell cycle entry (91). Translational studies in mouse and human β-cells confirmed its conserved role (91) indicating a universal mechanism inducing β-cell proliferation.

3.4.2 Trans-differentiation from other pancreatic cells

In vivo direct reprogramming studies to convert committed cell types in the pancreas (α-cell, δ-cell, γ-cell, and acinar cell) to β-cells have been extensively studied in the mouse model (92), (93), (94), (95), (96), (97), 29025873 (98), (99), (100). In zebrafish, several lineage tracing experiments reported direct α-to-β conversion events exist after β-cell ablation using *gcga:Cre* and *arx:Cre* lines (101), (102), (103), (79). These studies showed that glucagon, the inhibition of TGFβ signaling, artemether or aspterric acid treatment can promote such process. However, evidence from strict temporally-controlled lineage tracing experiments are still lacking. In addition, given the fact that we cannot rule out the potential leakage problem with these transgenics and whether other cell types, in particular pancreatic ductal cells, could also accounted for the replenished β-cells (data unpublished). Nevertheless, two recent studies, for the first time, identified a novel endocrine cell type featured by specific expression of a *sst* homolog, *sst1.1*, in the zebrafish islet (104), (74). *Sst1.1* (previously known as prss1) is an ortholog of mammalian somatostatin (with a conserved C-terminal sequence) and expresses in both the pancreas and the central nervous system; whereas *sst2*, another distinct version of somatostatin gene, shows exclusive expression in the pancreas (105), (106). *Sst1.1* positive cells, which were defined as a subpopulation of δ-cell, begin to form at 24hpf (107). Combining single-cell RNA-seq (cell cluster profiling, pseudo-time analysis), transgenic zebrafish lines as well as immunostaining, the two groups suggested that *sst1.1*$^{+}$ δ-cells are transcriptionally distinct from β-cells in the basal state, although they share several important transcription factors, such as *pdx1*. After β-cell ablation (by NTR/MTZ and DTA models), almost all regenerated insulin-producing cells are Insulin and Somatostatin double positive. However, due to a lack of *sst1.1*$^{+}$ δ-cell specific Creert2 line for lineage tracing, one still cannot rule out the possibility that regenerated hybrid cells may originate from ductal progenitors. Lastly, one very recent study indicated that the ghrelin positive ε-cells are also capable to trans-differentiate into β-cell in extreme β-cell loss condition (108). Such process can be accelerated by the deletion of *pax4*. Mechanistically, *Pax4* can bind to the cis-regulatory region of ghrelin and inhibit ghrelin expression. *Pax4* deletion can derepress ghrelin expression and promote ε-cells to β-cells trans-differentiation.

By analyzing a hypomorphic *ptf1a* mutant, Dong et al. showed that a normal level of ptf1a is critical in acinar cell fate determination, while a mild decrease of ptf1a expression can result in an increased number of endocrine cells, indicating that the gradient of ptf1a can differentially regulate pancreatic endocrine and exocrine cell fate (109). Further experiments showed that inhibition of *ptf1a* at later stages (after ventral bud formation) can lead to direct acinar-to-β cell conversion in zebrafish (110). In addition, the state-of-the-art chromatin epigenetic profiling provides novel inputs on the transcriptional regulation of pancreas development. A series of study done by Bessa and colleagues did interspecies analyses between zebrafish and human, and tried to elucidate the functional outcome of human disease related genetic variants located in cis-regulatory elements on the developmental defects of the pancreas (111), (112). Using genome-wide epigenetic profiling, one study provided an atlas of functionally equivalent cis-regulatory elements between zebrafish and human, regardless of sequence conservation. The deletion of one disease-relevant region located at the distal enhancer of ptf1a can lead to pancreatic agenesis, which is reminiscent of the phenotypes shown in human with mutations in a distal-enhancer of PTF1A. Such line of evidence further supports the conserved functional role of *ptf1a*/PTF1A in pancreas development (112).

Lastly, cells from a different germ layer might preserve a competence for trans-differentiation under certain extreme conditions. Using different mesodermal specific Cre/CreERT2 lines, Liu et al showed that *drl*$^{+}$/*etv2*$^{+}$ cells can become insulin-producing cells in avascular *npas4l* mutants upon β-cell ablation, although the detailed mechanism is still elusive (113).

3.4.3 β-cell neogenesis from a ductal origin

Immunostaining and lineage tracing experiment using tp1 promoter (a Notch-responsive element from the Epstein Barr virus) suggested that IPD, which are *nkx6.1* positive and highly Notch active ductal cells, can give rise to β-cells in the chemical ablation model. Delaspre et al firstly introduced the concept of centroacinar cells as Notch-responsive cells in adult zebrafish pancreas using *tp1* transgenic line (50). Using *Tg(tp1:CreERT2)* lines together with β-actin driven Cre responder lines with early 4-hydroxytamoxifen treatment, they showed that up to 75% of Notch-responsive ductal cells can be fluorescently labelled in the adult stage. At 10 days post ablation, 43% β-cell can be lineage traced, suggesting a large number of regenerative β-cells are of Notch-responsive duct origin. In partial pancreatectomy model, however, β-cells can hardly be traced back to *tp1* positive duct origin, suggesting that duct-to-β neogenesis event is injury model dependent and much less likely to happen when there remains a substantial number of pre-existing β-cells (50). Ghaye et al. further confirmed the latent progenitor potential of Notch-responsive IPD using *TgBAC(nkx6.1:EGFP)* lines (which normally label ductal cells) when co-stained with insulin in MTZ ablation model (29). On day 9 after ablation, scattered insulin positive cells can be observed at the core of islets and in the periphery of islet adjacent to the duct. Some insulin positive cells showed very weak EGFP signals, suggesting that these newly formed β-cells are of *nkx6.1*$^{+}$ duct origin.

Identifying small molecules boosting ductal-derived β-cell neogenesis is of great value, as it might be a promising strategy in the discovery of new drugs for the compensation of β-cell loss, i.e. from a duct origin and it might offer mechanistic insight into ductal-to-β differentiation. The first chemical screening searching for drugs inducing ductal-derived β-cell neogenesis was done by Rovira et al, with a focus on FDA-approved drugs for repurposing (56). Mechanistic studies showed that the suppression of cellular GTP levels and the inhibition of RA synthesis can induce precocious secondary islet formation (56). Another study conducted large-scale chemical screening to identify compounds demonstrating a synergistic effect with low-dose of Notch inhibition (114). In this study, Liu et al. discovered that a selective Cdk5 inhibitor can dramatically promote IPD to differentiate into β-cells in the secondary islet in both larvae and juvenile fish. Genetic loss-of-function experiments in zebrafish confirmed that *Cdk5* is the major drug target. Further experiments using the pancreatic ductal ligation model in adult mouse as well as human induced pluripotent stem (iPS) cells consolidate the findings showing that *Cdk5* performs as an endogenous suppressor of duct-to-β cell neogenesis across different species (114).

In addition, reverse genetic screening has also been done for the discovery of druggable targets (115). By analyzing RNA-seq data to identify differential expressed genes upregulated after β-cell ablation, Karampelias et al. carried out a small-scale reverse genetic screening of candidate genes, which code for soluble and membrane-bound gene products upregulated after β-cell ablation (115). This study found that both folate receptor 1 (*folr1*) overexpression and exogenous treatment with folinic acid can stimulate β-cell formation from a *tp1* positive duct origin. Interestingly, such effect was also seen in neonatal pig islet (116), suggesting that one-carbon metabolism is an evolutionarily conserved pathway for duct-to-β-cell neogenesis (115).

4 Transcriptional network, translational and epigenetic regulation in zebrafish pancreas development

4.1 Transcriptional regulation

The development of endodermal organs cells relies on a series of coordinated transcriptional regulatory events that take place at different stages (117). Such transcriptional regulatory map governed by transcription factors (TFs) is depicted by a large number of studies employing mutant lines or morpholino knock-down to disrupt TFs and associated signaling pathways. For example, *sox4b*, a member of the SRY-like HMG-box (SOX) family, is specifically required for α-cells differentiation, since knocking-down *sox4b* can lead to a downregulation of α-cell specific homeobox TF, *arxa* (118). The depletion of *pax6b* can markedly reduce β-cells and δ-cells, but significantly promote ε-cells. Nevertheless, a mild knock-down of pax6b can cause a rise in α-cells, suggesting that its distinct role in endocrine cell development is dosage-dependent (119). The expression of *pax6b* is transcriptionally regulated by homeoproteins *pdx1*, *pbx* and *prep* according to a gel shift assay showing the direct binding to regulatory elements (120). In addition, *pax6b* is a potent transcriptional activator of mnx1, which acts downstream of RA signaling to promote β-cells and repress α-cell fate (121), (122). *Isl1*, instead of *isl2a*/*isl2b*, has been shown to play distinct roles between the first and second wave of endocrine cell development, while the *isl2* genes can non-cell autonomously regulate exocrine pancreas expansion (123). *Cdx4* is specifically expressed in *sst2* positive δ-cells and is crucial for their specification (124). Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 mutagenesis and *myt1*$^{bulg029-/-}$ mutant showed prominent α-cell developmental impairments with no significant impact on insulin expression (124). The disruption of a maturity-onset diabetes of the young (MODY) gene, *rfx6*, can remarkably dampen non β-cell differentiation (125), (126).

Notably, there are also some major species differences between mouse and zebrafish. For example, *ascl1b* and *neurod1*, rather than *neurog3* in mammalian organisms, are endocrine progenitor markers and function as dual master regulators to confer ductal cells with differentiation competence towards the endocrine cell fate (127). It was shown that *neurod1* deficient fish fail to develop endocrine cells. In particular, α-cells are more sensitive to a mild reduction of *neurod1* (128). Unlike rodents and human, *pax4* is not required for β-cell and δ-cell development in zebrafish. Rather, it acts through a mutual repression model with *arxa* to negatively regulate α-cell formation (129). Other than that, most of TFs display conserved roles in endocrinogenesis across different species (127).

4.2 Posttranscriptional regulation

Posttranscriptional regulation, including non-coding RNA (microRNA, long non-coding RNA, circular RNA) and mRNA translational initiation and elongation, are also involved in the pancreas development. The overexpression of pre-miR-21 in zebrafish β-cells exhibited a reduced glucose tolerance, an attenuation of peak GSIS, a downregulation of β-cell identity and maturity markers, and an increase in insulin and glucagon hybrid cells. The overall effects were partially determined by targeting *tgfb2* and *smad2* mRNAs, implicating that the dysregulation of microRNAs can contribute to β-cell dysfunction and the impairment of β-cell differentiation (130).

Polyamine synthesis is essential in exocrine tissue growth and the maintenance of β-cell mass in zebrafish pancreas by the posttranslational modification of eIF5A, a mRNA translation initiator (131). The hyperactive protein synthesis is required for digestive organ development. One study reported that the *urb1*^{cq31} mutant, which demonstrated a disruption of a key ribosome protein biogenesis, showed hypoplastic liver and pancreas; the phenotypes also resembled the *mtor* and *raptor* morphants (132). Lastly, the overexpression of *urb1* can efficiently rescue *mtor* and *raptor* morphants phenotypes, suggesting that *urb1* acts downstream of mTOR complex 1 in liver and pancreas development (132).

4.3 Epigenetic regulation

Epigenetic modifications are key modulators in pancreas and islet cell differentiation. The histone deacetylase 1 (*hdac1*), which performs as a transcriptional repressor, is critical in several sequential steps in endoderm organogenesis (133). The *hdac1* mutant line displayed a wide range of developmental defects, including ectopic endocrine clusters displayed anterior to the main islet, exocrine pancreas dysplasia, an impairment of HPD morphogenesis, and an expansion of the foregut endoderm (133). Likewise, the *med12* mutant also demonstrated multiple digestive organ developmental defects. This is because med12 functions as a regulatory subunit of transcriptional Mediator complex activating the transcription of *sox17*, which is a master regulator in endoderm development (134). DNA methyltransferase 1(*dnmt1*) mutant lines can develop pancreas and liver at an early developmental stage, but acinar cells begin to degenerate after 84 hpf via apoptosis, whereas the endocrine cells and ductal cells can maintain intact (135). Most interestingly, the dnmt1 deficiency can enhance the restoration of insulin-producing cells in the NTR/MTZ model, indicating that epigenetic reprogramming is a crucial drug target for β-cell regeneration (135)).

5 Microenvironment -- vasculature and pancreas innervation

As described in the previous chapters, factors from neighboring tissues (such as mesoderm, notochord, and other endodermal organs) exert complex cell-cell signaling to regulate early pancreas specification and growth. Notably in later development, the vasculature and peripheral nervous system have been shown to play distinct roles either in the maintenance of stem cell quiescence or in the stimulation of stem cell activation across different stem cell niches. More specifically, in the zebrafish pancreas, β-cell specific overexpression of dominant negative of *vegfaa* (*dnVegfaa*) can lead to pancreas vascular deficiency and endocrine cell disruption without a severe impairment of β-cell function (136). In contrast, the overexpression of the soluble isoform of human Vegf receptor 1 (*sFlt1*) in β-cells can greatly suppress the formation of the vasculature in the pancreas. Meanwhile, sFlt1 can induce large sheets of β-cell formation. These β-cells reside along the pancreatic ducts, indicating accelerated duct-to-β cell neogenesis. Moreover, such alteration consequently disturbs the glucose response and tolerance. These phenotypes were not phenocopied in mutants lack of *vegfaa*, *vegfab*, *kdrl*, *kdr* or *flt1* function, indicating that such prominent phenotype was derived from the interference of multiple ligand/receptor pairs (136). Interestingly, the pancreas vasculature system is also required for the proper pancreatic innervation (137). These peripheral autonomic and sensory neurons have close connections with islets and then migrate away. Specific ablation of these neurons can partially inhibit other neurons moving away from the islet, which ultimately leads to a diminished islet innervation (137). In one study, combining live imaging, calcium imaging, chemogenic inhibition of neuronal activity, two-photon cell ablation, as well as optogenetic cell activity manipulation, Yang et al. performed a comprehensive analysis of neuronal-islet coupling status in acute and chronic neuronal activity loss condition (138). They, for the first time, describe that all endocrine cells are in connectivity with peripheral neurons. The abolishment of neural activity in either acute or chronic manner can dramatically diminish the β-to-β and α-to-β activity coupling. More interestingly, they identified that a subset of δ with detectable peri-islet neural activity coupling displayed higher homotypic coupling activity compared with their counterparts, suggesting that some δ-cell receiving the input from the peripheral nerves function as the hub in coordinating the δ-cell activity (138). And such functional pattern seems to be conserved and reminiscent of β-cells. As a previous study described, a subset of β-cells worked as leader cells in coordinating β-cells calcium influx as responsiveness to low- and high-blood glucose level in both zebrafish and rodents (62).

6 CRISPR-Cas9 genome editing in the construction of zebrafish models

With the advent of genome editing, various related techniques have been applied to generate genetically modified animals. Among these methods, the CRISPR-Cas9 system has become the most popular method to generate either knock-out mutant or knock-in animals due to its remarkable versatility compared with Zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) (139), (140). Knock-in animal models are particularly useful in the field the developmental and regenerative biology research, as the integrated genetic cassettes can be driven by the endogenous regulatory elements and thereby recapitulate the natural gene expression pattern, which is often hard to accomplish by classical promoter-cloning transgenics and Bacterial Artificial Chromosome (BAC) cloning. Currently, multiple knock-in strategies have been devised and refined for the construction of non-human primate (141), (142) , and zebrafish models (143), (144). In zebrafish, the knock-in approaches vary with the respect to the targeting regions (such as 5' non-coding region upstream of ATG, exon, intron, or 3' end) (145), (146), DNA double-stranded break repair mechanisms (homology directed repair [HDR] or non-homologous end joining [NHEJ]) (147), the types of donor (148), the injection of Cas9 protein or Cas9 mRNA, and the application of chemicals in promoting HDR (149), (150).

In zebrafish, the 5' knock-in methods have been explored independently in different labs. Researchers usually target the loci upstream of the ATG coupled with donor plasmids with or without in vivo linearization site(s) on either one side or both sides of insertion sequences (147), (151). The single linearization site upstream of the insertion sequence can facilitate the NHEJ-mediated integration (144), (152), (153); while the HDR can also be achieved by adding long homologous arms (HAs) flanked by two I-SceI/gRNA recognition sites on both sides (143). Currently, several fluorescent reporter lines, and CreERT2 lines, have been generated using such methods (154), the widespread applications are still hindered in several aspects, such as the disruption of one allele of the targeted gene, the cumbersome molecular cloning, low scalability and low efficiency of genetic integration. Similar to generating knock-in in mouse models and human cell lines, the 3' knock-in method has also been employed coupled with circular plasmids as the donor, with either long or short homologous arms flanked by two in vivo linearization sites (155), (156). The advantage of 3' knock-in using HDR method is that it can keep the knock-in cassettes in-frame and thereby more likely to retain the functionality of the targeted gene. However, in certain cases, insertion or deletion changes (of a few amino acids) might still occur when using the NHEJ strategy with donors in the absence of homologous arms (157). Several studies reported that the HDR mediated 3' knock-in efficiency is highly dependent on the length of the HAs (> 500 bp HAs are favorable), in particular when the gRNA targeting region is located upstream of the stop codon (143), (144). In addition, one recent study showed that the introduction of short HAs in the donor plasmids flanked by two linearization sites can facilitate microhomology-mediated end joining (MMEJ) and can also achieve promising knock-in efficiency (158). However, such methods are still limited in use due to the low scalability and the tedious constructs preparation steps. Recently, intron-based

and exon-based knock-in approaches have remarkably expanded the knock-in toolkits by targeting genetic loci beyond the 5' or 3' end (159), (160), (161). These methods rely on the NHEJ method and the targeted genes can be either destroyed or rescued (depending on whether or not the exon sequences downstream of the insertion site are added into the donor). Such methods are particularly useful to introduce loxP sites for generating genetic conditional knock-outs. Considering that all the above methods are compromised by certain drawbacks and are labor-intensive with limited scalability, the development of a straightforward and efficient knock-in methodology is still warranted in the zebrafish field. An ideal method should address on the following issues, including the disruption of the targeted gene allele (knock-in/knock-out), the complex pipeline in molecular cloning, low efficiency of precise recombination, limited germline transmission rate, and high workload in screening for founders.

Recent studies in mouse and in vitro systems have demonstrated several approaches that strikingly improve homology mediated end joining (HMEJ). The Tild-CRISPR (targeted integration with linearized dsDNA-CRISPR) strategy has been successfully applied in generating knock-in mouse lines (162). This method used PCR-amplified or enzymatic-cut donors with 800 base pairs HAs as the direct donor, indicating that nude double-stranded DNA (dsDNA) can serve as an effective donor in eukaryote embryos. Furthermore, 5' modified dsDNA with short HAs (roughly 50 base pairs) demonstrated impressive knock-in efficiency in an in vitro culture system (163). In this study, by systematic comparisons among 13 modifications on dsDNA with gRNA targeting the 3' untranslated region (UTR) of the GAPDH gene in HCT116 cells, the researchers observed that C6 linker (AmC6) or C12 linker (AmC12) as well as moieties by adding on secondary modifications out-performed the nude dsDNA as the donor with a marked increase of knock-in efficiency (> 5-fold). Although the mechanism is still elusive, it is proposed that the 5' modification can assist in preventing donor degradation and multimerization and thus circumvent stochastic NHEJ, indicated by less NHEJ events and random insertions.

Inspired by the progress of knock-in strategies in different systems, we tried to leverage the advantages of various improvements in an effort to generate knock-in zebrafish lines for multiple utility in a scalable fashion. Considering that during early development, the zebrafish zygotes undergo very rapid cell division indicating an unfavorable microenvironment for genetic integration, to tackle this problem, we combined 5' modified dsDNA with short homologous arms as the donor combined with Cas9 protein, and we manage to knock-in large piece of DNA (~ 3,000 bp) without disrupting the endogenous gene products. To date, we have successfully made ~30 knock-in lines targeting ~20 genetic loci and used these genetic tools to answer key questions in endodermal organ development and regeneration, which I elaborate more on in the result and summary session.

7 Single-cell technology in developmental biology and regenerative medicine

In the past decades, single-cell transcriptomics analyses have opened up new avenues in elucidating key biological questions, particularly in developmental biology and tissue regeneration. By profiling the transcriptional signatures of thousands of cells, researchers are able to identify novel cell types or cell states, interrogating the cellular behaviors in cell state conversion, cell communications and unravel the gene regulatory networks in key biological processes. The rationale of single-cell transcriptomics is to capture the messenger RNA (mRNAs) from individual cells, converting them to cDNA and amplifying them with PCR for sequencing. Starting from 2009, Tang and colleagues for the first time seek to provide the transcriptional signature of hundreds of cells in the human embryonic blastomere (164). In the following years, the major improvement of single-cell technologies appear in the aspect of (1), capturing more amount of mRNA (2), accomplishing more accurate quantification of number of transcripts with RNA spike-in or UMI (3), achieving higher throughput with the aim to sequence more than ten thousand or millions of cells with relatively low cost (from low- and intermediate-throughput well-based method to droplet based high-throughput techniques which can sequence millions of cells) (4), multiomics analyses involving epigenetic states profiling, such as chromatin accessibility (Assay for Transposase-Accessible Chromatin, ATAC-seq) (165) and histone modifications (Cleavage Under Targets and Tagmentation) (166), (167), metabolic states, lipidomics (168), genomics (169), as well as integrating tissue architecture and spatial information of the whole tissue in parallel (170). With the emergence of these techniques, a large variety of analytical tools have been created to disentangle the complexity of the data.

The single-cell technology has become a powerful tool and adds on novel information on the basis of various genetic tools. In the field of developmental biology and regenerative medicine, the single-cell transcriptomics can decode the composition of numerous cell types and states in various conditions (such as heathy states, disorders, drug treatment states). By comparing the transcriptomics among different cell states in different conditions, we can identify the key signaling pathways in driving the distinct molecular signatures and discovered favorable or detrimental factors in tissue regeneration. As the cell fate transition is a continuous process, capturing cells in transitional states enable us to infer the developing trajectory and gain more mechanistic insights on the key molecular event governing cell fate conversion and acquisition. For this purpose, enormously valuable tools have been created, such as monocle2, 3, PAGA, slingshot, RNA velocity, CellRank and the recently published dynamo. These tools applied mathematical modeling and machine learning algorithms. Some of them also leverage mRNA splicing/unsplicing or nascent RNA information to infer cell vectors and to predict the cell fate determination of each cell. Further, as the development of organs and tissue regeneration are precisely orchestrated processes involving numerous cell types to coordinate with each other, the cell-cell interplay based on the physically direct contact and secreted

factors play pivotal roles in directing the organization of tissue development and tissue recovery from injury. Currently, new strategies committed in sequencing neighboring cells with the downstream computational deconvolution step (such as PIC-seq, CIM-seq) allow researchers to identify the direct cell-cell contact and signaling pathways (171), (172). Also, other information such as ligand-receptor pairs, the expression the co-receptors, and the transcriptional activity of the signaling pathways' downstream genes have also been widely employed to screen for potential cell-cell communication (CellphoneDB, iTalk, Cellchat, Nichenet). This is particularly useful when researchers try to mimic the endogenous biological process in vitro. The spatial transcriptomics was firstly introduced by Stahl and colleagues. Although the commercialized version 10x visium still cannot reach single-cell resolution, the technique has been applied in elucidating the cell composition as a good complement to the tissue dissociation single-cell RNA-seq (173), (174). Apart from that, other spatial transcriptomics methods have been introduced (Slide-seq, Slide-seq2, in situ sequencing) and optimized (MERFISH, osm-FISH, ELL-FISH). Currently, a new technology named Stereo-seq manage to profile the transcriptomics signature in single-organelle resolution with preservation of spatial information. This technique can achieve roughly 4000 dots for each cell and has been used to decipher the mouse embryonic development, non-human primate brain development, zebrafish early development, salamander brain regeneration and etc. Multiomics assessments with epigenetic profiling in single-cell resolution have become a striving topic in single-cell technology as it can provide another layer of evidence accounting for how the transcriptomics is settled. The chromatin accessibility analysis in single-cell resolution (scATAC-seq) has been commercialized coupled with droplet based single-cell transcriptomics in 10x Genomics. This technique has been applied in investigating the development of brain, retina and limb regeneration. Many groups around the globe have been trying to go one step further in elucidating the histone modification and DNA methylation pattern in single-cell resolution. Two groups have combined the droplet-based microfluidics together with Tn5 transposase in conjunction of antibody/nanobody treatment and created the single-cell CUT & Tag technology. These integrative analyses can help us understand the conservation and differences in embryo and organ development as well as during tissue regeneration programs among different species.

The research of early zebrafish development has made great contributions in the application of single-cell technologies and nowadays we are able to portrait the cell developmental path within the first 24 hours with or without spatial information. As zebrafish is a powerful model organism in regenerative biology, large numbers of studies have been using single-cell transcriptomics in examining the lineage path of mesoderm, endodermal organ (including the pancreas, intestine, liver), central nervous system and pituitary gland, neural crest derive cells and hematopoietic stem cell. Tissue regeneration processes have also been intensively investigated, such as heart regeneration and epicardial trans-differentiation, β-cell regeneration after chemogenic β-cell regeneration, retina and fin regeneration as well as in various disease

models (alhzheimer’s disease, inflammatory bowel disease, liver fibrosis, tumorigenesis such as melanoma) (175), (176), (104), (177), (178), (179), (180), (181), (182).

8 Materials and methods

8.1 Animals

All animal work was permitted by the Ethical Committee on Animal Experiments (Stockholm North Committee) and conducted in accordance with the provisions and guidelines of the Swedish Animal Agency.

The detailed information of the transgenic and knock-in zebrafish lines is listed below.

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers or Reference**	**Used in study**
Strain, strain background (Danio rerio)	*Tüpfel long fin (TL)*	https://zfin.org/ZDB-GENO-990623-2		
Genetic reagent (Danio rerio)	*Tg(ptf1a:GFP)jh1*	PMID: 16258076	ZFIN: ZDB-ALT-070531-2	
Genetic reagent (Danio rerio)	*Tg(ela3l:H2BGFP)*	PMID: 28138096		
Genetic reagent (Danio rerio)	*Tg(Tp1bglob:eGFP)um14Tg*	PMID: 22492351		
Genetic reagent (Danio rerio)	*Tg(Tp1bglob:H2BmCherry)S939*	PMID: 22492351	ZFIN: ZDB-ALT-110503-3	
Genetic reagent (Danio rerio)	*Tg(-3.5ubb:loxP-EGFP-loxP-mCherry)cz1701*	PMID: 21138979	ZFIN: ZDB-ALT-110124-1	
Genetic reagent	*Tg(T2KTp1glob:creERT2)jh12*	PMID: 21208992		

(Danio rerio)				
Genetic reagent (Danio rerio)	*Tg(ubb:LOXP-CFP-LOXP-zgc:114046-mCherry)jh63*	PMID: 25773748	ZFIN: ZDB-ALT-151007-31	
Genetic reagent (Danio rerio)	*TgKI(krt92-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(krt4-p2A-mNeonGreen)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(krt4-p2A-mNeonGreen-t2A-iCre)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(krt4-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(nkx6.1-p2A-mNeonGreen)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(nkx6.1-p2A-mNeonGreen-t2A-iCre)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(nkx6.1-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(id2a-p2A-mNeonGreen)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(id2a-p2A-mNeonGreen-t2A-iCre)*	Generated by Jiarui Mi		

Genetic reagent (Danio rerio)	*TgKI(id2a-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(onecut1-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgKI(hes6-p2A-EGFP-t2A-CreERT2)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*TgBAC(cftr:gal4)pd1101*	PMID: 25592226		
Genetic reagent (Danio rerio)	*Tg(uas:GFP)zf82*	PMID: 18202183		
Genetic reagent (Danio rerio)	*Tg(4xUAS:Cre)*	PMID: 25214630		
Genetic reagent (Danio rerio)	*Tg(ins:FLAG-NTR,cryaa:mCherry)s950*	PMID: 22608007		
Genetic reagent (Danio rerio)	*TgBAC(neurod1:EGFP)nl1*	PMID: 18305245		
Genetic reagent (Danio rerio)	*TgBAC(pdx1:EGFP)bns13*	PMID: 31142539		
Genetic reagent (Danio rerio)	*TgBAC(ascl1b:EGFP-2A-Cre-ERT2)ulg006*	PMID: 26329351		
Genetic reagent	*Tg(kdrl:dsRed)*	PMID: 26109203		

(Danio rerio)				
Genetic reagent (Danio rerio)	*Tg(nkx2.2a:mEGFP)vu17*	PMID: 17099706		
Genetic reagent (Danio rerio)	*Tg(ins:LOXP-mCherry-LOXP-Hsa.HIST1H2BJ-GFP,cryaa:Cerulean)s934*	PMID: 21497092		
Genetic reagent (Danio rerio)	*Tg(ins:Hsa.HIST1H2BJ-GFP)s960*	PMID: 25117518		
Genetic reagent (Danio rerio)	*Tg(sst2:Cre; cryaa:YFP)s963*	PMID: 25852199		
Genetic reagent (Danio rerio)	*Tg(sst2:NTR,cryaa:Cerulean)KI102*	PMID: 27516442		
Genetic reagent (Danio rerio)	*Tg(gip:Cre)*			
Genetic reagent (Danio rerio)	*Tg(Hsa.CTGF:NLS-mCherry)ia49*	PMID: 29976931		
Genetic reagent (Danio rerio)	*Tg(-2.5cadherin17:mCherry)*	PMID: 25401745		
Genetic reagent (Danio rerio)	*Tg(-5.2foxj1a:EGFP)*	PMID: 27284198		
Genetic reagent (Danio rerio)	*Tg(neurod1:CreERT2, cryaa:Venus)*	Generated by Jiarui Mi		

Genetic reagent (Danio rerio)	*Tg(ins:CreERT2, cryaa:Venus)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*Tg(foxj1a:iCre; cryaa:Venus)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*Tg(ins:loxP-dsRed-terminator-stop-loxP-DTA)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*Tg(ins:loxP-CFP-stop-loxP-H2BmCherry)*	Generated by Jiarui Mi		
Genetic reagent (Danio rerio)	*Tg(ins:TagRFP-T-P2A-sflt1)bns286*	PMID: 31597659		
Genetic reagent (Danio rerio)	*Tg(ins:TagRFP-T-P2A-dnvegfaa)bns284*	PMID: 31597659		

Table 1. Zebrafish lines used in this thesis

8.2 Antibody list

The detailed information of antibodies used in this thesis is listed below

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Antibody	Anti-GFP (chicken polyclonal)	Aves Labs	GFP-1020	(1:500)
Antibody	Anti-mNeonGreen (mouse monoclonal)	Chromotek	32F6	(1:50)

Antibody	Anti-tdTomato (goat polyclonal)	MyBioSource	MBS448092	(1:500)
Antibody	Anti-Insulin (rabbit polyclonal)	Cambridge Research Biochemicals	Customised	(1:100)
Antibody	Anti-Glucagon (mouse monoclonal)	Sigma-Aldrich	G2654	(1:100)
Antibody	Anti-Vasnb (rabbit crude sera)	PMID: 26492970	Customised (a gift from Dr. Paolo Panza)	(1:1000)
Antibody	Anti-Cdh17 (rabbit polyclonal)	PMID: 27401686	Customised (a gift from Prof. Cao Ying)	(1:1000)
Antibody	Anti-Cldnc (rabbit polyclonal)	PMID: 27401686	Customised (a gift from Prof. Cao Ying)	(1:200)
Antibody	Anti-Sst1.1 (rat monoclonal)	PMID: 35088828	GTX39061	(1:50)

Table 2. Antibodies used in the thesis

8.3 Sequence reagent list

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
Recombinant DNA reagent	*krt92*-donor-p2A-EGFP-t2A-CreERT2 (plasmid)	GenScript	Synthesized	p2A-EGFP-t2A-CreERT2 flanked by 950 base pairs homologous arms to the *krt92* 3' end

Recombinant DNA reagent	*nkx6.1*-donor-p2A-EGFP-t2A-CreERT2 (plasmid)	GenScript	Synthesized	p2A-EGFP-t2A-CreERT2 flanked by 950 base pairs homologous arms to the *nkx6.1* 3' end
Recombinant DNA reagent	*nkx6.1*-donor-p2A-mNeonGreen-t2A-iCre (plasmid)	GenScript	Synthesized	p2A-mNeonGreen-t2A-iCre flanked by 950 base pairs homologous arms to the *nkx6.1* 3' end
Sequence-based reagent	long HA_common_FWD	This paper	PCR primer	CTCGGTACCCGGGGATC
Sequence-based reagent	Long HA_common_REV	This paper	PCR primer	GCAGGTCGACTCTAGAGGATC
Sequence-based reagent	*krt4*_common_FWD	This paper	PCR primer	CCAGTGTCACCACCGTCAGCAGTAAACGCTATGGAAGCGGAGCTACTAACTTCAG
Sequence-based reagent	*krt4*_ p2a-mNeonGreen _REV	This paper	PCR primer	CTGTGTCGGCTGGGGGTTTGGACGGGCTTCTCCTTACTTGTACAGCTCGTCCATGC
Sequence-based reagent	*krt4*_ p2A-mNeonGreen-t2A-iCre _REV	This paper	PCR primer	CTGTGTCGGCTGGGGGTTTGGACGGGCTTCTCCTTAGTCCCCATCCTCGAGCAG
Sequence-based reagent	*krt4*_ p2A-EGFP-t2A-CreERT2 _REV	This paper	PCR primer	CTGTGTCGGCTGGGGGTTTGGACGGGCTTCTCCTTAAGCTGTGGCAGGGAAACCC
Sequence-based reagent	*nkx6.1*_common_FWD	This paper	PCR primer	ACACAGCTCTTATCATTCATACGTCGGAAAACGAGAGCTCGGGAAGCGGAGCTACTAACTTC

Sequence-based reagent	*nkx6.1*_ p2a-mNeonGreen _REV	This paper	PCR primer	GAAGAATCTGACAACCCATTTCCCGTTTCCTTTTTACTTGTACAGCTCGTCCATGC
Sequence-based reagent	*nkx6.1*_ p2A-mNeonGreen-t2A-iCre _REV	This paper	PCR primer	GAAGAATCTGACAACCCATTTCCCGTTTCCTTTTCAGTCCCCATCCTCGAGCAG
Sequence-based reagent	*nkx6.1*_ p2A-EGFP-t2A-CreERT2 _REV	This paper	PCR primer	GAAGAATCTGACAACCCATTTCCCGTTTCCTTTTTAAGCTGTGGCAGGGAAACCC
Sequence-based reagent	*id2a*_common_FWD	This paper	PCR primer	ATCACAGAGGACAGCAGGACACTTTACCGTGGAAGCGGAGCTACTAACTTCAG
Sequence-based reagent	*id2a*_ p2a-mNeonGreen _REV	This paper	PCR primer	ACATTGTATTTTCAAGAAAACTTACCTGATTACTTGTACAGCTCGTCCATGC
Sequence-based reagent	*id2a*_ p2A-mNeonGreen-t2A-iCre _REV	This paper	PCR primer	ACATTGTATTTTCAAGAAAACTTACCTGATTAGTCCCCATCCTCGAGCAG
Sequence-based reagent	*id2a*_ p2A-EGFP-t2A-CreERT2 _REV	This paper	PCR primer	ACATTGTATTTTCAAGAAAACTTACCTGATTAAGCTGTGGCAGGGAAACCC
Sequence-based reagent	*hes6*_common_FWD	This paper	PCR primer	ACATTATACACCAACAAGTCCATTTGGAGACCCTGGGGAAGCGGAGCTACTAACTTCAGC
Sequence-based reagent	*hes6*_ p2A-EGFP-t2A-CreERT2 _REV	This paper	PCR primer	ACTTGAATGTTCATGAATCGGCGGAGGTGAGCATCTGTCTAAGCTGTGGCAGGGAAACCC

Sequence-based reagent	*onecut1*_common_FWD	This paper	PCR primer	GCCAACTCCTCCTCCTCCAGCACTTGTACCAAAGCAGGAAGCGGAGCTACTAACTTCAGC
Sequence-based reagent	*onecut1*_ p2A-EGFP-t2A-CreERT2_REV	This paper	PCR primer	TTTTTTCTTTTTTTCCCCACAATGCAGCCGTTACTCCATCAAGCTGTGGCAGGGAAACCC
Sequence-based reagent	*krt92*_fwd	This paper	qPCR primer	CCGAAACCCTCACCAAGGAA
Sequence-based reagent	*krt92*_rev	This paper	qPCR primer	CCTCGCTCGTAGATTGGGAG
Sequence-based reagent	*krt4*_fwd	This paper	qPCR primer	AACAAGCGTGCTTCCGTAGA
Sequence-based reagent	*krt4*_rev	This paper	qPCR primer	GCGATCATGCGGTTGAGTTC
Sequence-based reagent	*nkx6.1*_fwd	This paper	qPCR primer	CGTGCTCACATCAAAAC
Sequence-based reagent	*nkx6.1*_rev	This paper	qPCR primer	CGGTTTTGAAACCACACCTT
Sequence-based reagent	*id2a*_fwd	This paper	qPCR primer	CAGATCGCGCTCGACTCCAA

Sequence-based reagent	*id2a*_rev	This paper	qPCR primer	CAGGGGTGTTCTGGATGTCCC
Sequence-based reagent	*b-actin*_fwd	This paper	qPCR primer	CGAGCAGGAGATGGGAACC
Sequence-based reagent	*b-actin*_rev	This paper	qPCR primer	CAACGGAAACGCTCATTGC
Sequence-based reagent	EGFP	This paper	Sequencing primer	CATGTGGTCGGGGTAGCG
Sequence-based reagent	mNeonGreen	This paper	Sequencing primer	ACTGATGGAAGCCATACCCG
Sequence-based reagent	Chemically synthesized Alt-R®-Modified_tracrRNA	IDT	tracrRNA	
Sequence-based reagent	Chemically synthesized Alt-R®-modified_*krt92*_crRNA	IDT	crRNA	5'-AACCTCGCTCGTAGATTGGG(AGG)-3'
Sequence-based reagent	Chemically synthesized Alt-R®-modified_*krt4*_crRNA	IDT	crRNA	5'-GTCAGCAGTAAACGCTATTA(AGG)-3'
Sequence-based reagent	Chemically synthesized Alt-R®-modified_*nkx6.1*_crRNA	IDT	crRNA	5'-AGAGCTCGTAAAAAGGAAAC(GGG)-3'
Sequence-based reagent	Chemically synthesized Alt-R®-modified_*id2a*_crRNA	IDT	crRNA	5'-AGGACACTTTACCGTTAATC(AGG)-3'
Sequence-based reagent	Chemically synthesized Alt-R®-modified_hes6_crRNA	IDT	sgRNA	5'-AGGTGAGCATCTGTCTACCA(GGG)-3'

Sequence-based reagent	Chemically synthesized Alt-R®-modified_onecut1_crRNA	IDT	sgRNA	5'-GCACTTGTACCAAAGCATGA(TGG)-3'

Table 3. Sequence-based reagents used in the thesis

8.4 Methodological approaches

The following methodologies were applied (for details, please see the attached papers):

- Animal experiment and sample preparation
- Confocal microscopy and image analysis
- Immunofluorescence staining
- Hybridization chain reaction (HCR)3.0 in situ hybridization
- Molecular cloning (Gateway, Infusion)
- Flow cytometry
- Quantitative real-time polymerase chain reaction
- Genotyping
- Bacteria culture and plasmid purification
- Microinjection
- Statistical analysis
- CRISPR-Cas9 3' knock-in

The gRNA selection was based on the CHOPCHOP web-based tool (http://chopchop.cbu.uib.no/) with the reference genome "danRer11/GRCz11". We selected "CRISPR/Cas9" and "knock-in" module after typing in the targeted gene. This tool would scan through the gene exon regions and order the gRNA based on 1) efficiency score, 2) self-complementarity, and 3) the number of mismatches. Apart from the above in silico prediction results, we also manually examined the 3' end of the targeted locus in Ensembl database (https://www.ensembl.org/Danio_rerio/Info/Index) to avoid polymorphisms included in the gRNA targeting site and to minimize the polymorphisms in the flanking regions.

The chemically synthesized Alt-R-modified crRNA, tracrRNA, high-fidelity (Hifi) Cas9 protein, and nuclease free duplex buffer were all ordered from IDT. The crRNA and tracrRNA powder were diluted into 100 μM with nuclease free duplex buffer. The 10 μL Hifi Cas9 protein were aliquoted in 1:5 dilution with Opti-MEM (ThermoFisher Scientific, 31985062) before use.

Next, we prepared 10 μM crRNA:tracrRNA duplex solution by mixing 1 μL crRNA stock solution, 1 μL tracrRNA stock solution and 8 μL nuclease free duplex buffer and then incubated in PCR machine, in 95 °C for 3 minutes followed by natural cooling in room temperature for 15 minutes. Afterwards, we prepared the Cas9/gRNA RNP by mixing 2 μL Cas9 protein solution and 2 μL crRNA:tracrRNA duplex solution in 37 °C for 10 minutes. In the last step,

we mixed 2 μL Cas9/gRNA RNP, 5 μL donor dsDNA and 0.8 μL phenol red (Sigma, P0290) and stored in 4 °C. Such mixed solution can be reused several times for up to 2 weeks if stored in 4 °C.

The 5' modified primers were ordered with AmC6 5' modification from Integrated DNA Technologies (IDT). The primer powders were diluted with distilled water into 100 mM as stock solution. The 50 μL PCR mixture include:

Forward primer: 2.5 μL

Reverse primer: 2.5 μL

Template plasmid: 1 μL

Distilled water: 19 μL

Q5 Hot Star high-fidelity 2× master mix: 25 μL

We use the following PCR cycle setting:

Pre-denaturing: 98 °C, 30 sec

Denaturing: 98 °C, 10 sec

Annealing: 58-60 °C, 20 sec

Extension: 72 °C, 90 sec

Final extension: 72 °C, 2 minutes and hold on 4 °C

Next, we ran the PCR products in 1% agarose gel with 100V for 45-60 minutes. The corresponding bands were cut out and purified accordingly (Promega, A9282). The concentrations of purified PCR products were measured and diluted into 70-100 ng/uL with distilled water. The purified PCR products were stored in -20 °C before injection.

We injected 1-2 nL Cas9 RNP and donor dsDNA (50-70 pg/nL) into zebrafish embryos at one-cell stage. We suggest to inject at the early one-cell stage (i.e. before the dome stage) based on our experience. The overall mortality rate should be around 30%-50% at the end of the injection day and we sorted out all dead embryos in the following days. We selected mosaic F0 at 2-3 dpf based on the fluorescence in the skin (*krt92* and *krt4*), hindbrain and spinal cord (*nkx6.1*) and hindbrain, spinal cord, and olfactory organ (*id2a*) under a widefield fluorescence microscope LEICA M165 FC (Leica Microsystems) using either the GFP (EGFP or mNeonGreen) or YFP (mNeonGreen) channel. Positive mosaic F0 were put into the fish husbandry system at 6 dpf.

- Tissue dissection, Single-cell suspension preparation and FACS

We sacrificed the adult fish by putting them into 4 °C and used blunt forceps to remove the skin, kidney, eggs (female), liver and gallbladder to expose the pancreata. This step is critical to avoid *krt4* positive cells contamination from other organs. We also removed adipocytes in order to have a better enzymatic digestion in the following step. Then, we cut at the anterior region of intestinal bulb and the hindgut, and moved the intestine and pancreas together to a new dish. We performed blunt dissection carefully to separate the pancreata from the intestine. Meanwhile, we also removed the spleen (shown in dark red) which is also attached to the intestine. We moved and immersed the whole pancreata into 5 mL HBSS at 4 °C on ice. In each condition, we pooled 4-8 samples and enzymatic digested with 1×TrypLE™ 600 µL (ThermoFisher Scientific) supplemented with 100× Pluronic™ F-68 (ThermoFisher Scientific) 60 µL (to prevent tissue adhesion and cell aggregates) at 37 °C on a shaker with 125 rpm for 45 – 60 minutes. We also pipetted the solution every 5-10 minutes to promote a more complete digestion. After 1 hour digestion, we added pre-cooled 2% BSA 6 mL to terminate the digestion reactions and centrifuged the sample at 500 g for 5 minutes. After removing the supernatant, we washed the pellets with 100-300 µL pre-cooled 1% BSA + 0.1% Pluronic F-68 + 0.1% DAPI and used Corning™ Falcon™ cell strainer (Corning™, 352235) to filter out undigested tissues and large cell aggregates.

During the FACS, we selected single-cell populations based on forward scatter and side scatter signals. Next, we performed negative selection in BV421/DAPI channel to remove dead cells and cell debris. Lastly, we used PE/Cherry channel for the subsequent gating to collect all *krt4* lineage cells (H2BmCherry positive) in a collecting tube as the single-cell suspension.

- Library preparation, single-cell RNA-seq and data analysis

Droplet-based scRNA-seq was performed using the Chromium Single Cell 3′ Library and Gel Bead Kit v3 (10× Genomics) and Chromium Single Cell 3′ Chip G (10× Genomics). Approximately 8,000-10,000 cells from each condition were loaded and encapsulated in a single v3 reaction. GEM generation and library preparation were performed according to manufacturer's instructions. The 10× scRNA-Seq libraries were PCR amplified (13 cycles), pooled, denatured, and diluted in prior to paired-end sequencing on a NextSeq 500 according to manufacturer's recommendations. Sequencing data was aligned to zebrafish reference genome (GRCz11) with the addition of mNeonGreen and mCherry sequence using Cell Ranger v5.0.1 (10× Genomics) to generate gene-by-cell count matrix with default parameters.

The single-cell RNA-Seq data of adult zebrafish principal islet datasets were downloaded from the Gene Expression Omnibus (GEO) under accession number GSE106121 and GSE166052. The UMI counts matrix was imported into R and processed using the Seurat R package version 4.0.4 (183). Cells with a detected gene number less than 400 or above 7000 genes were considered as low-quality cells or doublets and removed prior to downstream analysis. The counts matrix was then normalized and scaled with default parameters, followed by highly variable genes (HVGs) selection. Further doublet filtering was performed using

Doubletfinder R package (184). After regular dimension reduction using principal component analysis (PCA), we selected 8-30 PCs (depending on the anticipated cell types) for graph-based clustering (Louvain). We used the Uniform Manifold Approximation and Projection (UMAP) algorithm to display the relationships within and between different clusters and Harmony algorithm for batch correction (185). To unravel the molecular details and cellular trajectory in *krt4* derived duct-to-endocrine and endocrine lineages, we manually annotated each cell clusters based on well-known verified marker genes and subset the clusters of interest followed by a series of normalization, scaling, dimensional reduction, batch correction and graph-based clustering. Average expression level for each gene was normalized to determine the proportion of expression in each cell states for ternary plotting using the R package "ggtern".

The descriptions for SCENIC, scVelo, CellRank and Enrichment analyses are shown in **Paper III**, with customized code deposited in Jiarui Mi's Github webpage (https://github.com/JiaruiMi/zebrafish_Neogenesis_2022). The interactive web browser of zebrafish endodermal organ single-cell data query portal is accessible via the link https://olovanderssonlab.shinyapps.io/neogenesis/

9 Short summary and discussion

9.1 Paper I

Identifying chemicals which can promote ductal progenitors to become Insulin-producing cells might be a promising strategy to cure diabetes. From a previous large-scale chemical screening, we identified a compound, CID661578, which can markedly increase the amounts of β-cells in extreme β-cell loss condition. The yeast hybrid assay suggested that the zebrafish *Mknk2b* and human MNK2 have direct binding with the compound. The *mknk2b* gene is highly expressed in zebrafish pancreatic duct. Lineage tracing experiments using *tp1* tracers indicated that the regenerative Insulin-producing cells are originated from a ductal origin. To genetically validate the biological functions of *mknk2b* in β-cell regeneration in vivo, we tested *mknk2b* and *mknk2b* morpholino knock-down and observed the increased number of β-cells after ablation. To investigate the molecular mechanism of CID661578, we performed metabolomics and O-propargyl-puromycin (OPP) incorporation analysis and demonstrated that the levels of protein synthesis is altered after the drug treatment. Pull-down experiments showed that CID661578 can directly bind to MNK2 and consequently prevent it from interacting with EIF4G. Ultimately, such drug-target binding can enhance the formation of EIF4E and EIF4G complex at the mRNA cap region to promote mRNA translation. Lastly, we tested the effect of the compound in neonatal pig islet aggregates and showed that treatment with CID661578 can induce an increased amount of CK7 and Insulin double positive cells, indicating β-cell differentiation.

9.2 Paper II

Although various genome editing tools have been applied to generate zebrafish knock-in strains, such methods are not yet widely used due to the following limitations: low integration efficiency, low scalability, disruption of the targeted genes and the complicated molecular cloning pre-processing to obtain the right construct as template. To tackle these issues, we developed a novel straightforward, clone-free 3' knock-in strategy, which enable us to integrate large piece of DNA with multiple cassettes in a scalable fashion. Using the newly devised tools, we delineate the developmental paths of several endodermal organ and confirmed the duct-to-hepatocyte trans-differentiation in an extreme liver injury model.

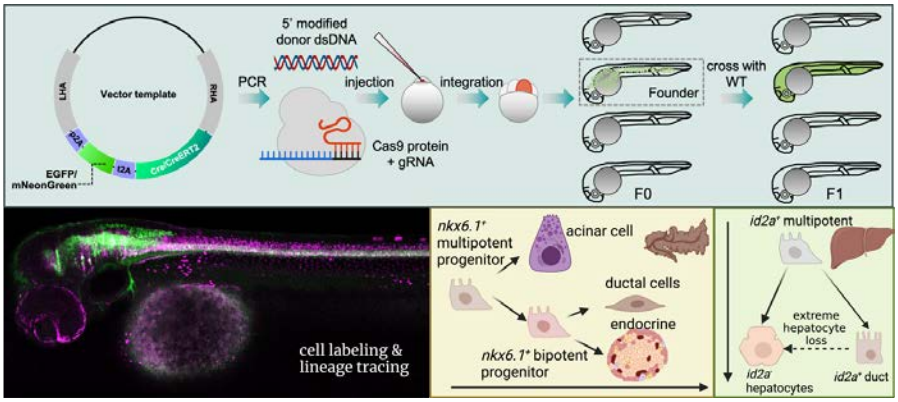

Figure 4 Schematics of the knock-in strategy and the discoveries

1, In brief, we used PCR amplified double-stranded DNA with 5' modification (5' AmC6) as the direct donor. The 5' modification was added by ordering primers with the corresponding modification. To facilitate early integration, we performed in vitro Cas9 protein and gRNA pre-assembly and co-inject with the double-strand donor into early one cell stage embryos.

2, We did a systematic comparison and confirmed that the double-stranded DNA with 5' modification out-performed the circular plasmid as the donor in the selected loci. Furthermore, the highly efficient early integration is aided by the 5' modification as the nude double-stranded DNA showed much less efficient integration in F0 generation, as shown in the *krt4* locus. In addition, when the gRNA spans over the stop codon region, the short homologous arm (~ 30-40 nt) demonstrate better performance than the long homologous arm (~ 1000 nt).

3, We successfully targeted four genetic loci and generated 10 knock-in zebrafish lines. Sanger sequencing results confirmed the precise integration without insertions and deletion in the 5' region of the insertion site. Using immunostaining, in situ hybridization and publicly accessible single-cell RNA-sequencing results, we demonstrated that our knock-in lines are reminiscent of the endogenous gene expression without ectopic labeling and lack of labeling, which indicated great advantages compared to the conventional transgenics (as shown for *krt4*).

4, Using *nkx6.1 knock-in EGFP-t2a-CreERT2* and *mNeonGreen-t2a-iCre* lines, we, for the first time, provide concrete evidence indicating that the nkx6.1 positive cells are multipotent progenitors which can give rise to endocrine, ductal and acinar cells before 13 hpf; while these cells become gradually restricted to the bipotent progenitors, which can only give rise to endocrine cells and ductal cells in the pancreas.

5, Lineage tracing experiments using *id2a* knock-in EGFP-t2a-CreERT2 and mNeonGreen-t2a-iCre suggested that *id2a* positive cells are multipotent progenitors that can give rise to different types of ductal cells in the liver and hepatocytes during normal development and become gradually restricted to the ductal lineage upon 36 hpf. In alcohol induced severe liver injury, we observed few duct-to-hepatocyte trans-differentiation during liver regeneration; however, in the extreme hepatocyte loss model using NTR/MTZ system, we confirmed that

the duct-to-hepatocyte conversion takes place and make great contribution to replenish the loss of hepatocytes.

9.3 Paper III

Unlike mammals, zebrafish has very high potential in organ regeneration. In particular, zebrafish can regenerate Insulin-producing cells within a short time period and hence it has become an excellent model organism to investigate the β-cell regeneration. However, previous studies and putative concepts are still compromised by the lack of concrete and consistent evidence based on lineage tracing experiment. Thus, the real progenitor of developing endocrine cells is still undetermined. In addition, we still lack knowledge in the major cellular events during the process of β-cell regeneration.

1, Analyzing public single-cell RNA-seq datasets of isolated principal islets from adult zebrafish, we, for the first time, characterize the unrecognized ductal heterogeneity within the zebrafish pancreas. Using immunostaining, in situ hybridization and a couple of transgenics lines, we confirmed that such heterogeneity indeed exists in both larvae and adult fish. We observed that in zebrafish larvae, the *krt4* positive ductal cells are located anterior to the principal islet, while the canonical Notch-responsive ductal cells, which is labeled and visualized by *tp1* driven transgenics, are *krt4* negative. In the adult fish, we observed that the luminal ductal cells lining across the whole pancreata and feature a distinct molecular signature compared with Notch-responsive duct.

2, Short-term lineage tracing experiments using *tp1* tracers can barely label *krt4* positive ducts in zebrafish larvae; while early-labeling long-term tracing suggested that a subset of *krt4* positive luminal ducts in the juveniles can be lineage traced back to the Notch-responsive duct origin. Lineage tracing using *cftr* tracer, which is able to label a subset of luminal duct anterior to the principal islet, showed a great amount of developing endocrine cells that are derived from the ductal lineage.

3, Non-inducible and spatial temporal lineage tracing experiments using *krt4* knock-in tracers confirmed that the endocrine cells in the anterior part of principal islet and the majority of endocrine cells in the secondary islet are originated from *krt4* positive duct. Such ductal cells showed elevated endocrine progenitor master regulator, *ascl1b* and *neurod1*, expression at juvenile stage without β-cell ablation or larvae stage 0-2 days after β-cell ablation.

4, Spatial temporal labeling using pan-endocrine tracer, *neurod1:CreERT2*, confirmed the existence of trans-differentiation upon a single period of β-cell ablation. Based on a variety of endocrine cell tracers, we concur that the *sst1.1* positive cells can upregulate Insulin expression as the major compensatory strategy for the loss of β-cells.

5, Combining various ductal tracers, we, for the first time, demonstrated a ductal remodeling process that is coupled with the neogenic event during development and growth. Such

remodeling process takes place at a critical period, i.e. roughly 1 month post-fertilization. In brief, the *tp1* driven Notch-responsive ductal cells in the pancreatic tail region can gradually assemble together and form luminal structures and eventually become neogenic *krt4* positive duct. The *krt4* positive ductal cells expand and form large ducts in the pancreatic head region and ultimately connect to the remodelled *tp1* derived *krt4* positive luminal duct for the final construction of the pancreatic ductal tree.

6, Using single-cell RNA-seq based in combination with the lineage tracing, we are able to characterize the molecular signatures of single cells in the *krt4* lineage. We identified the endocrine precursor cells are *dlb* positive, showed multi-hormonal states and upregulate a wide range of endocrine-related transcription factors. These cells also upregulate the negative regulator of Notch signaling, *hes6*, indicating that the downregulation of Notch is a critical step in ductal-to-endocrine cells neogenesis. Furthermore, we delineate the *dlb*-to-Ins/*Sst1.1* hybrid cell trajectory upon β-cell ablation.

7, Regulon analyses showed that *fev* regulon is critical in the early transition cells state and various injury responsive regulons dominate in the late transition stage. In the Ins/Sst1.1 hybrid cells, *pdx1*, *klf11b* and *tp53* are the major enriched regulon. RNA velocity analyses suggested a β-to-transition de-differentiation process takes place upon β-cell injury, while the differentiation process is accelerated in the acute phase after double ablation. The cells. undergoing differentiation upregulate genes that are involved in inflammatory response and EMT. Lastly, using pharmacological approach, we proposed the negative feedback loop of Insulin signaling in preventing endocrine precursors to differentiate into committed β-cells.

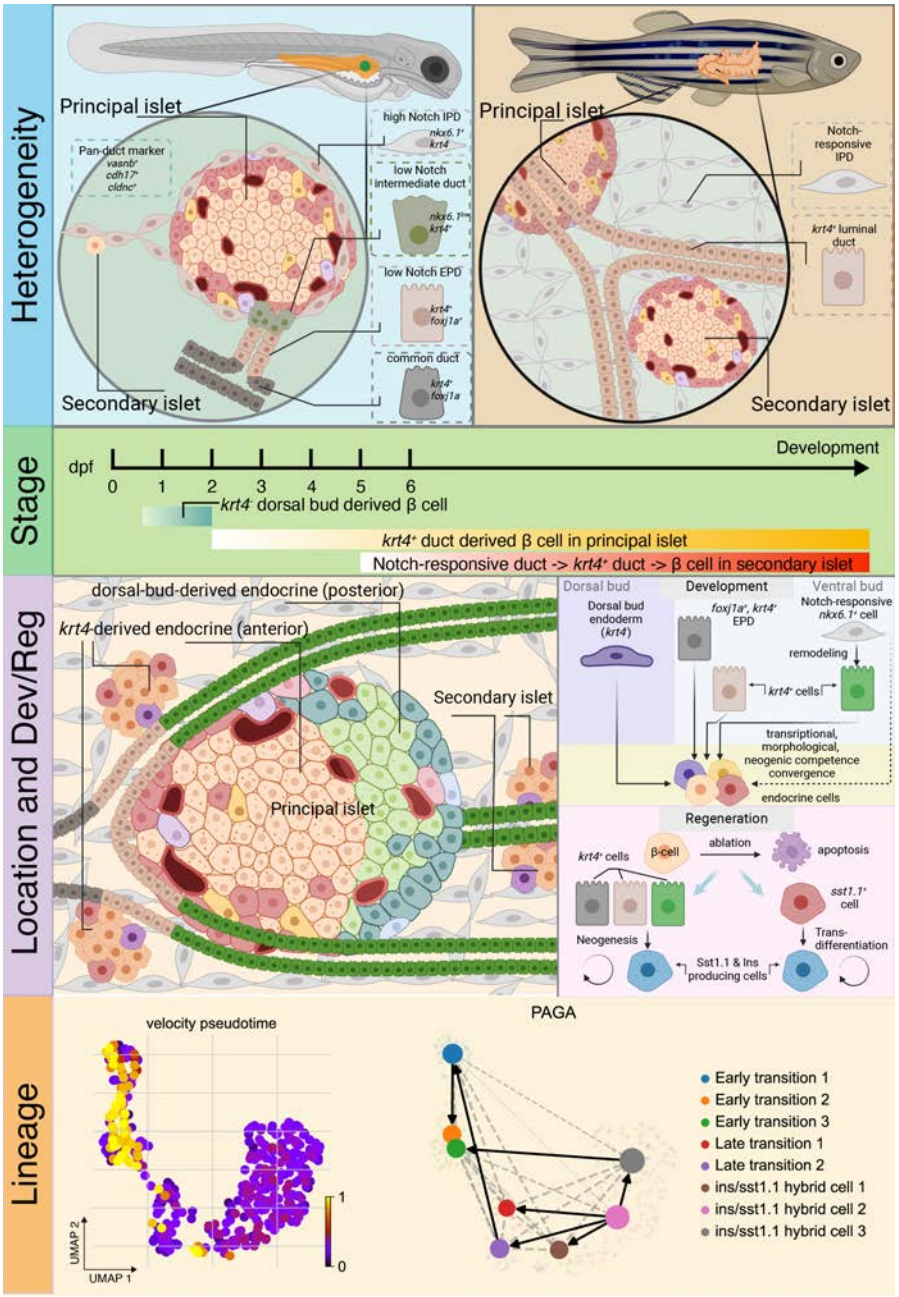

Figure 5 The summary of the major discoveries in ***Paper III***

10 Concluding remarks and future perspective

In this thesis,

1). In **Paper I**, we characterized a chemical, CID661578, which can greatly induce the duct-to-β cell neogenesis. Using a yeast hybrid assay, biochemistry experiments, polysome-profiling and genetic zebrafish mutants, we determined that *mknk2b* (and MNK2 in human) is the target of CID661578. We also confirmed the efficacy of the compound in neonatal pig islet, indicating a translational potential to induce β cell differentiation from the duct.

2). We devised a novel CRISPR/Cas9 knock-in strategy in zebrafish. Such clone-free strategy is based on PCR amplified DNA donors with 5' modifications on each side as the donor. Moreover, we combined fluorescent protein with Cre recombinase together for genetic integration, which allow us to both label and lineage trace the cell type of interest. Injecting pre-assembled Cas9/gRNA complex into early single-cell stage embryos, we are able to generate knock-in zebrafish lines in parallel in a scalable fashion. In **Paper II**, we introduced 10 newly generated zebrafish lines. By systematic comparisons, we concluded that the 5' modified double-stranded DNA out-perform the circular plasmid in integration efficiency. Moreover, when the gRNA spanning over the stop codon, the short homologous arm has better integration efficiency than the long homologous arm. In additionally, we applied these lines to delineate developmental/regenerative paths in zebrafish pancreas and liver. We demonstrated that the *nkx6.1* positive cells are multipotent and can give rise to endocrine, ductal and acinar cells at 13 hpf stage; while it becomes bipotent shortly afterwards. In the late larvae stage, *nkx6.1* positive cells can only specifically label the ductal cells. Likewise, the *id2a* positive cells are bipotent progenitors which can give rise to hepatocytes and liver duct but gradually restricted to the duct at later stages. Lineage tracing experiments showed that the *id2a* positive duct can convert to hepatocytes in extreme hepatocyte ablation condition.

3). In **Paper III**, using in silico single-cell RNA-seq, immunofluorescent staining and in situ hybridization, we identified a previously unrecognized ductal cell type in zebrafish pancreas. Such ductal cells are featured by *krt4* expression and are *tp1:EGFP* negative. Lineage tracing experiments using the novel *krt4* knock-in zebrafish lines indicated that such ductal cells are endocrine progenitors, which can give rise to the islet cells in the anterior region of principal islet and most endocrine cells in the secondary islet. Moreover, we observed that a ductal modeling process, i.e., the *tp1* Notch-responsive duct-to-*krt4* duct transition, is coupled with ductal-to-β cell neogenesis. Furthermore, we for the first time, using lineage tracing methodology and confirmed the existence of an endocrine cell trans-differentiation process taking place to replenish the loss of β-cells after β-cell ablation. Combining the lineage tracing and single-cell RNA-seq, we are able to delineate the developmental path from an unappreciated dlb positive endocrine precursor to Insulin-producing cell trajectory and

pinpoint the distinct regulon profile in each developmental stage. RNA velocity analysis suggested both differentiation and de-differentiation happen and cells shuffle during the process of β-cell regeneration. Blocking the Insulin signaling pathway can disrupt a potential negative feedback loop and promote endocrine precursor to β-cell differentiation.

Together, these studies make up an analytical pipeline for characterizing and making use of an epithelial progenitor population for regenerative biology, a pipeline that may be of widespread use for other progenitor populations across the animal kingdom.

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12 References

1. Y. Yang, H. Wang, J. He, W. Shi, Z. Jiang, L. Gao, Y. Jiang, R. Ni, Q. Yang, L. Luo, A single-cell-resolution fate map of endoderm reveals demarcation of pancreatic progenitors by cell cycle. Proc Natl Acad Sci U S A 118, (2021).
2. N. Tiso, A. Filippi, S. Pauls, M. Bortolussi, F. Argenton, BMP signalling regulates anteroposterior endoderm patterning in zebrafish. Mech Dev 118, 29-37 (2002).
3. W. S. Chung, C. H. Shin, D. Y. Stainier, Bmp2 signaling regulates the hepatic versus pancreatic fate decision. Dev Cell 15, 738-748 (2008).
4. W. S. Chung, D. Y. Stainier, Intra-endodermal interactions are required for pancreatic beta cell induction. Dev Cell 14, 582-593 (2008).
5. S. Kumar, G. Duester, SnapShot: retinoic acid signaling. Cell 147, 1422-1422 e1421 (2011).
6. S. Shimozono, T. Iimura, T. Kitaguchi, S. Higashijima, A. Miyawaki, Visualization of an endogenous retinoic acid gradient across embryonic development. Nature 496, 363-366 (2013).
7. H. L. Larsen, A. Grapin-Botton, The molecular and morphogenetic basis of pancreas organogenesis. Semin Cell Dev Biol 66, 51-68 (2017).
8. A. M. Zorn, J. M. Wells, Vertebrate endoderm development and organ formation. Annu Rev Cell Dev Biol 25, 221-251 (2009).
9. D. Stafford, V. E. Prince, Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol 12, 1215-1220 (2002).
10. D. Stafford, R. J. White, M. D. Kinkel, A. Linville, T. F. Schilling, V. E. Prince, Retinoids signal directly to zebrafish endoderm to specify insulin-expressing beta-cells. Development 133, 949-956 (2006).
11. M. D. Kinkel, E. M. Sefton, Y. Kikuchi, T. Mizoguchi, A. B. Ward, V. E. Prince, Cyp26 enzymes function in endoderm to regulate pancreatic field size. Proc Natl Acad Sci U S A 106, 7864-7869 (2009).
12. A. R. Lopez-Perez, P. J. Balwierz, B. Lenhard, F. Muller, F. C. Wardle, I. Manfroid, M. L. Voz, B. Peers, Identification of downstream effectors of retinoic acid specifying the zebrafish pancreas by integrative genomics. Sci Rep 11, 22717 (2021).
13. E. Zecchin, A. Filippi, F. Biemar, N. Tiso, S. Pauls, E. Ellertsdottir, L. Gnugge, M. Bortolussi, W. Driever, F. Argenton, Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. Dev Biol 301, 192-204 (2007).
14. E. A. Ober, H. Verkade, H. A. Field, D. Y. Stainier, Mesodermal Wnt2b signalling positively regulates liver specification. Nature 442, 688-691 (2006).
15. M. Poulain, E. A. Ober, Interplay between Wnt2 and Wnt2bb controls multiple steps of early foregut-derived organ development. Development 138, 3557-3568 (2011).
16. H. Lu, J. Ma, Y. Yang, W. Shi, L. Luo, EpCAM is an endoderm-specific Wnt derepressor that licenses hepatic development. Dev Cell 24, 543-553 (2013).
17. J. J. Lancman, N. Zvenigorodsky, K. P. Gates, D. Zhang, K. Solomon, R. K. Humphrey, T. Kuo, L. Setiawan, H. Verkade, Y. I. Chi, U. S. Jhala, C. V. Wright, D. Y. Stainier, P. D. Dong, Specification of hepatopancreas progenitors in zebrafish by hnf1ba and wnt2bb. Development 140, 2669-2679 (2013).
18. Z. Tehrani, S. Lin, Antagonistic interactions of hedgehog, Bmp and retinoic acid signals control zebrafish endocrine pancreas development. Development 138, 631-640 (2011).
19. P. J. diIorio, J. B. Moss, J. L. Sbrogna, R. O. Karlstrom, L. G. Moss, Sonic hedgehog is required early in pancreatic islet development. Dev Biol 244, 75-84 (2002).
20. S. Korzh, C. L. Winata, Z. Gong, V. Korzh, The development of zebrafish pancreas affected by deficiency of Hedgehog signaling. Gene Expr Patterns 41, 119185 (2021).
21. S. L. Yang, S. S. Aw, C. Chang, S. Korzh, V. Korzh, J. Peng, Depletion of Bhmt elevates sonic hedgehog transcript level and increases beta-cell number in zebrafish.

Endocrinology 152, 4706-4717 (2011).
22. M. D. Kinkel, S. C. Eames, M. R. Alonzo, V. E. Prince, Cdx4 is required in the endoderm to localize the pancreas and limit beta-cell number. Development 135, 919-929 (2008).
23. J. Xu, J. Cui, A. Del Campo, C. H. Shin, Four and a Half LIM Domains 1b (Fhl1b) Is Essential for Regulating the Liver versus Pancreas Fate Decision and for beta-Cell Regeneration. PLoS Genet 12, e1005831 (2016).
24. S. Nissim, R. I. Sherwood, J. Wucherpfennig, D. Saunders, J. M. Harris, V. Esain, K. J. Carroll, G. M. Frechette, A. J. Kim, K. L. Hwang, C. C. Cutting, S. Elledge, T. E. North, W. Goessling, Prostaglandin E2 regulates liver versus pancreas cell-fate decisions and endodermal outgrowth. Dev Cell 28, 423-437 (2014).
25. D. Shin, C. H. Shin, J. Tucker, E. A. Ober, F. Rentzsch, K. D. Poss, M. Hammerschmidt, M. C. Mullins, D. Y. Stainier, Bmp and Fgf signaling are essential for liver specification in zebrafish. Development 134, 2041-2050 (2007).
26. J. P. Amorim, A. Gali-Macedo, H. Marcelino, R. Bordeira-Carrico, S. Naranjo, S. Rivero-Gil, J. Teixeira, M. Galhardo, J. Marques, J. Bessa, A Conserved Notochord Enhancer Controls Pancreas Development in Vertebrates. Cell Rep 32, 107862 (2020).
27. E. Zecchin, A. Mavropoulos, N. Devos, A. Filippi, N. Tiso, D. Meyer, B. Peers, M. Bortolussi, F. Argenton, Evolutionary conserved role of ptf1a in the specification of exocrine pancreatic fates. Dev Biol 268, 174-184 (2004).
28. H. A. Field, P. D. Dong, D. Beis, D. Y. Stainier, Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. Dev Biol 261, 197-208 (2003).
29. A. P. Ghaye, D. Bergemann, E. Tarifeno-Saldivia, L. C. Flasse, V. Von Berg, B. Peers, M. L. Voz, I. Manfroid, Progenitor potential of nkx6.1-expressing cells throughout zebrafish life and during beta cell regeneration. BMC Biol 13, 70 (2015).
30. W. S. Chung, O. Andersson, R. Row, D. Kimelman, D. Y. Stainier, Suppression of Alk8-mediated Bmp signaling cell-autonomously induces pancreatic beta-cells in zebrafish. Proc Natl Acad Sci U S A 107, 1142-1147 (2010).
31. F. Naye, M. L. Voz, N. Detry, M. Hammerschmidt, B. Peers, I. Manfroid, Essential roles of zebrafish bmp2a, fgf10, and fgf24 in the specification of the ventral pancreas. Mol Biol Cell 23, 945-954 (2012).
32. G. Dong, Y. Huang, H. Ding, L. Luo, Y. Zhang, H. Huang, H. Ruan, Mypt1 regulates Bmp signaling to promote embryonic exocrine pancreas growth in zebrafish. Genesis 58, e23345 (2020).
33. H. Huang, H. Ruan, M. Y. Aw, A. Hussain, L. Guo, C. Gao, F. Qian, T. Leung, H. Song, D. Kimelman, Z. Wen, J. Peng, Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. Development 135, 3209-3218 (2008).
34. M. Hernandez-Perez, A. Kulkarni, N. Samala, C. Sorrell, K. El, I. Haider, A. Mukhtar Aleem, T. R. Holman, G. Rai, S. A. Tersey, R. G. Mirmira, R. M. Anderson, A 12-lipoxygenase-Gpr31 signaling axis is required for pancreatic organogenesis in the zebrafish. FASEB J 34, 14850-14862 (2020).
35. I. Manfroid, F. Delporte, A. Baudhuin, P. Motte, C. J. Neumann, M. L. Voz, J. A. Martial, B. Peers, Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. Development 134, 4011-4021 (2007).
36. I. Cebola, S. A. Rodriguez-Segui, C. H. Cho, J. Bessa, M. Rovira, M. Luengo, M. Chhatriwala, A. Berry, J. Ponsa-Cobas, M. A. Maestro, R. E. Jennings, L. Pasquali, I. Moran, N. Castro, N. A. Hanley, J. L. Gomez-Skarmeta, L. Vallier, J. Ferrer, TEAD and YAP regulate the enhancer network of human embryonic pancreatic progenitors. Nat Cell Biol 17, 615-626 (2015).
37. X. Yi, J. Yu, C. Ma, L. Li, L. Luo, H. Li, H. Ruan, H. Huang, Yap1/Taz are essential for the liver development in zebrafish. Biochem Biophys Res Commun 503, 131-137

(2018).
38. P. D. Dong, C. A. Munson, W. Norton, C. Crosnier, X. Pan, Z. Gong, C. J. Neumann, D. Y. Stainier, Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nat Genet 39, 397-402 (2007).
39. M. Delous, C. Yin, D. Shin, N. Ninov, J. Debrito Carten, L. Pan, T. P. Ma, S. A. Farber, C. B. Moens, D. Y. Stainier, Sox9b is a key regulator of pancreaticobiliary ductal system development. PLoS Genet 8, e1002754 (2012).
40. I. Manfroid, A. Ghaye, F. Naye, N. Detry, S. Palm, L. Pan, T. P. Ma, W. Huang, M. Rovira, J. A. Martial, M. J. Parsons, C. B. Moens, M. L. Voz, B. Peers, Zebrafish sox9b is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration. Dev Biol 366, 268-278 (2012).
41. M. I. Thestrup, S. Caviglia, J. Cayuso, R. L. S. Heyne, R. Ahmad, W. Hofmeister, L. Satriano, D. G. Wilkinson, J. B. Andersen, E. A. Ober, A morphogenetic EphB/EphrinB code controls hepatopancreatic duct formation. Nat Commun 10, 5220 (2019).
42. S. Pauls, E. Zecchin, N. Tiso, M. Bortolussi, F. Argenton, Function and regulation of zebrafish nkx2.2a during development of pancreatic islet and ducts. Dev Biol 304, 875-890 (2007).
43. R. A. Kimmel, L. Onder, A. Wilfinger, E. Ellertsdottir, D. Meyer, Requirement for Pdx1 in specification of latent endocrine progenitors in zebrafish. BMC Biol 9, 75 (2011).
44. D. Zhang, K. P. Gates, L. Barske, G. Wang, J. J. Lancman, X. I. Zeng, M. Groff, K. Wang, M. J. Parsons, J. G. Crump, P. D. S. Dong, Endoderm Jagged induces liver and pancreas duct lineage in zebrafish. Nat Commun 8, 769 (2017).
45. R. M. Anderson, M. Delous, J. A. Bosch, L. Ye, M. A. Robertson, D. Hesselson, D. Y. Stainier, Hepatocyte growth factor signaling in intrapancreatic ductal cells drives pancreatic morphogenesis. PLoS Genet 9, e1003650 (2013).
46. M. J. Parsons, H. Pisharath, S. Yusuff, J. C. Moore, A. F. Siekmann, N. Lawson, S. D. Leach, Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mech Dev 126, 898-912 (2009).
47. N. Ninov, D. Hesselson, P. Gut, A. Zhou, K. Fidelin, D. Y. Stainier, Metabolic regulation of cellular plasticity in the pancreas. Curr Biol 23, 1242-1250 (2013).
48. Y. Wang, M. Rovira, S. Yusuff, M. J. Parsons, Genetic inducible fate mapping in larval zebrafish reveals origins of adult insulin-producing beta-cells. Development 138, 609-617 (2011).
49. S. P. Singh, S. Janjuha, T. Hartmann, O. Kayisoglu, J. Konantz, S. Birke, P. Murawala, E. A. Alfar, K. Murata, A. Eugster, N. Tsuji, E. R. Morrissey, M. Brand, N. Ninov, Different developmental histories of beta-cells generate functional and proliferative heterogeneity during islet growth. Nat Commun 8, 664 (2017).
50. F. Delaspre, R. L. Beer, M. Rovira, W. Huang, G. Wang, S. Gee, C. Vitery Mdel, S. J. Wheelan, M. J. Parsons, Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration. Diabetes 64, 3499-3509 (2015).
51. N. Ninov, M. Borius, D. Y. Stainier, Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. Development 139, 1557-1567 (2012).
52. L. A. Maddison, W. Chen, Nutrient excess stimulates beta-cell neogenesis in zebrafish. Diabetes 61, 2517-2524 (2012).
53. M. Li, P. Page-McCaw, W. Chen, FGF1 Mediates Overnutrition-Induced Compensatory beta-Cell Differentiation. Diabetes 65, 96-109 (2016).
54. M. Li, L. A. Maddison, P. Page-McCaw, W. Chen, Overnutrition induces beta-cell differentiation through prolonged activation of beta-cells in zebrafish larvae. Am J Physiol Endocrinol Metab 306, E799-807 (2014).

55. W. Huang, R. L. Beer, F. Delaspre, G. Wang, H. E. Edelman, H. Park, M. Azuma, M. J. Parsons, Sox9b is a mediator of retinoic acid signaling restricting endocrine progenitor differentiation. Dev Biol 418, 28-39 (2016).
56. M. Rovira, W. Huang, S. Yusuff, J. S. Shim, A. A. Ferrante, J. O. Liu, M. J. Parsons, Chemical screen identifies FDA-approved drugs and target pathways that induce precocious pancreatic endocrine differentiation. Proc Natl Acad Sci U S A 108, 19264-19269 (2011).
57. M. Rovira, G. Atla, M. A. Maestro, V. Grau, J. Garcia-Hurtado, M. Maqueda, J. L. Mosquera, Y. Yamada, J. Kerr-Conte, F. Pattou, J. Ferrer, REST is a major negative regulator of endocrine differentiation during pancreas organogenesis. Genes Dev 35, 1229-1242 (2021).
58. M. Li, L. A. Maddison, Z. Crees, W. Chen, Targeted overexpression of CKI-insensitive cyclin-dependent kinase 4 increases functional beta-cell number through enhanced self-replication in zebrafish. Zebrafish 10, 170-176 (2013).
59. D. Hesselson, R. M. Anderson, M. Beinat, D. Y. Stainier, Distinct populations of quiescent and proliferative pancreatic beta-cells identified by HOTcre mediated labeling. Proc Natl Acad Sci U S A 106, 14896-14901 (2009).
60. E. Ahmed Alfar, D. Kirova, J. Konantz, S. Birke, J. Mansfeld, N. Ninov, Distinct Levels of Reactive Oxygen Species Coordinate Metabolic Activity with Beta-cell Mass Plasticity. Sci Rep 7, 3994 (2017).
61. S. Janjuha, S. P. Singh, A. Tsakmaki, S. N. Mousavy Gharavy, P. Murawala, J. Konantz, S. Birke, D. J. Hodson, G. A. Rutter, G. A. Bewick, N. Ninov, Age-related islet inflammation marks the proliferative decline of pancreatic beta-cells in zebrafish. Elife 7, (2018).
62. V. Salem, L. D. Silva, K. Suba, E. Georgiadou, S. Neda Mousavy Gharavy, N. Akhtar, A. Martin-Alonso, D. C. A. Gaboriau, S. M. Rothery, T. Stylianides, G. Carrat, T. J. Pullen, S. P. Singh, D. J. Hodson, I. Leclerc, A. M. J. Shapiro, P. Marchetti, L. J. B. Briant, W. Distaso, N. Ninov, G. A. Rutter, Leader beta-cells coordinate Ca(2+) dynamics across pancreatic islets in vivo. Nat Metab 1, 615-629 (2019).
63. H. Matsuda, S. T. Mullapudi, Y. Zhang, D. Hesselson, D. Y. R. Stainier, Thyroid Hormone Coordinates Pancreatic Islet Maturation During the Zebrafish Larval-to-Juvenile Transition to Maintain Glucose Homeostasis. Diabetes 66, 2623-2635 (2017).
64. H. Matsuda, Zebrafish as a model for studying functional pancreatic beta cells development and regeneration. Dev Growth Differ 60, 393-399 (2018).
65. S. Curado, R. M. Anderson, B. Jungblut, J. Mumm, E. Schroeter, D. Y. Stainier, Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev Dyn 236, 1025-1035 (2007).
66. S. Curado, D. Y. Stainier, R. M. Anderson, Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat Protoc 3, 948-954 (2008).
67. A. Kulkarni, A. R. Pineros, M. A. Walsh, I. Casimiro, S. Ibrahim, M. Hernandez-Perez, K. S. Orr, L. Glenn, J. L. Nadler, M. A. Morris, S. A. Tersey, R. G. Mirmira, R. M. Anderson, 12-Lipoxygenase governs the innate immune pathogenesis of islet inflammation and autoimmune diabetes. JCI Insight 6, (2021).
68. A. A. Kulkarni, A. M. Conteh, C. A. Sorrell, A. Mirmira, S. A. Tersey, R. G. Mirmira, A. K. Linnemann, R. M. Anderson, An In Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic beta-Cell Injury, Response, and Prevention. Oxid Med Cell Longev 2018, 1324739 (2018).
69. A. V. Sharrock, T. S. Mulligan, K. R. Hall, E. M. Williams, D. T. White, L. Zhang, K. Emmerich, F. Matthews, S. Nimmagadda, S. Washington, K. D. Le, D. Meir-Levi, O. L. Cox, M. T. Saxena, A. L. Calof, M. E. Lopez-Burks, A. D. Lander, D. Ding, H. Ji, D. F. Ackerley, J. S. Mumm, NTR 2.0: a rationally engineered prodrug-converting

enzyme with substantially enhanced efficacy for targeted cell ablation. Nat Methods 19, 205-215 (2022).
70. D. Bergemann, L. Massoz, J. Bourdouxhe, C. A. Carril Pardo, M. L. Voz, B. Peers, I. Manfroid, Nifurpirinol: A more potent and reliable substrate compared to metronidazole for nitroreductase-mediated cell ablations. Wound Repair Regen 26, 238-244 (2018).
71. N. Schmitner, K. Kohno, D. Meyer, ptf1a(+) , ela3l(-) cells are developmentally maintained progenitors for exocrine regeneration following extreme loss of acinar cells in zebrafish larvae. Dis Model Mech 10, 307-321 (2017).
72. J. Kang, J. Hu, R. Karra, A. L. Dickson, V. A. Tornini, G. Nachtrab, M. Gemberling, J. A. Goldman, B. L. Black, K. D. Poss, Modulation of tissue repair by regeneration enhancer elements. Nature 532, 201-206 (2016).
73. Y. Kimura, S. I. Higashijima, Regulation of locomotor speed and selection of active sets of neurons by V1 neurons. Nat Commun 10, 2268 (2019).
74. C. A. Carril Pardo, L. Massoz, M. A. Dupont, D. Bergemann, J. Bourdouxhe, A. Lavergne, E. Tarifeno-Saldivia, C. S. Helker, D. Y. Stainier, B. Peers, M. M. Voz, I. Manfroid, A delta-cell subpopulation with a pro-beta-cell identity contributes to efficient age-independent recovery in a zebrafish model of diabetes. Elife 11, (2022).
75. L. Ye, M. Bae, C. D. Cassilly, S. V. Jabba, D. W. Thorpe, A. M. Martin, H. Y. Lu, J. Wang, J. D. Thompson, C. R. Lickwar, K. D. Poss, D. J. Keating, S. E. Jordt, J. Clardy, R. A. Liddle, J. F. Rawls, Enteroendocrine cells sense bacterial tryptophan catabolites to activate enteric and vagal neuronal pathways. Cell Host Microbe 29, 179-196 e179 (2021).
76. U. B. Pajvani, M. E. Trujillo, T. P. Combs, P. Iyengar, L. Jelicks, K. A. Roth, R. N. Kitsis, P. E. Scherer, Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. Nat Med 11, 797-803 (2005).
77. J. B. Moss, P. Koustubhan, M. Greenman, M. J. Parsons, I. Walter, L. G. Moss, Regeneration of the pancreas in adult zebrafish. Diabetes 58, 1844-1851 (2009).
78. K. Wang, X. M. Yin, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, C. L. Keck, D. B. Zimonjic, N. C. Popescu, S. J. Korsmeyer, BID, a proapoptotic BCL-2 family member, is localized to mouse chromosome 6 and human chromosome 22q11. Genomics 53, 235-238 (1998).
79. J. Jia, Q. Kang, S. Liu, Y. Song, F. S. Wong, Y. Qiu, M. Li, Artemether and aspterric acid induce pancreatic alpha cells to transdifferentiate into beta cells in zebrafish. Br J Pharmacol, (2021).
80. B. Yang, L. A. Maddison, K. E. Zaborska, C. Dai, L. Yin, Z. Tang, L. Zang, D. A. Jacobson, A. C. Powers, W. Chen, RIPK3-mediated inflammation is a conserved beta cell response to ER stress. Sci Adv 6, (2020).
81. L. F. Delgadillo-Silva, A. Tsakmaki, N. Akhtar, Z. J. Franklin, J. Konantz, G. A. Bewick, N. Ninov, Modelling pancreatic beta-cell inflammation in zebrafish identifies the natural product wedelolactone for human islet protection. Dis Model Mech 12, (2019).
82. S. Ibrahim, A. Harris-Kawano, I. Haider, R. G. Mirmira, E. K. Sims, R. M. Anderson, A novel Cre-enabled tetracycline-inducible transgenic system for tissue-specific cytokine expression in the zebrafish: CETI-PIC3. Dis Model Mech 13, (2020).
83. H. Zhao, X. Huang, Z. Liu, W. Pu, Z. Lv, L. He, Y. Li, Q. Zhou, K. O. Lui, B. Zhou, Pre-existing beta cells but not progenitors contribute to new beta cells in the adult pancreas. Nat Metab 3, 352-365 (2021).
84. X. Xu, J. D'Hoker, G. Stange, S. Bonne, N. De Leu, X. Xiao, M. Van de Casteele, G. Mellitzer, Z. Ling, D. Pipeleers, L. Bouwens, R. Scharfmann, G. Gradwohl, H. Heimberg, Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132, 197-207 (2008).

85. N. Tsuji, N. Ninov, M. Delawary, S. Osman, A. S. Roh, P. Gut, D. Y. Stainier, Whole organism high content screening identifies stimulators of pancreatic beta-cell proliferation. PLoS One 9, e104112 (2014).
86. J. Xu, Y. F. Jia, S. Tapadar, J. D. Weaver, I. O. Raji, D. J. Pithadia, N. Javeed, A. J. Garcia, D. S. Choi, A. V. Matveyenko, A. K. Oyelere, C. H. Shin, Inhibition of TBK1/IKKepsilon Promotes Regeneration of Pancreatic beta-cells. Sci Rep 8, 15587 (2018).
87. G. Wang, S. K. Rajpurohit, F. Delaspre, S. L. Walker, D. T. White, A. Ceasrine, R. Kuruvilla, R. J. Li, J. S. Shim, J. O. Liu, M. J. Parsons, J. S. Mumm, First quantitative high-throughput screen in zebrafish identifies novel pathways for increasing pancreatic beta-cell mass. Elife 4, (2015).
88. O. Andersson, B. A. Adams, D. Yoo, G. C. Ellis, P. Gut, R. M. Anderson, M. S. German, D. Y. Stainier, Adenosine signaling promotes regeneration of pancreatic beta cells in vivo. Cell Metab 15, 885-894 (2012).
89. N. Schulz, K. C. Liu, J. Charbord, C. L. Mattsson, L. Tao, D. Tworus, O. Andersson, Critical role for adenosine receptor A2a in beta-cell proliferation. Mol Metab 5, 1138-1146 (2016).
90. J. P. Annes, J. H. Ryu, K. Lam, P. J. Carolan, K. Utz, J. Hollister-Lock, A. C. Arvanites, L. L. Rubin, G. Weir, D. A. Melton, Adenosine kinase inhibition selectively promotes rodent and porcine islet beta-cell replication. Proc Natl Acad Sci U S A 109, 3915-3920 (2012).
91. J. Charbord, L. Ren, R. B. Sharma, A. Johansson, R. Agren, L. Chu, D. Tworus, N. Schulz, P. Charbord, A. F. Stewart, P. Wang, L. C. Alonso, O. Andersson, In vivo screen identifies a SIK inhibitor that induces beta cell proliferation through a transient UPR. Nat Metab 3, 682-700 (2021).
92. H. Chakravarthy, X. Gu, M. Enge, X. Dai, Y. Wang, N. Damond, C. Downie, K. Liu, J. Wang, Y. Xing, S. Chera, F. Thorel, S. Quake, J. Oberholzer, P. E. MacDonald, P. L. Herrera, S. K. Kim, Converting Adult Pancreatic Islet alpha Cells into beta Cells by Targeting Both Dnmt1 and Arx. Cell Metab 25, 622-634 (2017).
93. V. Cigliola, L. Ghila, F. Thorel, L. van Gurp, D. Baronnier, D. Oropeza, S. Gupta, T. Miyatsuka, H. Kaneto, M. A. Magnuson, A. B. Osipovich, M. Sander, C. E. V. Wright, M. K. Thomas, K. Furuyama, S. Chera, P. L. Herrera, Pancreatic islet-autonomous insulin and smoothened-mediated signalling modulate identity changes of glucagon(+) alpha-cells. Nat Cell Biol 20, 1267-1277 (2018).
94. N. Ben-Othman, A. Vieira, M. Courtney, F. Record, E. Gjernes, F. Avolio, B. Hadzic, N. Druelle, T. Napolitano, S. Navarro-Sanz, S. Silvano, K. Al-Hasani, A. Pfeifer, S. Lacas-Gervais, G. Leuckx, L. Marroqui, J. Thevenet, O. D. Madsen, D. L. Eizirik, H. Heimberg, J. Kerr-Conte, F. Pattou, A. Mansouri, P. Collombat, Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis. Cell 168, 73-85 e11 (2017).
95. J. Li, T. Casteels, T. Frogne, C. Ingvorsen, C. Honore, M. Courtney, K. V. M. Huber, N. Schmitner, R. A. Kimmel, R. A. Romanov, C. Sturtzel, C. H. Lardeau, J. Klughammer, M. Farlik, S. Sdelci, A. Vieira, F. Avolio, F. Briand, I. Baburin, P. Majek, F. M. Pauler, T. Penz, A. Stukalov, M. Gridling, K. Parapatics, C. Barbieux, E. Berishvili, A. Spittler, J. Colinge, K. L. Bennett, S. Hering, T. Sulpice, C. Bock, M. Distel, T. Harkany, D. Meyer, G. Superti-Furga, P. Collombat, J. Hecksher-Sorensen, S. Kubicek, Artemisinins Target GABAA Receptor Signaling and Impair alpha Cell Identity. Cell 168, 86-100 e115 (2017).
96. M. Courtney, E. Gjernes, N. Druelle, C. Ravaud, A. Vieira, N. Ben-Othman, A. Pfeifer, F. Avolio, G. Leuckx, S. Lacas-Gervais, F. Burel-Vandenbos, D. Ambrosetti, J. Hecksher-Sorensen, P. Ravassard, H. Heimberg, A. Mansouri, P. Collombat, The inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion

into functional beta-like cells. PLoS Genet 9, e1003934 (2013).
97. S. Chera, D. Baronnier, L. Ghila, V. Cigliola, J. N. Jensen, G. Gu, K. Furuyama, F. Thorel, F. M. Gribble, F. Reimann, P. L. Herrera, Diabetes recovery by age-dependent conversion of pancreatic delta-cells into insulin producers. Nature 514, 503-507 (2014).
98. N. Druelle, A. Vieira, A. Shabro, M. Courtney, M. Mondin, S. Rekima, T. Napolitano, S. Silvano, S. Navarro-Sanz, B. Hadzic, F. Avolio, M. Rassoulzadegan, H. A. Schmid, A. Mansouri, P. Collombat, Ectopic expression of Pax4 in pancreatic delta cells results in beta-like cell neogenesis. J Cell Biol 216, 4299-4311 (2017).
99. M. Perez-Frances, L. van Gurp, M. V. Abate, V. Cigliola, K. Furuyama, E. Bru-Tari, D. Oropeza, T. Carreaux, Y. Fujitani, F. Thorel, P. L. Herrera, Pancreatic Ppy-expressing gamma-cells display mixed phenotypic traits and the adaptive plasticity to engage insulin production. Nat Commun 12, 4458 (2021).
100. W. Li, C. Cavelti-Weder, Y. Zhang, K. Clement, S. Donovan, G. Gonzalez, J. Zhu, M. Stemann, K. Xu, T. Hashimoto, T. Yamada, M. Nakanishi, Y. Zhang, S. Zeng, D. Gifford, A. Meissner, G. Weir, Q. Zhou, Long-term persistence and development of induced pancreatic beta cells generated by lineage conversion of acinar cells. Nat Biotechnol 32, 1223-1230 (2014).
101. L. Ye, M. A. Robertson, D. Hesselson, D. Y. Stainier, R. M. Anderson, Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. Development 142, 1407-1417 (2015).
102. J. Lu, K. C. Liu, N. Schulz, C. Karampelias, J. Charbord, A. Hilding, L. Rautio, P. Bertolino, C. G. Ostenson, K. Brismar, O. Andersson, IGFBP1 increases beta-cell regeneration by promoting alpha- to beta-cell transdifferentiation. EMBO J 35, 2026-2044 (2016).
103. C. S. M. Helker, S. T. Mullapudi, L. M. Mueller, J. Preussner, S. Tunaru, O. Skog, H. B. Kwon, F. Kreuder, J. J. Lancman, R. Bonnavion, P. D. S. Dong, M. Looso, S. Offermanns, O. Korsgren, F. M. Spagnoli, D. Y. R. Stainier, A whole organism small molecule screen identifies novel regulators of pancreatic endocrine development. Development 146, (2019).
104. S. P. Singh, P. Chawla, A. Hnatiuk, M. Kamel, L. D. Silva, B. Spanjaard, S. E. Eski, S. Janjuha, P. Olivares-Chauvet, O. Kayisoglu, F. Rost, J. Blasche, A. Krankel, A. Petzold, T. Kurth, S. Reinhardt, J. P. Junker, N. Ninov, A single-cell atlas of de novo beta-cell regeneration reveals the contribution of hybrid beta/delta-cells to diabetes recovery in zebrafish. Development 149, (2022).
105. N. Devos, G. Deflorian, F. Biemar, M. Bortolussi, J. A. Martial, B. Peers, F. Argenton, Differential expression of two somatostatin genes during zebrafish embryonic development. Mech Dev 115, 133-137 (2002).
106. F. Biemar, F. Argenton, R. Schmidtke, S. Epperlein, B. Peers, W. Driever, Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Dev Biol 230, 189-203 (2001).
107. F. Argenton, E. Zecchin, M. Bortolussi, Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. Mech Dev 87, 217-221 (1999).
108. J. Yu, J. Ma, Y. Li, Y. Zhou, L. Luo, Y. Yang, Pax4-Ghrelin mediates the conversion of pancreatic epsilon-cells to beta-cells after extreme beta-cell loss in zebrafish. Development 150, (2023).
109. P. D. Dong, E. Provost, S. D. Leach, D. Y. Stainier, Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. Genes Dev 22, 1445-1450 (2008).
110. D. Hesselson, R. M. Anderson, D. Y. Stainier, Suppression of Ptf1a activity induces acinar-to-endocrine conversion. Curr Biol 21, 712-717 (2011).
111. A. Eufrasio, C. Perrod, F. J. Ferreira, M. Duque, M. Galhardo, J. Bessa, In Vivo Reporter Assays Uncover Changes in Enhancer Activity Caused by Type 2 Diabetes-

Associated Single Nucleotide Polymorphisms. Diabetes 69, 2794-2805 (2020).
112. R. Bordeira-Carrico, J. Teixeira, M. Duque, M. Galhardo, D. Ribeiro, R. D. Acemel, P. N. Firbas, J. J. Tena, A. Eufrasio, J. Marques, F. J. Ferreira, T. Freitas, F. Carneiro, J. L. Gomez-Skarmeta, J. Bessa, Multidimensional chromatin profiling of zebrafish pancreas to uncover and investigate disease-relevant enhancers. Nat Commun 13, 1945 (2022).
113. K. C. Liu, A. Villasenor, M. Bertuzzi, N. Schmitner, N. Radros, L. Rautio, K. Mattonet, R. L. Matsuoka, S. Reischauer, D. Y. Stainier, O. Andersson, Insulin-producing beta-cells regenerate ectopically from a mesodermal origin under the perturbation of hemato-endothelial specification. Elife 10, (2021).
114. K. C. Liu, G. Leuckx, D. Sakano, P. A. Seymour, C. L. Mattsson, L. Rautio, W. Staels, Y. Verdonck, P. Serup, S. Kume, H. Heimberg, O. Andersson, Inhibition of Cdk5 Promotes beta-Cell Differentiation From Ductal Progenitors. Diabetes 67, 58-70 (2018).
115. C. Karampelias, H. Rezanejad, M. Rosko, L. Duan, J. Lu, L. Pazzagli, P. Bertolino, C. E. Cesta, X. Liu, G. S. Korbutt, O. Andersson, Reinforcing one-carbon metabolism via folic acid/Folr1 promotes beta-cell differentiation. Nat Commun 12, 3362 (2021).
116. H. Yang, D. Qin, S. Xu, C. He, J. Sun, J. Hua, S. Peng, Folic acid promotes proliferation and differentiation of porcine pancreatic stem cells into insulin-secreting cells through canonical Wnt and ERK signaling pathway. J Steroid Biochem Mol Biol 205, 105772 (2021).
117. P. S. McGrath, J. M. Wells, SnapShot: GI tract development. Cell 161, 176-176 e171 (2015).
118. A. Mavropoulos, N. Devos, F. Biemar, E. Zecchin, F. Argenton, H. Edlund, P. Motte, J. A. Martial, B. Peers, sox4b is a key player of pancreatic alpha cell differentiation in zebrafish. Dev Biol 285, 211-223 (2005).
119. V. Verbruggen, O. Ek, D. Georlette, F. Delporte, V. Von Berg, N. Detry, F. Biemar, P. Coutinho, J. A. Martial, M. L. Voz, I. Manfroid, B. Peers, The Pax6b homeodomain is dispensable for pancreatic endocrine cell differentiation in zebrafish. J Biol Chem 285, 13863-13873 (2010).
120. F. M. Delporte, V. Pasque, N. Devos, I. Manfroid, M. L. Voz, P. Motte, F. Biemar, J. A. Martial, B. Peers, Expression of zebrafish pax6b in pancreas is regulated by two enhancers containing highly conserved cis-elements bound by PDX1, PBX and PREP factors. BMC Dev Biol 8, 53 (2008).
121. V. Arkhipova, B. Wendik, N. Devos, O. Ek, B. Peers, D. Meyer, Characterization and regulation of the hb9/mnx1 beta-cell progenitor specific enhancer in zebrafish. Dev Biol 365, 290-302 (2012).
122. G. Dalgin, A. B. Ward, T. Hao le, C. E. Beattie, A. Nechiporuk, V. E. Prince, Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas. Development 138, 4597-4608 (2011).
123. A. Wilfinger, V. Arkhipova, D. Meyer, Cell type and tissue specific function of islet genes in zebrafish pancreas development. Dev Biol 378, 25-37 (2013).
124. E. Tarifeno-Saldivia, A. Lavergne, A. Bernard, K. Padamata, D. Bergemann, M. L. Voz, I. Manfroid, B. Peers, Transcriptome analysis of pancreatic cells across distant species highlights novel important regulator genes. BMC Biol 15, 21 (2017).
125. J. Lu, C. Cheng, Z. C. Cheng, Q. Wu, H. Shen, M. X. Yuan, B. Zhang, J. K. Yang, The dual role of RFX6 in directing beta cell development and insulin production. J Mol Endocrinol 66, 129-140 (2021).
126. J. Soyer, L. Flasse, W. Raffelsberger, A. Beucher, C. Orvain, B. Peers, P. Ravassard, J. Vermot, M. L. Voz, G. Mellitzer, G. Gradwohl, Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. Development 137, 203-212 (2010).
127. L. C. Flasse, J. L. Pirson, D. G. Stern, V. Von Berg, I. Manfroid, B. Peers, M. L. Voz,

Ascl1b and Neurod1, instead of Neurog3, control pancreatic endocrine cell fate in zebrafish. BMC Biol 11, 78 (2013).
128. G. Dalgin, V. E. Prince, Differential levels of Neurod establish zebrafish endocrine pancreas cell fates. Dev Biol 402, 81-97 (2015).
129. J. Djiotsa, V. Verbruggen, J. Giacomotto, M. Ishibashi, E. Manning, S. Rinkwitz, I. Manfroid, M. L. Voz, B. Peers, Pax4 is not essential for beta-cell differentiation in zebrafish embryos but modulates alpha-cell generation by repressing arx gene expression. BMC Dev Biol 12, 37 (2012).
130. S. Ibrahim, M. Johnson, C. H. Stephens, J. Xu, R. Moore, A. Mariani, C. Contreras, F. Syed, R. G. Mirmira, R. M. Anderson, E. K. Sims, beta-Cell pre-mir-21 induces dysfunction and loss of cellular identity by targeting transforming growth factor beta 2 (Tgfb2) and Smad family member 2 (Smad2) mRNAs. Mol Metab 53, 101289 (2021).
131. T. L. Mastracci, M. A. Robertson, R. G. Mirmira, R. M. Anderson, Polyamine biosynthesis is critical for growth and differentiation of the pancreas. Sci Rep 5, 13269 (2015).
132. J. He, Y. Yang, J. Zhang, J. Chen, X. Wei, J. He, L. Luo, Ribosome biogenesis protein Urb1 acts downstream of mTOR complex 1 to modulate digestive organ development in zebrafish. J Genet Genomics 44, 567-576 (2017).
133. E. S. Noel, A. Casal-Sueiro, E. Busch-Nentwich, H. Verkade, P. D. Dong, D. L. Stemple, E. A. Ober, Organ-specific requirements for Hdac1 in liver and pancreas formation. Dev Biol 322, 237-250 (2008).
134. C. H. Shin, W. S. Chung, S. K. Hong, E. A. Ober, H. Verkade, H. A. Field, J. Huisken, D. Y. Stainier, Multiple roles for Med12 in vertebrate endoderm development. Dev Biol 317, 467-479 (2008).
135. R. M. Anderson, J. A. Bosch, M. G. Goll, D. Hesselson, P. D. Dong, D. Shin, N. C. Chi, C. H. Shin, A. Schlegel, M. Halpern, D. Y. Stainier, Loss of Dnmt1 catalytic activity reveals multiple roles for DNA methylation during pancreas development and regeneration. Dev Biol 334, 213-223 (2009).
136. S. T. Mullapudi, G. L. M. Boezio, A. Rossi, M. Marass, R. L. Matsuoka, H. Matsuda, C. S. M. Helker, Y. H. C. Yang, D. Y. R. Stainier, Disruption of the pancreatic vasculature in zebrafish affects islet architecture and function. Development 146, (2019).
137. Y. H. C. Yang, K. Kawakami, D. Y. Stainier, A new mode of pancreatic islet innervation revealed by live imaging in zebrafish. Elife 7, (2018).
138. Y. H. C. Yang, L. J. B. Briant, C. A. Raab, S. T. Mullapudi, H. M. Maischein, K. Kawakami, D. Y. R. Stainier, Innervation modulates the functional connectivity between pancreatic endocrine cells. Elife 11, (2022).
139. H. Wang, H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, R. Jaenisch, One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910-918 (2013).
140. H. Yang, H. Wang, C. S. Shivalila, A. W. Cheng, L. Shi, R. Jaenisch, One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370-1379 (2013).
141. X. Yao, Z. Liu, X. Wang, Y. Wang, Y. H. Nie, L. Lai, R. Sun, L. Shi, Q. Sun, H. Yang, Generation of knock-in cynomolgus monkey via CRISPR/Cas9 editing. Cell Res 28, 379-382 (2018).
142. E. Zuo, Y. J. Cai, K. Li, Y. Wei, B. A. Wang, Y. Sun, Z. Liu, J. Liu, X. Hu, W. Wei, X. Huo, L. Shi, C. Tang, D. Liang, Y. Wang, Y. H. Nie, C. C. Zhang, X. Yao, X. Wang, C. Zhou, W. Ying, Q. Wang, R. C. Chen, Q. Shen, G. L. Xu, J. Li, Q. Sun, Z. Q. Xiong, H. Yang, One-step generation of complete gene knockout mice and monkeys by CRISPR/Cas9-mediated gene editing with multiple sgRNAs. Cell Res 27, 933-945 (2017).

143. K. Hoshijima, M. J. Jurynec, D. J. Grunwald, Precise Editing of the Zebrafish Genome Made Simple and Efficient. Dev Cell 36, 654-667 (2016).
144. U. Irion, J. Krauss, C. Nusslein-Volhard, Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. Development 141, 4827-4830 (2014).
145. B. Han, Y. Zhang, X. Bi, Y. Zhou, C. J. Krueger, X. Hu, Z. Zhu, X. Tong, B. Zhang, Bi-FoRe: an efficient bidirectional knockin strategy to generate pairwise conditional alleles with fluorescent indicators. Protein Cell 12, 39-56 (2021).
146. D. S. Levic, N. Yamaguchi, S. Wang, H. Knaut, M. Bagnat, Knock-in tagging in zebrafish facilitated by insertion into non-coding regions. Development 148, (2021).
147. T. O. Auer, K. Duroure, A. De Cian, J. P. Concordet, F. Del Bene, Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res 24, 142-153 (2014).
148. W. A. Wierson, J. M. Welker, M. P. Almeida, C. M. Mann, D. A. Webster, M. E. Torrie, T. J. Weiss, S. Kambakam, M. K. Vollbrecht, M. Lan, K. C. McKeighan, J. Levey, Z. Ming, A. Wehmeier, C. S. Mikelson, J. A. Haltom, K. M. Kwan, C. B. Chien, D. Balciunas, S. C. Ekker, K. J. Clark, B. R. Webber, B. S. Moriarity, S. L. Solin, D. F. Carlson, D. L. Dobbs, M. McGrail, J. Essner, Efficient targeted integration directed by short homology in zebrafish and mammalian cells. Elife 9, (2020).
149. S. Albadri, F. Del Bene, C. Revenu, Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. Methods 121-122, 77-85 (2017).
150. Y. A. Aksoy, D. T. Nguyen, S. Chow, R. S. Chung, G. J. Guillemin, N. J. Cole, D. Hesselson, Chemical reprogramming enhances homology-directed genome editing in zebrafish embryos. Commun Biol 2, 198 (2019).
151. Y. Hisano, T. Sakuma, S. Nakade, R. Ohga, S. Ota, H. Okamoto, T. Yamamoto, A. Kawahara, Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. Sci Rep 5, 8841 (2015).
152. G. Kesavan, A. Chekuru, A. Machate, M. Brand, CRISPR/Cas9-Mediated Zebrafish Knock-in as a Novel Strategy to Study Midbrain-Hindbrain Boundary Development. Front Neuroanat 11, 52 (2017).
153. Y. Kimura, Y. Hisano, A. Kawahara, S. Higashijima, Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. Sci Rep 4, 6545 (2014).
154. G. Kesavan, J. Hammer, S. Hans, M. Brand, Targeted knock-in of CreER (T2) in zebrafish using CRISPR/Cas9. Cell Tissue Res 372, 41-50 (2018).
155. P. Gillotay, M. Shankar, B. Haerlingen, E. Sema Elif, M. Pozo-Morales, I. Garteizgogeascoa, S. Reinhardt, A. Krankel, J. Blasche, A. Petzold, N. Ninov, G. Kesavan, C. Lange, M. Brand, A. Lefort, F. Libert, V. Detours, S. Costagliola, S. Sumeet Pal, Single-cell transcriptome analysis reveals thyrocyte diversity in the zebrafish thyroid gland. EMBO Rep 21, e50612 (2020).
156. A. Eschstruth, S. Schneider-Maunoury, F. Giudicelli, Creation of zebrafish knock-in reporter lines in the nefma gene by Cas9-mediated homologous recombination. Genesis 58, e23340 (2020).
157. M. R. Cronan, D. M. Tobin, Endogenous Tagging at the cdh1 Locus for Live Visualization of E-Cadherin Dynamics. Zebrafish 16, 324-325 (2019).
158. J. J. Luo, W. P. Bian, Y. Liu, H. Y. Huang, Q. Yin, X. J. Yang, D. S. Pei, CRISPR/Cas9-based genome engineering of zebrafish using a seamless integration strategy. FASEB J 32, 5132-5142 (2018).
159. J. Li, B. B. Zhang, Y. G. Ren, S. Y. Gu, Y. H. Xiang, J. L. Du, Intron targeting-mediated and endogenous gene integrity-maintaining knockin in zebrafish using the CRISPR/Cas9 system. Cell Res 25, 634-637 (2015).
160. W. Li, Y. Zhang, B. Han, L. Li, M. Li, X. Lu, C. Chen, M. Lu, Y. Zhang, X. Jia, Z. Zhu, X. Tong, B. Zhang, One-step efficient generation of dual-function conditional

knockout and geno-tagging alleles in zebrafish. Elife 8, (2019).
161. J. M. Welker, W. A. Wierson, M. P. Almeida, C. M. Mann, M. E. Torrie, Z. Ming, S. C. Ekker, K. J. Clark, D. L. Dobbs, J. J. Essner, M. McGrail, GeneWeld: Efficient Targeted Integration Directed by Short Homology in Zebrafish. Bio Protoc 11, e4100 (2021).
162. X. Yao, M. Zhang, X. Wang, W. Ying, X. Hu, P. Dai, F. Meng, L. Shi, Y. Sun, N. Yao, W. Zhong, Y. Li, K. Wu, W. Li, Z. J. Chen, H. Yang, Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells. Dev Cell 45, 526-536 e525 (2018).
163. Y. Yu, Y. Guo, Q. Tian, Y. Lan, H. Yeh, M. Zhang, I. Tasan, S. Jain, H. Zhao, An efficient gene knock-in strategy using 5'-modified double-stranded DNA donors with short homology arms. Nat Chem Biol 16, 387-390 (2020).
164. F. Tang, C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B. B. Tuch, A. Siddiqui, K. Lao, M. A. Surani, mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods 6, 377-382 (2009).
165. C. A. Lareau, F. M. Duarte, J. G. Chew, V. K. Kartha, Z. D. Burkett, A. S. Kohlway, D. Pokholok, M. J. Aryee, F. J. Steemers, R. Lebofsky, J. D. Buenrostro, Droplet-based combinatorial indexing for massive-scale single-cell chromatin accessibility. Nat Biotechnol 37, 916-924 (2019).
166. M. Bartosovic, M. Kabbe, G. Castelo-Branco, Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. Nat Biotechnol 39, 825-835 (2021).
167. S. J. Wu, S. N. Furlan, A. B. Mihalas, H. S. Kaya-Okur, A. H. Feroze, S. N. Emerson, Y. Zheng, K. Carson, P. J. Cimino, C. D. Keene, J. F. Sarthy, R. Gottardo, K. Ahmad, S. Henikoff, A. P. Patel, Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. Nat Biotechnol 39, 819-824 (2021).
168. L. Capolupo, I. Khven, A. R. Lederer, L. Mazzeo, G. Glousker, S. Ho, F. Russo, J. P. Montoya, D. R. Bhandari, A. P. Bowman, S. R. Ellis, R. Guiet, O. Burri, J. Detzner, J. Muthing, K. Homicsko, F. Kuonen, M. Gilliet, B. Spengler, R. M. A. Heeren, G. P. Dotto, G. La Manno, G. D'Angelo, Sphingolipids control dermal fibroblast heterogeneity. Science 376, eabh1623 (2022).
169. V. Zachariadis, H. Cheng, N. Andrews, M. Enge, A Highly Scalable Method for Joint Whole-Genome Sequencing and Gene-Expression Profiling of Single Cells. Mol Cell 80, 541-553 e545 (2020).
170. P. L. Stahl, F. Salmen, S. Vickovic, A. Lundmark, J. F. Navarro, J. Magnusson, S. Giacomello, M. Asp, J. O. Westholm, M. Huss, A. Mollbrink, S. Linnarsson, S. Codeluppi, A. Borg, F. Ponten, P. I. Costea, P. Sahlen, J. Mulder, O. Bergmann, J. Lundeberg, J. Frisen, Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. Science 353, 78-82 (2016).
171. M. Cohen, A. Giladi, O. Barboy, P. Hamon, B. Li, M. Zada, A. Gurevich-Shapiro, C. G. Beccaria, E. David, B. B. Maier, M. Buckup, I. Kamer, A. Deczkowska, J. Le Berichel, J. Bar, M. Iannacone, A. Tanay, M. Merad, I. Amit, The interaction of CD4(+) helper T cells with dendritic cells shapes the tumor microenvironment and immune checkpoint blockade response. Nat Cancer 3, 303-317 (2022).
172. N. Andrews, J. T. Serviss, N. Geyer, A. B. Andersson, E. Dzwonkowska, I. Sutevski, R. Heijboer, N. Baryawno, M. Gerling, M. Enge, An unsupervised method for physical cell interaction profiling of complex tissues. Nat Methods 18, 912-920 (2021).
173. C. Ortiz, J. F. Navarro, A. Jurek, A. Martin, J. Lundeberg, K. Meletis, Molecular atlas of the adult mouse brain. Sci Adv 6, eabb3446 (2020).
174. M. Asp, S. Giacomello, L. Larsson, C. Wu, D. Furth, X. Qian, E. Wardell, J. Custodio, J. Reimegard, F. Salmen, C. Osterholm, P. L. Stahl, E. Sundstrom, E. Akesson, O. Bergmann, M. Bienko, A. Mansson-Broberg, M. Nilsson, C. Sylven, J. Lundeberg, A

Spatiotemporal Organ-Wide Gene Expression and Cell Atlas of the Developing Human Heart. Cell 179, 1647-1660 e1619 (2019).
175. M. Weinberger, F. C. Simoes, R. Patient, T. Sauka-Spengler, P. R. Riley, Functional Heterogeneity within the Developing Zebrafish Epicardium. Dev Cell 52, 574-590 e576 (2020).
176. B. Hu, S. Lelek, B. Spanjaard, H. El-Sammak, M. G. Simoes, J. Mintcheva, H. Aliee, R. Schafer, A. M. Meyer, F. Theis, D. Y. R. Stainier, D. Panakova, J. P. Junker, Origin and function of activated fibroblast states during zebrafish heart regeneration. Nat Genet 54, 1227-1237 (2022).
177. T. Hoang, J. Wang, P. Boyd, F. Wang, C. Santiago, L. Jiang, S. Yoo, M. Lahne, L. J. Todd, M. Jia, C. Saez, C. Keuthan, I. Palazzo, N. Squires, W. A. Campbell, F. Rajaii, T. Parayil, V. Trinh, D. W. Kim, G. Wang, L. J. Campbell, J. Ash, A. J. Fischer, D. R. Hyde, J. Qian, S. Blackshaw, Gene regulatory networks controlling vertebrate retinal regeneration. Science 370, (2020).
178. B. Laplace-Builhe, A. Barthelaix, S. Assou, C. Bohaud, M. Pratlong, D. Severac, G. Tejedor, P. Luz-Crawford, M. Nguyen-Chi, M. Mathieu, C. Jorgensen, F. Djouad, NRG1/ErbB signalling controls the dialogue between macrophages and neural crest-derived cells during zebrafish fin regeneration. Nat Commun 12, 6336 (2021).
179. M. I. Cosacak, P. Bhattarai, S. Reinhardt, A. Petzold, A. Dahl, Y. Zhang, C. Kizil, Single-Cell Transcriptomics Analyses of Neural Stem Cell Heterogeneity and Contextual Plasticity in a Zebrafish Brain Model of Amyloid Toxicity. Cell Rep 27, 1307-1318 e1303 (2019).
180. S. Nayar, J. K. Morrison, M. Giri, K. Gettler, L. S. Chuang, L. A. Walker, H. M. Ko, E. Kenigsberg, S. Kugathasan, M. Merad, J. Chu, J. H. Cho, A myeloid-stromal niche and gp130 rescue in NOD2-driven Crohn's disease. Nature 593, 275-281 (2021).
181. J. K. Morrison, C. DeRossi, I. L. Alter, S. Nayar, M. Giri, C. Zhang, J. H. Cho, J. Chu, Single-cell transcriptomics reveals conserved cell identities and fibrogenic phenotypes in zebrafish and human liver. Hepatol Commun 6, 1711-1724 (2022).
182. M. V. Hunter, R. Moncada, J. M. Weiss, I. Yanai, R. M. White, Spatially resolved transcriptomics reveals the architecture of the tumor-microenvironment interface. Nat Commun 12, 6278 (2021).
183. Y. Hao, S. Hao, E. Andersen-Nissen, W. M. Mauck, 3rd, S. Zheng, A. Butler, M. J. Lee, A. J. Wilk, C. Darby, M. Zager, P. Hoffman, M. Stoeckius, E. Papalexi, E. P. Mimitou, J. Jain, A. Srivastava, T. Stuart, L. M. Fleming, B. Yeung, A. J. Rogers, J. M. McElrath, C. A. Blish, R. Gottardo, P. Smibert, R. Satija, Integrated analysis of multimodal single-cell data. Cell 184, 3573-3587 e3529 (2021).
184. C. S. McGinnis, L. M. Murrow, Z. J. Gartner, DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. Cell Syst 8, 329-337 e324 (2019).
185. I. Korsunsky, N. Millard, J. Fan, K. Slowikowski, F. Zhang, K. Wei, Y. Baglaenko, M. Brenner, P. R. Loh, S. Raychaudhuri, Fast, sensitive and accurate integration of single-cell data with Harmony. Nat Methods 16, 1289-1296 (2019).