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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis

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ABSTRACT

The interconversion of cell lineages via transdifferentiation is an adaptive mode of tissue regeneration and an appealing therapeutic target. However, its clinical exploitation is contingent upon the discovery of contextual regulators of cell fate acquisition and maintenance. In murine models of diabetes, glucagon-secreting alpha cells transdifferentiate into insulin-secreting beta cells following targeted beta cell depletion, regenerating the form and function of the pancreatic islet. However, the molecular triggers of this mode of regeneration are unknown. Here, using lineage-tracing assays in a transgenic zebrafish model of beta cell ablation, we demonstrate conserved plasticity of alpha cells during islet regeneration. In addition, we show that *glucagon* expression is upregulated after injury. Through gene knockdown and rescue approaches, we also find that peptides derived from the *glucagon* gene are necessary for alpha-to-beta cell fate switching. Importantly, whereas beta cell neogenesis was stimulated by glucose, alpha-to-beta cell conversion was not, suggesting that transdifferentiation is not mediated by glucagon/GLP-1 control of hepatic glucose production. Overall, this study supports the hypothesis that alpha cells are an endogenous reservoir of potential new beta cells. It further reveals that *glucagon* plays an important role in maintaining endocrine cell homeostasis through feedback mechanisms that govern cell fate stability.

KEY WORDS: Transdifferentiation, Alpha cell, Beta cell, Regeneration, Glucagon, Gcga, GLP-1, Insulin, Pancreas, Zebrafish, Arxa, Arx, Pancreatic progenitor

INTRODUCTION

Glucagon and insulin, secreted by pancreatic alpha (α) and beta (β) cells, respectively, exert counter-regulatory actions on glucose metabolism. Glucagon increases circulating glucose levels by stimulating hepatic glycogenolysis and gluconeogenesis. Conversely, insulin decreases glucose levels by eliciting cellular influx and storage of glucose while dampening hepatic glucose production. In the pancreatic islet, α and β cells are physically juxtaposed; each cell type receives and responds to secreted signals from the other to modulate secretory activities (i.e. cell function). For instance, insulin represses glucagon expression and release from the α cell, whereas glucagon stimulates β cell insulin secretion (Kawai et al., 1995; Kawamori and Kulkarni, 2009). Dysregulation of insulin and glucagon signaling consequent to β cell dysfunction or destruction causes diabetes mellitus, a devastating disease that afflicts more than 360 million people worldwide (Whiting et al., 2011). With the loss of insulin signaling, unchecked glucagon signaling drives hyperglycemia and, thereby, the complications associated with diabetes (Unger and Cherrington, 2012). However, the mechanisms by which acute changes in intra-islet hormone signaling affect islet cell composition or islet cell fates are largely unknown.

Restoration of β cell mass is a conceptually proven approach to cure diabetes (Shapiro et al., 2000), but its widespread implementation will require a vast supply of functional β cells derived from either exogenous (e.g. stem cells) or endogenous (e.g. facultative progenitors) sources. It is clear from models of β cell regeneration in the mouse that the mode and extent of pancreatic injury influence mechanisms of regeneration, and these models have revealed that new β cells can arise from several endogenous sources. First, β cell regeneration may be mediated by the proliferation of surviving β cells (Dor et al., 2004). Second, neogenesis from duct or duct-associated progenitor cells may occur spontaneously or in response to injury via pancreatic duct ligation (Inada et al., 2008; Xu et al., 2008), although contradictory reports indicate that this process might be rare (Solar et al., 2009; Kopp et al., 2011; Xiao et al., 2013). Third, spontaneous transdifferentiation of β cells from α or δ cells occurs in conditions of extreme β cell loss (Thorel et al., 2010; Chera et al., 2014). Likewise, with some limitations, α and β cells can be interconverted through gain or loss of key pancreatic transcription factors, including Arx, Pax4 and Pdx1 (Collombat et al., 2007, 2009; Yang et al., 2011). Together, these studies have revealed plasticity in the endocrine pancreas and indicate that other pancreatic endocrine cells might be an exploitable source of new, functional β cells. However, the endogenous extracellular cues triggering α-to-β cell transdifferentiation are unknown.

Here, using transgenic zebrafish models of β cell ablation, we have investigated the cellular and molecular mechanisms of new β cell formation during regeneration. The zebrafish is an attractive and useful model for studying mechanisms of pancreas formation and disease repair because of its conserved architecture, composition, development, and attributes that permit rapid loss-of-function analyses (Biemar et al., 2001; Field et al., 2003; Kinkel and Prince, 2009; Jurczyk et al., 2011). In addition, the heightened regenerative capacity of the zebrafish facilitates the discovery of novel organ repair mechanisms that might be present, but dormant, in mammals (Aguirre et al., 2013). We use lineage-tracing tools to demonstrate conserved plasticity of α cells during regeneration in the zebrafish islet. Importantly, and for the first time, we show that *glucagon* gene activation is responsible for this α cell fate switch; blockade of this signaling pathway via *glucagon* knockdown nearly extinguishes β cell regeneration. Importantly, our data further suggest that transdifferentiation is not solely dependent on the

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gluconeogenic properties of glucagon. Overall, this study supports the hypothesis that α cells constitute an endogenous reservoir of new β cells that is pharmacologically exploitable.

RESULTS

β cell regeneration occurs by neogenesis in zebrafish

To investigate the origin of regenerating β cells, we used transgenic models of conditional β cell ablation. In *Tg(ins:CFP-NTR)*^{s892} and *Tg(ins:Flag-NTR)*^{s950} nitroreductase converts Metronidazole (MTZ) into a toxic compound that rapidly induces β cell apoptosis (Curado et al., 2007). Treatment of embryos with MTZ from 3 to 4 days post fertilization (dpf) ablated all β cells, and after its removal β cell mass rapidly recovered at a rate greater than that of normal larval neogenesis (Fig. 1A-F). We observed that free glucose levels were elevated in β cell-ablated larvae (Fig. 1G), confirming the functionality of larval β cells. Free glucose levels peaked one day into the recovery period [1 day post ablation (dpa)], but, importantly, by 8 dpf there was no difference in glucose levels between the ablated and control groups. This restoration of sufficient overall β cell function, despite only partial recovery of β cell mass, indicates that individual β cells may be hyperfunctional.

To determine whether surviving β cells contributed to islet regeneration in our model, we used a genetic lineage-tracing approach. Specifically, *Tg(ins:Cre;cryaa:YFP)*^{s924} (hereafter *ins:Cre*) was crossed with the inducible reporter line *Tg(hsp70l:loxp-mcherry-stop-loxp-H2B-GFP;cryaa:CFP)*^{s923} (hereafter *hs:CSH*) (Hesselson et al., 2009). First, as a control, we heat pulsed *ins:Cre; hs:CSH* larvae at 3 dpf to mark *insulin* (*ins*)$^{+}$ β cells with histone H2B-GFP and found that 41% of β cells were labeled at 4 dpf (supplementary material Fig. S1A,B,G). In accord with previous studies (Hesselson et al., 2009), we saw no change in the number of H2B-GFP^{+} β cells between 1 and 2 days after labeling (supplementary material Fig. S1A-D,H). Next, we heat pulsed *ins:Flag-NTR; ins:Cre*; *hs:CSH* embryos at 3 dpf, shortly before MTZ treatment, and found that in 1-dpa regenerating islets only 2% of all post-ablation *ins*$^{+}$ β cells were labeled (Fig. 1H,I; supplementary material Fig. S1I; *n*=13). This indicates that pre-existing β cells do not significantly contribute to regeneration in our model.

To further exclude a significant contribution of pre-existing β cells to islet regeneration in our model, we used *Tg(ins:Kaede)*^{s949} fish, in which β cells are labeled by the green-to-red photoconvertible fluorescent protein Kaede (Andersson et al., 2012). When Kaede was converted to red at 72 hours post fertilization (hpf), control (unablated) islets were composed of two populations of β cells at 96 hpf. Most exhibited yellow (green plus red) fluorescence, indicating *ins*$^{+}$ cells that existed during labeling, whereas some β cells exhibited only green fluorescence, indicating that they were generated in the 24-h period after labeling (supplementary material Fig. S1J,K). In regenerating *Tg(ins:CFP-NTR)*^{s892} islets, when Kaede was converted at 72 hpf immediately after MTZ treatment, the 1-dpa islets contained only unconverted green *ins*$^{+}$ cells (supplementary material Fig. S1L,M).

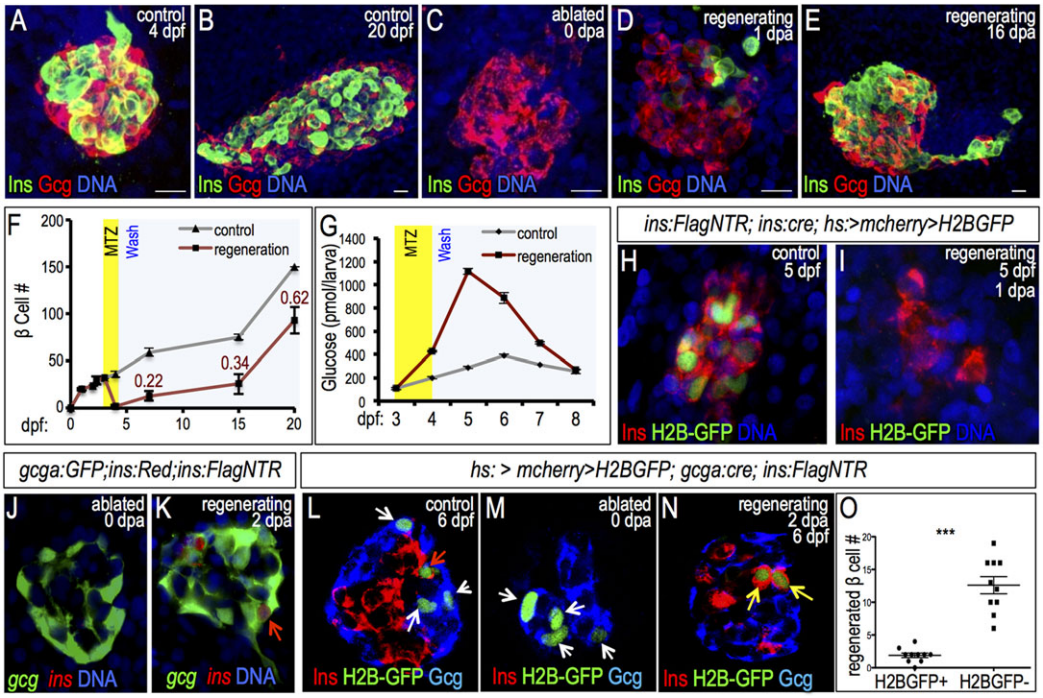

Fig. 1. β cell neogenesis from α cell transdifferentiation in zebrafish. (A-E) Confocal projections showing α (red) and β (green) cells in the principal islet of intact (A,B) and ablated (C-E) *Tg(ins:CFP-NTR)* larvae at 0, 1 and 16 days post ablation (dpa). Scale bars: 10 µm. (F) Quantification of *insulin*$^{+}$ cells in intact (gray line) and regenerating (red line) islets from 0 to 20 dpf (*n*≥3 for all groups except control 20 dpf, *n*=1). The ratio of β cells in regenerating versus control islets is indicated. (G) Blood glucose measurements of non-ablated larvae (gray) and larvae in which β cells were ablated from 3-4 dpf (red) (*n*=3). (H,I) Confocal projections of β cell lineage-marked islets in 5-dpf control and 1-dpa *Tg(ins:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* larvae. β cells were labeled by inducible H2B-GFP at 3 dpf before ablation and stained for GFP (green) and insulin (red). (J,K) Confocal planes of ablated (J) and regenerating (K) islets in *Tg(ins:Flag-NTR); Tg(gcga:GFP); Tg(ins:dsRed)* larvae. Red arrow in K indicates *gcga:GFP*$^{+}$ *ins:dsRed*$^{+}$ β cells in regenerating islet. (L-N) Confocal planes of *Tg(gcga:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets labeled by H2B-GFP before ablation, and stained for GFP (green), insulin (red) and glucagon (blue). α cells are indicated by white arrows and β cells by the red arrow. (L) 6-dpf non-ablated islet, (M) 4-dpf ablated islet at 0 dpa, and (N) 6-dpf islet at 2 dpa. H2B-GFP^{+} regenerating β cells are indicated with yellow arrows. (O) Quantification of H2B-GFP^{+} and H2B-GFP^{-} β cells in 2-dpa islets (*n*=10). ***P≤0.001 (Student's *t*-test).

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Together, our *ins*:*Cre* and *ins*:*Kaede* data demonstrate that essentially all β cells are ablated by MTZ treatment in the *NTR* model, and that islet regeneration occurs through β cell neogenesis.

β cells transdifferentiate from α cells during regeneration

In mice, severe β cell ablation triggers α-to-β cell conversion (Chung et al., 2010; Thorel et al., 2010). We reasoned that if this switch occurred in our model, then intermediate cell phenotypes would be detected as α cell character gives way to β cell character. To test this hypothesis we used triple-transgenic *Tg(ins:Flag-NTR); Tg(gcga:GFP); Tg(ins:dsRed)* zebrafish, in which α and β cells are marked in green and red, respectively. Although no β cells remained after MTZ treatment at 0 dpa, several GFP$^+$ dsRed$^+$ double-positive cells were detected at 1 and 2 dpa (Fig. 1J,K; supplementary material Fig. S2).

Next, to distinguish between α-to-β cell transdifferentiation and *de novo* co-expression of glucagon and insulin during the differentiation of β cell progenitors, we used temporally restricted α cell lineage tracing. *Tg(gcga:Cre; cryaa:YFP)*^{s962} (hereafter *gcga:Cre*) fish were crossed with *hs:CSH*, and *gcga:Cre; hs:CSH* embryos were heat pulsed at 3 dpf and analyzed at 4 dpf. This approach marked 17% of glucagon (Gcga)$^+$ α cells and 8.5% of β cells with H2B-GFP, but no delta (δ) cells (Fig. 1L; supplementary material Fig. S3A-E). This might reflect activity of the *glucagon* promoter in some β cells or their progenitors during neogenesis in embryos/larvae and adults (supplementary material Fig. S4). Even so, when we heat pulsed *gcga:Cre; hs:CSH; ins:Flag-NTR* embryos at 3 dpf to mark *gcga*$^+$ cells, then ablated β cells with MTZ treatment from 3-4 dpf, Gcga$^+$ α cells were the only cells that remained marked by H2B-GFP (Fig. 1M). At 2 dpa, ∼13.1% of newly generated β cells (1.9 cells out of 14.5 cells/islet) were H2B-GFP marked (Fig. 1N,O). When this is normalized to the efficiency of *gcga:Cre* it suggests that ∼11 β cells (77%) per recovering islet are derived from *gcga:Cre*-expressing cells. Since all β cells, including those labeled by *gcga:Cre*, are destroyed by MTZ treatment, this observation indicates that new β cells originated from pre-labeled α cells.

Additionally, it was recently shown that somatostatin$^+$ δ cells spontaneously convert into insulin-producing cells in young, β cell-ablated mice (Chera et al., 2014). To determine whether δ cells could also switch identity in zebrafish, we used *Tg(sst2:Cre;cryaa:Venus)*^{s963} (hereafter *sst2:Cre*) to similarly trace δ cells. *sst2:Cre* labeled 45% of δ cells and 1.5% of insulin$^+$ cells in intact islets, whereas in regenerating islets only 1% of the new insulin$^+$ β cells were labeled (Fig. 2A-E). Concordantly, regenerating islets rarely showed colocalization of insulin and somatostatin (supplementary material Fig. S5). These data indicate that δ cells do not typically share the capacity of α cells to transdifferentiate to β cells in response to β cell ablation in zebrafish.

α-to-β cell transdifferentiation can be marked by label retention in the zebrafish principal islet

In the pancreata of both *gcga:GFP* and *gcga:Cre; hs:CSH* fish, we consistently observed a small fraction of GFP$^+$ cells outside the islet that did not stain for either insulin or glucagon (supplementary material Fig. S6). These might represent immature cells that express the *glucagon* promoter construct, but are not yet committed to the α or β cell fate (pro-α cells) (Habener and Stanojevic, 2012). Our genetic tools would not distinguish between β cells formed from such pro-α cells or from the conversion of mature α cells inside the islet. Thus, to further test whether mature hormone-expressing α cells can transdifferentiate to β cells during regeneration, we used a label-retaining cell (LRC) assay to mark a discrete population of endocrine cells in the principal islet (Hesselson et al., 2009; Wilfinger et al., 2013). For these experiments, embryos were injected with mRNA encoding H2B-RFP, which is a stable fluorescent protein that is exponentially diluted by cell division (Fig. 3A). Zebrafish endocrine cells derived from the dorsal pancreatic bud differentiate, cluster into an islet, and become largely quiescent by 24 hpf, and therefore retain H2B-RFP fluorescence (Hesselson et al., 2009). Conversely, ventral pancreatic bud-derived

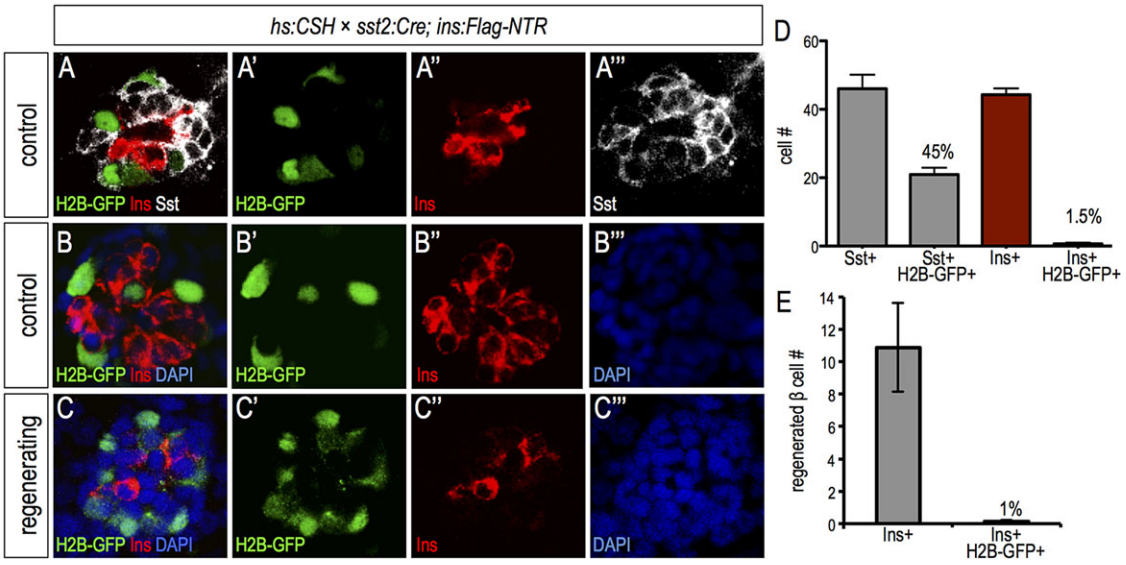

Fig. 2. δ cells, but not regenerating β cells, are lineage marked by *sst2:Cre*. (A-A‴) Merged and single-channel images of 5-dpf *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets that were heat shocked at 3 dpf and stained for GFP (green), insulin (red) and somatostatin (white). Somatostatin$^+$ δ cells are labeled by H2B-GFP. (B-C‴) Merged and single-channel images of 5-dpf non-ablated (B) or 1 dpa (C) *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets that were heat shocked at 3 dpf and stained for GFP (green), insulin (red) and DNA (blue). (D) Quantification of total somatostatin$^+$, somatostatin$^+$/H2B-GFP$^+$, insulin$^+$, and insulin$^+$/H2B-GFP$^+$ cells in non-ablated 5-dpf *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets. 45% of somatostatin$^+$ cells and 1.5% of insulin$^+$ cells are marked by *sst2:Cre* (n=15). (E) Quantification of insulin$^+$ and insulin$^+$/H2B-GFP$^+$ cells in 5-dpf regenerating islets. 1% of insulin$^+$ cells are marked by *sst2:Cre* (n=9).

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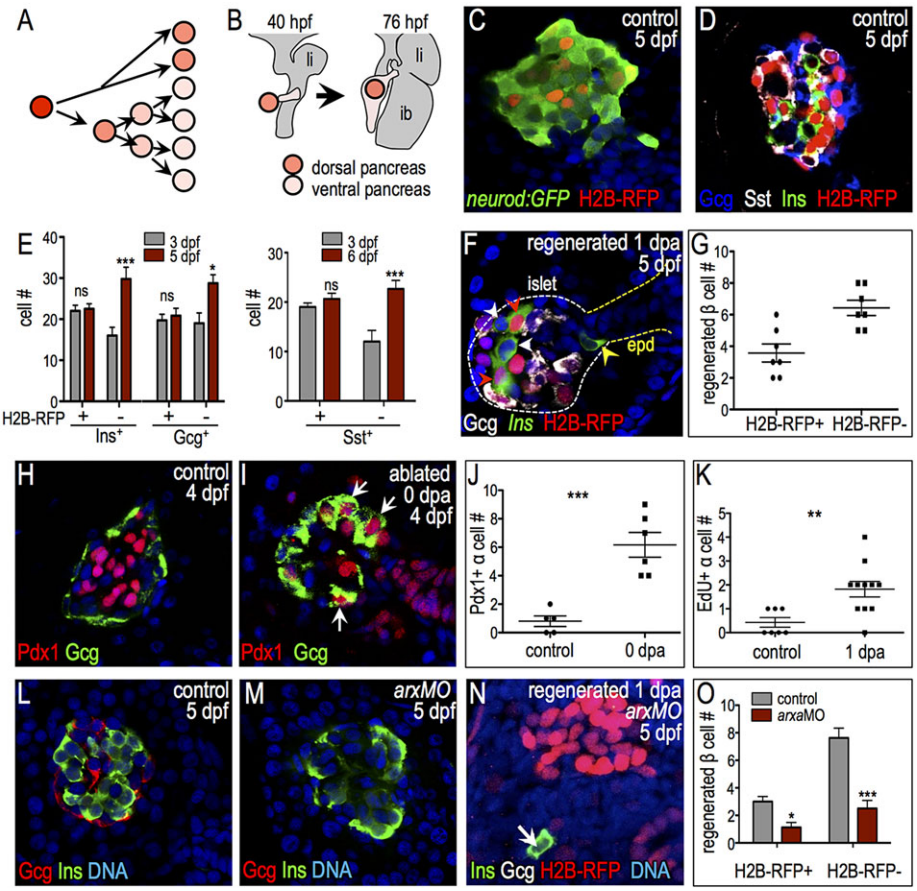

Fig. 3. α-to-β cell transdifferentiation can be marked by the label-retaining cell (LRC) assay in the zebrafish principal islet. (A,B) Schematic of the LRC assay. (A) Zygotes were injected with *H2B-RFP* mRNA. Fluorescent signal from H2B-RFP protein was diluted by mitosis. (B) The LRC assay marks quiescent, early differentiated pancreatic endocrine cells derived from the dorsal bud. li, liver; ib, intestinal bulb. (C,D) Confocal planes of H2B-RFP-labeled 5-dpf islets. (C) *TgBAC(neurod1:EGFP)* (n=4). (D) Wild-type islet stained for insulin (green), glucagon (blue) and somatostatin (white) (n=5). (E) Quantification of H2B-RFP$^+$ and H2B-RFP$^-$ cells expressing insulin, glucagon and somatostatin ($n\geq3$ for all). (F) H2B-RFP-labeled regenerating islet of 5-dpf/1-dpa *Tg(ins:CFP-NTR)* larva stained for CFP (insulin, green) and glucagon (white). Red arrowheads indicate H2B-RFP$^+$ regenerating β cells, white arrowheads indicate H2B-RFP$^-$ regenerating β cells in the islet, and yellow arrowhead indicates H2B-RFP$^-$ β cells in the extra-pancreatic duct region (epd). (G) Quantification of 1-dpa H2B-RFP$^+$ and H2B-RFP$^-$ β cells (n=7). (H,I) Confocal planes showing Pdx1 (red) and glucagon (green) expression in 4-dpf non-ablated (H) and ablated (I) islets. Pdx1$^+$ α cells are indicated by arrows. (J) Quantification of α cells expressing Pdx1 in 4-dpf non-ablated (n=5) and ablated (n=6) islets. (K) Quantification of proliferating (EdU$^+$) α cells in 5-dpf non-ablated and 1-dpa islets ($n\geq7$ islets examined). (L,M) Confocal planes showing glucagon (red) and insulin (green) staining in control MO-injected (L) and *arxa*MO-injected (M) 5-dpf larvae. Glucagon$^+$ cells are absent from *arxa* morphant islets. (N) Confocal projection of *arxa*MO-injected 5-dpf/1-dpa *Tg(ins:CFP-NTR)* islet labeled with H2B-RFP lacking most β cell regeneration. Arrow indicates regenerated β cell. (O) Quantification of 1-dpa β cells in control MO-injected (gray, n=16) and *arxa*MO-injected (red, n=19) islets. *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$; ns, not significant (Student's t-test in J,K; two-way ANOVA followed by Bonferroni post-test in E,O).

progenitor cells undergo intensive proliferation before differentiating (Wang et al., 2011), and thus do not retain the fluorescent signal (H2B-RFP$^-$). Using this approach therefore makes it possible to discern the population of dorsal pancreatic bud-derived endocrine cells as H2B-RFP$^+$ (Fig. 3B).

We used multiple criteria to demonstrate that the label-retaining cells in the principal islet were committed endocrine cells. First, we determined that nearly all H2B-RFP$^+$ cells in the islet were positive for *neurod1:*GFP (a pan-endocrine marker) and *sox17:*GFP (an endoderm marker) and expressed insulin, glucagon or somatostatin (Fig. 3C,D; supplementary material Fig. S7A-C). A small number of H2B-RFP$^+$ cells in the principal islet were *kdrl:*GFP positive and this fraction was similar in size to that of the *sox17*-negative and *neurod1*-negative fractions (supplementary material Fig. S7D,E), suggesting that the non-endocrine H2B-RFP$^+$ cells are endothelial cells. The number of H2B-RFP$^+$ α, β and δ cells remained constant during development (Fig. 3E), which indicates that dorsal bud-derived α and δ cells are also largely quiescent, as previously reported for β cells (Hesselson et al., 2009). Taken together, our data show that the LRC assay marks differentiated, quiescent pancreatic endocrine cells in the principal islet. Moreover, H2B-RFP$^-$ ventral bud-derived pancreatic progenitor cells continuously differentiate into endocrine cells and contribute to principal islet formation in zebrafish larvae (Fig. 3E), in accord with previous findings (Hesselson et al., 2009; Wilfinger et al., 2013).

Next, we used the LRC assay to investigate the origin of new β cells in β cell-ablated *Tg(ins:CFP-NTR)*^{s892} larvae. We found that 35.7% of new β cells in 1-dpa regenerating islets were H2B-RFP$^+$, indicating that they arose from pre-labeled dorsal bud-derived endocrine cells (Fig. 3F,G, red arrowhead). Given our data above that δ cells and surviving β cells do not contribute to β cell regeneration, these H2B-RFP$^+$ β cells most likely arise from H2B-RFP$^+$ α cells. In further support of this assertion, a subset of H2B-RFP$^+$ β cells was also glucagon positive (supplementary material Fig. S7F, white arrow).

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Finally, we examined the expression of Pdx1 in putative transdifferentiating α cells. Initially, Pdx1 is expressed in all pancreatic progenitors, and later in differentiated β and δ cells (Offield et al., 1996). Importantly, the role of Pdx1 as a transcription factor crucial for β cell development and maturation is conserved in zebrafish (Yee et al., 2001; Kimmel et al., 2011). In the 4-dpf pancreas, Pdx1 was expressed in β and duct cells, but not in Gcga$^+$ α cells (Fig. 3H). However, after β cells were ablated, many Pdx1$^+$ Gcga$^+$ α cells were detected in the islet (Fig. 3I,J), consistent with previous observations in mouse models that Pdx1 is expressed in α cells during α-to-β cell transdifferentiation (Chung et al., 2010; Thorel et al., 2010).

Since pre-existing α cells switched to β cells in our ablation model, we hypothesized that α cell mass would decrease unless compensated by proliferation or neogenesis. We found no difference in α cell mass at any of the stages examined through 20 dpf (supplementary material Fig. S8A). To measure proliferation rate, we injected 1-dpa larvae with EdU and found that α cell labeling was significantly increased in the regenerating group (Fig. 3K; supplementary material Fig. S8B,C). We observed similar results using the M-phase marker phospho-histone H3 (supplementary material Fig. S8D-I). No significant β cell proliferation was detected at 1 dpa (supplementary material Fig. S8J-L). This further supports our deduction above that neogenesis, rather than proliferation of pre-existing β cells, is the primary source during the initial stage of β cell regeneration in our model. Subsequently, however, β cell proliferation may also contribute to the restoration of β cell mass in the later stages of regeneration.

Next, we excluded the possibility that any replication of dorsal bud derivatives confounded the LRC assay by titrating the injected *H2B-RFP* mRNA. RFP fluorescence was completely lost only with a 16-fold dilution, suggesting that labeled cells can divide three times ($1/2^3$) and remain detectable (supplementary material Fig. S9A-E). Since we found no significant change in H2B-RFP$^+$ islet cell number between 0 and 1 dpa (supplementary material Fig. S9F-H), and since we only detected ≤2 EdU$^+$ H2B-RFP$^+$ cells per regenerating islet (supplementary material Fig. S9I), these data indicate that dilution of H2B-RFP signal by proliferation is negligible. Moreover, among the proliferating α cells, most were found to be H2B-RFP$^-$ (supplementary material Fig. S9J). Altogether, our data suggest that dorsal bud-derived endocrine cells do not proliferate, but that H2B-RFP$^+$ α cells directly convert to β cells. The increased rate of H2B-RFP$^-$ α cell proliferation may compensate for the loss of α cell mass due to α-to-β cell conversion during the regeneration phase.

In sum, our data show that β cells formed during islet regeneration arise both from α cell transdifferentiation (H2B-RFP$^+$) and from the differentiation of ventral bud-derived pancreatic cells (H2B-RFP$^-$), which could be naïve progenitors or ventral bud-derived endocrine cells. These findings prompt the question of how these two cellular regeneration sources are coordinately regulated.

α cells and *glucagon* are required for β cell regeneration

To test the necessity of α cells in islet regeneration, we eliminated them by zygotic injection of *arxa* morpholino (*arxa*MO). The homeobox transcriptional repressor Arx is conserved between humans and zebrafish, and orthologs exhibit 68% identity in their peptide sequences. Importantly, loss of *Arx* results in depletion of α cells from the endocrine pancreas in human, mouse and fish (Collombat et al., 2003; Itoh et al., 2010; Mastracci et al., 2011; Djiotsa et al., 2012). In *arxa*MO-injected, but not control MO-injected, islets, glucagon protein and mRNA were undetectable, although neither MO generated general morphological defects (Fig. 3L,M; supplementary material Fig. S10A-C; data not shown). β cell number was statistically unaffected, whereas δ cell number increased (supplementary material Fig. S10D,E).

Next, we tested how the loss of Arx influenced β cell regeneration from dorsal and ventral bud-derived sources by co-injecting *arxa*MO and *H2B-RFP* mRNA into *Tg(ins:CFP-NTR)* embryos. In *arxa*MO-injected larvae, the number of H2B-RFP$^+$ regenerated β cells was significantly reduced compared with control MO-injected larvae (Fig. 3N,O), showing that Arxa is required for β cell transdifferentiation from α cells. Interestingly, regenerating islets in *arxa*MO-injected larvae had an increased number of insulin$^+$ somatostatin$^+$ cells, suggesting that α cell loss might facilitate δ-to-β cell transdifferentiation (supplementary material Fig. S10F-H). Surprisingly, loss of Arxa also resulted in a dramatic decrease in H2B-RFP$^-$ β cell regeneration (Fig. 3N,O). Thus, in addition to our earlier findings showing that α cells can be direct precursors of β cells, α cells might also provide local or systemic cues that regulate neogenesis from ventral bud-derived precursors.

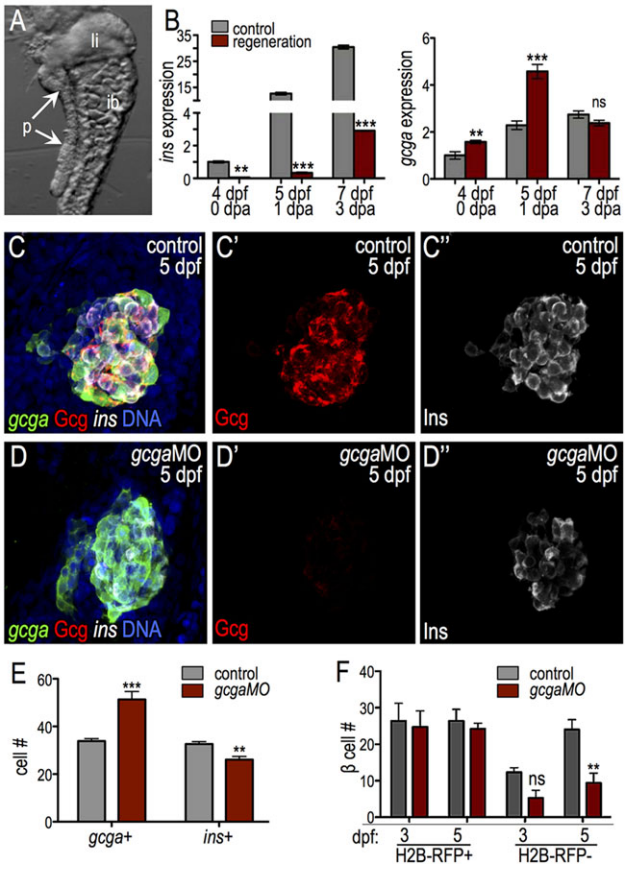

Fig. 4. *glucagon* is required for islet development. (A) Endodermal organs isolated from 4-dpf larvae. p, pancreas; li, liver; ib, intestinal bulb. (B) qPCR of *insulin* (*ins*) and *glucagon* (*gcga*) in control and ablated larvae. *ins* expression was diminished and *gcga* expression transiently increased during regeneration (n=3 independent experiments). (C-E) *gcga*MO blocks glucagon protein expression and increases the α to β cell ratio. (C-D″) Confocal projections of 5-dpf control MO-injected (C) and *gcga*MO-injected (D) *Tg(gcga:GFP); Tg(ins:dsRed)* larvae stained for glucagon (red). (E) Quantification of *gcga:GFP*$^+$ α cells and *ins*$^+$ β cells in control MO-injected (n=15) and *gcga*MO-injected (n=10) islets. (F) Quantification of H2B-RFP$^+$ and H2B-RFP$^-$ β cells at 3 and 5 dpf in control MO-injected (n≥3) and *gcga*MO-injected (n=5) islets. Ventral bud-derived H2B-RFP$^-$ β cells are specifically diminished in *gcga* morphants. *P≤0.05, **P≤0.01, ***P≤0.001; ns, not significant (two-way ANOVA followed by Bonferroni post-test).

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To test whether glucagon could be such a cue, we isolated endodermal organs from ablated and control embryos (Fig. 4A; supplementary material Fig. S11), and then used quantitative PCR (qPCR) to examine *insulin* (*ins*) and *glucagon* (*gcga*) expression (other genes examined are listed in supplementary material Table S1). As expected, *ins* was depleted at 0 dpa, but showed recovery at 1 and 3 dpa (Fig. 4B). By contrast, *gcga* expression mirrored free glucose measurements: elevated at 0 and 1 dpa, but returned to baseline by 3 dpa (Fig. 1G and Fig. 4B). *In situ* hybridization at 4 and 5 dpf showed that *gcga* expression was restricted to the pancreas and that expression in control and regenerating islets followed the same trend, which qualitatively supports the qPCR data (supplementary material Fig. S12A-H). Zygotic injection of translation-blocking *glucagon* MO (*gcga*MO) into *Tg(gcga:GFP)* zygotes caused no gross developmental defects (supplementary material Fig. S12I); however, it eliminated glucagon protein expression, increased *gcga:GFP*$^{+}$ cell number, and decreased β cell formation (Fig. 4C-E). Moreover, using the LRC assay we found that *gcga*MO impaired ventral but not dorsal bud-derived β cell formation (Fig. 4F; supplementary material Fig. S13). Conversely, the expansion of α cell mass was associated with increased α cell proliferation and neogenesis from duct-associated progenitors (supplementary material Fig. S14), consistent with the endocrine phenotypes reported in mutant mice deficient in the glucagon pathway (Prasadan et al., 2002; Webb et al., 2002; Gelling et al., 2003; Hayashi et al., 2009).

We next asked if β cell regeneration was impacted following *glucagon* knockdown by co-injecting *gcga*MO and *H2B-RFP* mRNA into *Tg(ins:CFP-NTR)* zygotes, and found that β cell regeneration from both H2B-RFP^{+} and H2B-RFP^{-} sources was significantly reduced (Fig. 5A,D,G). The effect of *gcga*MO on β cell regeneration and α cell transdifferentiation was further confirmed by *gcga:Cre; hs:CSH* α cell lineage tracing. We found a significant reduction of total β cells and of H2B-GFP^{+} β cells in *gcga*MO-injected regenerating islets (Fig. 5H-J). These results mirror our *arxa*MO results in which α cell loss influences β cell regeneration from both cellular sources, and are consistent with both local and systemic functions of glucagon.

Glucagon or Exendin-4 infusion restores β cell regeneration

Selective proteolysis results in the production of several peptide products from the *glucagon* gene, including glucagon and Glucagon-like peptide 1 (GLP-1), which signal through distinct receptors. Using *glucagon* gene knockdown, we could not distinguish between the actions of the different peptides on β cell regeneration. Because human glucagon binds and activates zebrafish Glucagon receptor (Gcgra), and the GLP-1 receptor agonist Exendin-4 (Ex-4) activates the zebrafish GLP-1 receptor (Gcgrb) (Mojsov, 2000; Yeung et al., 2002), we therefore parsed their roles by infusing recombinant human glucagon and Ex-4 into the circulation of β cell-ablated zebrafish larvae. When we infused glucagon or Ex-4 into control β cell-ablated embryos, glucagon increased H2B-RFP^{-} β cell production but had no significant effect on transdifferentiating H2B-RFP^{+} cells (Fig. 5A-C,K). Neither Ex-4 nor the GLP-1 receptor antagonist Ex-9-39 significantly affected β

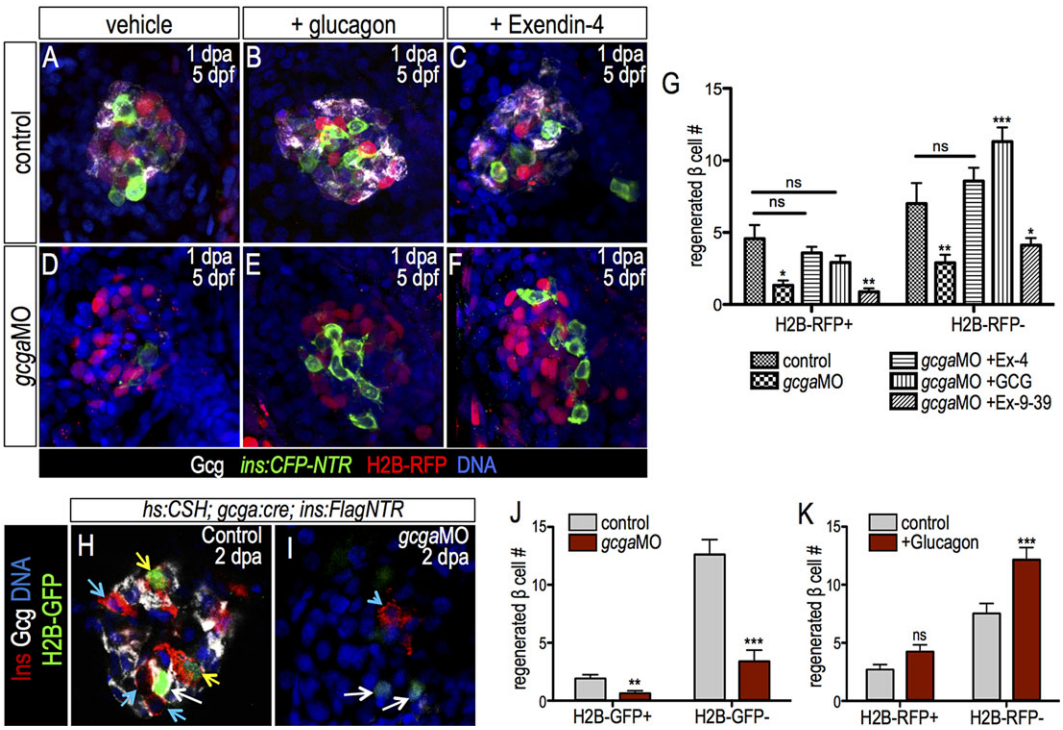

Fig. 5. Differential regulation of β cell progenitor pools by *glucagon* gene products. (A-F) Confocal projections of 1-dpa H2B-RFP-labeled *Tg(ins:CFP-NTR)* control islets (A-C) and *gcga*MO-injected islets (D-F) that were treated with vehicle (A,D), recombinant glucagon (B,E) or Ex-4 (C,F). Islets were immunostained for CFP (insulin, green) and glucagon (white). β cell regeneration was restored with glucagon or Ex-4 injection, but not with Ex-9-39 injection. (G) Quantification of H2B-RFP^{+} and H2B-RFP^{-} β cell number in control regenerating (n=7) and *gcga*MO-injected regenerating islets treated with vehicle (n=9), glucagon (n=13), Ex-4 (n=12) or Ex-9-39 (n=17). (H,I) Confocal planes of 1-dpa *Tg(gcga:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* control and *gcga*MO-injected regenerating islets stained for glucagon (white) and insulin (red). α cells were labeled (white arrows) at 3 dpf by H2B-GFP before MTZ treatment. Yellow arrows indicate H2B-GFP^{+} β cells and blue arrows/arrowhead indicate H2B-GFP^{-} β cells. (J) Quantification of H2B-GFP^{+} and H2B-GFP^{-} β cells in regenerating control (n=10) and *gcga*MO-injected (n=13) islets. (K) Quantification of H2B-RFP^{+} and H2B-RFP^{-} β cells in regenerating islets treated with vehicle or glucagon (n=14). *P≤0.05, **P≤0.01, ***P≤0.001; ns, not significant (two-way ANOVA followed by Bonferroni post-test in G; Student's t-test in J,K).

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cell regeneration (supplementary material Fig. S15). By contrast, in *gcga*MO-injected β cell-ablated embryos, either glucagon or Ex-4 infusion rescued β cell regeneration from both H2B-RFP$^+$ α cells and H2B-RFP$^-$ ventral bud-derived sources (Fig. 5A,D-G; supplementary material Fig. S16). Together, these data suggest that glucagon and GLP-1 might have permissive effects on α-to-β cell transdifferentiation. In addition, both pathways can modulate β cell neogenesis from the ventral bud-derived sources, which are likely to be duct-derived progenitors. In accord with these interpretations, we found that *gcgra* and *gcgrb* were expressed widely in zebrafish endodermal organs at 4 and 5 dpf, including the pancreas and liver (supplementary material Fig. S17).

A primary action of glucagon signaling is the stimulation of hepatic glucose production. Previous studies showed that glucose is crucial for β cell differentiation (Guillemain et al., 2007) and is a potent β cell mitogen (Bonner-Weir et al., 1989; Alonso et al., 2007; Porat et al., 2011). We performed glucose infusions to determine whether glucagon might influence β cell production indirectly through liver-derived glucose. Injected glucose was cleared from larvae by 6 h, and free glucose was decreased in *gcga*MO-injected larvae (supplementary material Fig. S18). In accord with a previous report (Maddison and Chen, 2012), glucose treatment modestly increased β cell mass (Fig. 6A-C). Similarly, when we infused regenerating larvae with glucose there was an increase in the number of β cells (Fig. 6D-F). Quantification of label-retaining populations showed that the increased regeneration was confined to the ventral bud-derived H2B-RFP$^-$ progenitors, indicating that glucose did not directly affect α cell transdifferentiation (Fig. 6F). In support of this interpretation, we found no increase in the expression of Pdx1 in α cells after treatment with glucose (supplementary material Fig. S19).

Finally, to further dissect the influence of hyperglycemia and glucagon signaling on regeneration from each β cell source, we tested whether glucose could rescue β cell regeneration in *gcga* morphants. Although we observed an increase in regenerating β cells in both glucose-treated and glucagon-treated larvae, only glucagon could restore H2B-RFP$^+$ β cell formation (Fig. 6G-K). Together, our data support a model in which *glucagon* per se is necessary for α-to-β cell conversion, while glucose levels regulated by glucagon or GLP-1 signaling drive β cell neogenesis from ventral bud progenitors (Fig. 6L).

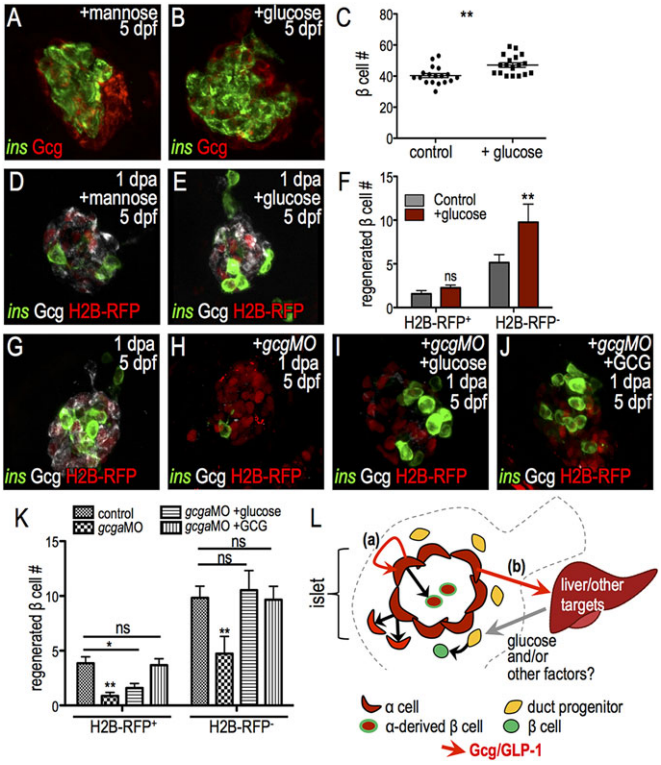

Fig. 6. Differential regulation of β cell progenitor pools by glucagon and glucose. (A,B) Confocal projections of *Tg(ins:CFP-NTR)* islets with 1-day treatment with mannose (osmolality control, A) or glucose (B) and staining for CFP (insulin, green) and glucagon (red). (C) Quantification of *insulin*$^+$ cells showed that glucose treatment increases β cell mass (n=18). (D,E) Confocal projections of 5-dpf/1-dpa *H2B-RFP* mRNA-injected islets that were treated with mannose (D) or glucose (E) during regeneration and stained for CFP (green) and glucagon (white). (F) Quantification of H2B-RFP$^+$ and H2B-RFP$^-$ β cells in control (n=7) and glucose-treated (n=7) islets shows a specific increase in the H2B-RFP$^-$ population. (G-J) Confocal projections of 5-dpf/1-dpa *Tg(ins:CFP-NTR)* islets injected with *H2B-RFP* mRNA alone (G) or co-injected with *gcga*MO (H-J). Samples were treated with vehicle (G,H), glucose (I) or glucagon (J). (K) Quantification of H2B-RFP$^+$ and H2B-RFP$^-$ cells from islets in control (n=27), *gcga*MO (n=15), *gcga*MO +glucose (n=17), *gcga*MO+glucagon (n=22) treatments. Only glucagon rescued H2B-RFP$^+$ β cell regeneration. (L) Model integrating distinct roles of glucagon in β cell formation. Loss of β cells triggers α cell proliferation and activation of *glucagon*. Increased output of glucagon and GLP-1 drives regeneration from two sources: (a) glucagon/GLP-1 acts autonomously in α cells to permit their transdifferentiation to β cells; and (b) glucagon/GLP-1 acts non-autonomously through intermediates (e.g. liver-derived glucose) to drive β cell formation from duct-associated progenitors. *P≤0.05, **P≤0.01; ns, not significant (Student's t-test in C,F; one-way ANOVA followed by Tukey's post-hoc test in K).

DISCUSSION

Diabetes results from the depletion or dysfunction of β cells, and their replacement will be central to a cure. Even though β cell replacement strategies can be effective in principle, they are limited by β cell availability (McCall and Shapiro, 2012), necessitating the identification of novel exogenous or endogenous sources of therapeutic β cells. Here, we have exploited a zebrafish model of conditional β cell ablation, in which functional β cell mass rapidly recovers, to study mechanisms of vertebrate β cell regeneration. We have investigated the origins of regenerating pancreatic β cells in this zebrafish β cell ablation model using two approaches: a Cre-lox-based genetic lineage-tracing approach that indelibly marks endocrine cells with temporal precision; and a non-genetic cell-tracing approach that marks quiescent, differentiated endocrine cells (Hesselson et al., 2009). Using these approaches, we distinguished two sources of new β cells: a ventral pancreatic bud-derived source, which may include the 'naïve' duct-associated progenitor cells that have been described previously (Wang et al., 2011; Ninov et al., 2013); and non-β endocrine cells (α cells in particular) that transdifferentiate into β cells. Thus, we have shown for the first time the spontaneous facultative conversion of α cells into β cells in an organism other than mouse. Most importantly, we identified that activation of *glucagon* is required for β cell regeneration from both precursor pools. Furthermore, our results demonstrate that key physiological actions of *glucagon* in regulating blood glucose are conserved with mammals, just as for insulin (Kinkel and Prince, 2009; Jurczyk et al., 2011; Andersson et al., 2012); in particular, we found that in zebrafish glucagon activity is correlated with blood glucose levels, and that *glucagon* expression is upregulated in the absence of β cells. These findings further validate zebrafish as a model for studying human metabolic disease.

In our study, we found that α-to-β cell transdifferentiation was strongly diminished in *glucagon* knockdown embryos and that this process could be restored by infusion of either glucagon or Ex-4. Thus, we have uncovered a novel mechanism that regulates

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spontaneous α-to-β cell transdifferentiation through the action of physiologically relevant peptide hormone signaling pathways; in particular, peptides derived from the *glucagon* gene. However, since exogenous treatment with glucagon/Ex-4 did not further increase α-to-β transdifferentiation, these results suggest that *glucagon* gene products are important permissive signals facilitating the destabilization of α cell identity. Moreover, our data suggest that activation of the *glucagon* gene might coordinately regulate β cell regeneration from α cells and pancreatic duct-associated progenitor cells. The effect of *glucagon* on the latter source might act indirectly through increased gluconeogenesis, as glucose potently increased the number of H2B-RFP^{-} β cells, even in *gcga* knockdown islets. Interestingly, we found that newly formed H2B-RFP^{+} β cells in the principal islet often form contacts with extra-islet H2B-RFP^{-} β cells via long cellular extensions. This suggests that α cell transdifferentiation within the islet might help recruit new β cells to the core of the islet and thus to regenerate normal islet structure (L.Y. and R.M.A., unpublished).

The *glucagon* gene gives rise to multiple active endocrine peptides through selective proteolysis of Preproglucagon by prohormone convertases (PCs). The α cells yield glucagon via PC2 activity, whereas GLP-1 is generated mainly in intestinal L cells by PC1/3 activity (Holst, 2007). In mammals, glucagon and GLP-1 have counter-regulatory actions on blood glucose: glucagon stimulates hepatic glucose production, whereas GLP-1 inhibits it through stimulation of β cell and inhibition of α cell functions (Habener and Stanojevic, 2013). However, in our zebrafish ablation model the regeneration defect observed in *glucagon* morphants could be rescued by injection of either recombinant human glucagon or the GLP-1 receptor agonist Ex-4. Indeed, glucagon and GLP-1 have similar effects on cAMP generation and hepatic glucose production in teleost fish (Chow et al., 2004). This might be due to loss of the ancestral GLP-1 receptor, together with the evolved capacity of a duplicated glucagon receptor to bind GLP-1 (Irwin and Wong, 2005). Thus, we speculate that in fish the glucagon signaling pathway executes some of the functions of GLP-1 in mammals.

In mammalian animal models of diabetes and in human diabetic patients, upregulation of *Pc1/3* (*Pcsk1*) expression and GLP-1 production in α cells was observed together with increased glucagon secretion (Nie et al., 2000; Thyssen et al., 2006; Marchetti et al., 2012). Furthermore, GLP-1 production in α cells is associated with β cell compensation and regeneration in response to β cell injury (Kilimnik et al., 2010; Hansen et al., 2011; Donath and Burcelin, 2013). Thus, it is possible that the increased glucagon/GLP-1 signaling observed in our zebrafish β cell ablation model reflects a conserved function of GLP-1 signaling in mammalian β cell regeneration. GLP-1 receptor is abundantly expressed in pancreatic ducts, β cells and embryonic α cells (Kedees et al., 2009). Although it is unclear whether adult α cells express GLP-1 receptors, these receptors are expressed in insulin/glucagon dual hormone-positive cells in the rat pancreas (Tornehave et al., 2008; Kedees et al., 2009). Thus, we anticipate that GLP-1 receptor expression increases in α cells during transdifferentiation; this will be an area of future study. In addition to the demonstrated functions of GLP-1 signaling during β cell regeneration, we cannot exclude the role of glucagon signaling in this process given that glucagon plays a role in the maturation and function of β cells. For instance, injection of glucagon into chick embryos increases β cell mass and decreases α cell mass (Anderson and Gibson, 1981). Furthermore, insulin expression is delayed and ultimately reduced in the islets of *Gcgr*$^{-/-}$ mice (Gelling et al., 2003; Vuguin et al., 2006), and fetal mice treated with glucagon MOs show impaired differentiation of early β cells (Prasadan et al., 2002). Indeed, we found that both glucagon receptor (*gcgra*) and GLP-1 receptor (*gcgrb*) were expressed throughout the pancreas in zebrafish larvae. Thus, further research is needed to delineate how different peptides derived from the *glucagon* gene work together to restore islet cell composition and function following loss of β cells.

Owing to the limitations of our current tools, we cannot discern whether ventral bud-derived H2B-RFP^{-} α cells also trans-differentiated into β cells, and if these precursors are mature or immature α cells. β cell neogenesis might involve a glucagon-positive intermediate that may initially exhibit some characteristics of α cells but subsequently matures into a β cell. Intriguingly, the presence of *insulin*:mCherry and *glucagon*:GFP dual-positive cells in the non-ablated larval and adult zebrafish pancreas suggests that this process might be active in the homeostasis of the islet cell mass. Indeed, it has been proposed that a pro-α cell population exists as a facultative progenitor that exhibits features of β and α cells in mammals (Habener and Stanojevic, 2012), although this proposition contradicts earlier lineage-tracing studies (Herrera, 2000) that showed that mature β and α cells arise from distinct lineages. As ∼20-65% of α cells are not marked in glucagon:Cre transgenic mice (Herrera, 2000; Quoix et al., 2007; Magnuson and Osipovich, 2013; Mastracci et al., 2013), it is possible that relevant subpopulations of α cells have gone undetected in these studies. The development of novel mouse and zebrafish transgenic strains in which Cre is more faithfully regulated by the endogenous glucagon locus might shed light on this possibility.

Consistent with the hypothesis that α cells are a relevant source of new β cells in regenerating islets, the loss of α cells due to *arxa* knockdown strongly diminished the regeneration of β cell mass. In *arxa*MO-injected islets, we expect that α cell progenitors are diverted to δ and β cell identities, as in the mouse *Arx* knockout, where increases of 200% and 31%, respectively, were observed (Collombat et al., 2003). In *arxa*MO-injected islets, we found that δ cell numbers were indeed increased, as was the number of β cells, although the latter was not statistically significant (supplementary material Fig. S10D,E). In regenerating islets, *gcga* knockdown phenocopies the loss of α cells observed in the *arxa* knockdown, providing strong evidence that peptides derived from the *glucagon* gene are crucial regulators of α cell conversion. As in murine disruptions of the glucagon signaling pathway (Gelling et al., 2003; Hayashi et al., 2009; Vuguin and Charron, 2011; Longuet et al., 2013), we found that α cell mass was not only maintained, but also expanded through proliferation and neogenesis. That the number of potential α cell progenitors of β cells was expanded, yet β cell regeneration was depressed, further underscores the importance of *glucagon* for cell fate switching. Finally, as hypoglycemia is expected to result from α cell/glucagon depletion, we hypothesize that the observed reduction of β cell neogenesis from ventral pancreatic bud-derived tissues in *arxa* morphants results from a combination of at least two factors: (1) loss of glucose, which acts as a stimulator of β cell differentiation (Maddison and Chen, 2012); and (2) fewer α cells acting as progenitors of β cells.

Regeneration studies in mice have shown that the mode and extent of β cell regeneration are dependent on the injury model. Proliferation of surviving β cells predominates after targeted ablation of 70-80% of β cells (Dor et al., 2004; Nir et al., 2007), while conversion of α or δ cells to β cells occurs only with extreme β cell loss (>99%) (Thorel et al., 2010; Chera et al., 2014). Likewise, we hypothesize that lower levels of β cell ablation in zebrafish might mitigate transdifferentiation in this model. Additionally, the plasticity of α and δ cells is not constant with age. In prepubescent

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mice, δ cells, but not α cells, are competent to transform into β cells after injury (Chera et al., 2014). By contrast, fetal α cells can be reprogrammed to β cells by misexpression of *Pdx1*, whereas α cells of newborn mice cannot (Yang et al., 2011). Intriguingly, regenerating islets in streptozotocin-treated adult zebrafish show increased PCNA staining in the mantle region (Moss et al., 2009), which is consistent with an induced proliferation of α cells, just as we observe in regenerating larvae (Fig. 3K). Whether the endocrine plasticity of the zebrafish larval pancreas is maintained in juveniles, and adults, and whether this is dependent on extreme β cell loss are important questions that are being addressed in our ongoing studies.

Our data do not distinguish between the possibilities that peptides derived from the *glucagon* gene act directly on α cells, or that they act indirectly through other tissues (such as the liver) to produce non-glucose intermediate messengers that feed back to α cells. Indeed, it has recently been shown that hyperplasia of α cells in *Gcgr* knockouts can be phenocopied by tissue-specific knockdown of the glucagon receptor in hepatocytes (Longuet et al., 2013), revealing that a circulating factor derived from the liver may partially mediate this hyperplastic effect. Thus, it will be important to investigate how α and β cell mass are affected in this system when challenged by β cell depletion and hyperglycemia. In addition, given the multiple paracrine actions of glucagon in the islet, it will be important to examine the role of the glucagon receptor, as well as the GLP-1 receptor, specifically within the α cells using tissue-specific knockdown or hyperactivation approaches.

In summary, our data are consistent with a model whereby the glucagon/GLP-1 signaling pathway works through two routes to elicit new β cell formation: (1) non-autonomously through stimulation of hepatic glucose production, which can stimulate β cell formation from naïve duct-associated progenitor cells (Guillemain et al., 2007; Ninov et al., 2013); and (2) autonomously within the α cell compartment to regulate α cell mass and cell identity (Fig. 6L). Arx is a potent reprogramming factor that is essential for α cell development and maintenance (Collombat et al., 2003, 2007; Courtney et al., 2013). As such, suppression of *Arx* in α cells will be essential for their conversion to β cells. Therefore, we hypothesize that *arxa* expression is negatively regulated by excessive glucagon/GLP-1 signaling in α cells. Loss of glucagon/GLP-1 signaling by multiple approaches results in hyperplasia of α cells and increased glucagon content concomitant with increased expression of *Arx* (Hayashi et al., 2009; Unger and Cherrington, 2012). Our data provide a connection between glucagon/GLP-1 signaling and the maintenance of α cell fate.

As α cells appear to be epigenetically programmed to enable their conversion to β cells in humans (Bramswig et al., 2013), investigating how glucagon/GLP-1 signaling pathways link to epigenetic regulators will be an exciting area for further study. Understanding how such extrinsic signals can regulate α cell stability will facilitate novel diabetes therapies that promote β cell replenishment. Furthermore, together with the observation that α cells appear to have a heightened capacity for regeneration by neogenesis from duct-associated progenitors (Collombat et al., 2009), our results support the hypothesis that the α cell pool constitutes an extensive reserve of new β cells. Our data indicate that peptides derived from the *glucagon* gene are crucial for regulation of this endogenous repair mechanism and suggest that stimulation of endocrine transdifferentiation might unlock unlimited sources of new β cells.

MATERIALS AND METHODS

Zebrafish maintenance and strains

Zebrafish were raised under standard laboratory conditions at 28°C. For HotCre lineage tracing, heat shock activations were performed at 38.5°C for 20 min at 3 dpf. If β cell ablation was required, larvae were recovered at 28.5°C for 3 h before MTZ was added. All animal procedures were conducted in accordance with Office of Laboratory Animal Welfare guidelines and were approved by the Indiana University Institutional Animal Care and Use Committee. Details of strains can be found in the supplementary materials and methods.

Detection of protein, mRNA and cell proliferation

Whole-mount immunofluorescent staining, *in vitro* hybridization, qPCR and EdU incorporation assays were performed as previously described (Anderson et al., 2009). For details, see the supplementary materials and methods. Primer sets are listed in supplementary material Tables S2 and S3.

Microinjections

100 pg *in vitro* transcribed *H2B-RFP* mRNA and/or the following antisense MOs (Gene Tools) were injected into zygotes: control MO (4 or 8 ng), *arxa*MO (4 ng) and *gcga*MO (8 ng). Detailed experimental procedures are provided in the supplementary materials and methods and Table S4.

Drug/chemical treatments

For β cell ablation, *Tg(ins:CFP-NTR)*^{s892} or *Tg(ins:Flag-NTR)*^{s950} animals were incubated in 0.1% DMSO (Sigma) ±10 mM Metronidazole (MTZ, Sigma) in egg water. For injections, a mixture of KCl (0.2 M), Phenol Red (0.1%) and either vehicle, recombinant human glucagon (Sigma), Exendin-4 (Sigma), Exendin9-39 (Sigma), glucose or mannose was injected into the pericardial sac of each embryo. Further details are given in the supplementary materials and methods.

Glucose measurements

Glucose measurement in zebrafish embryos was performed as described (Andersson et al., 2012) using a glucose assay kit (Biovision, K606-100).

Statistical analysis

Data are expressed as mean±s.d. Statistical significance was tested by ANOVA analysis or Student's *t*-test (as noted in the figure legends).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.Y. and R.M.A. conceived the project. L.Y., R.M.A. and M.A.R. designed experiments, and analyzed the data. R.M.A., D.H. and D.Y.R.S. generated *gcga:Cre* and *sst2:Cre* transgenic fish. L.Y. and R.M.A. wrote and edited the manuscript with editorial input from D.Y.R.S. and D.H.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.117911/-/DC1

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Ye et al. *glucagon* is essential for alpha cell transdifferentiation and beta cell neogenesis.

Supplementary Materials

Supplementary Figures

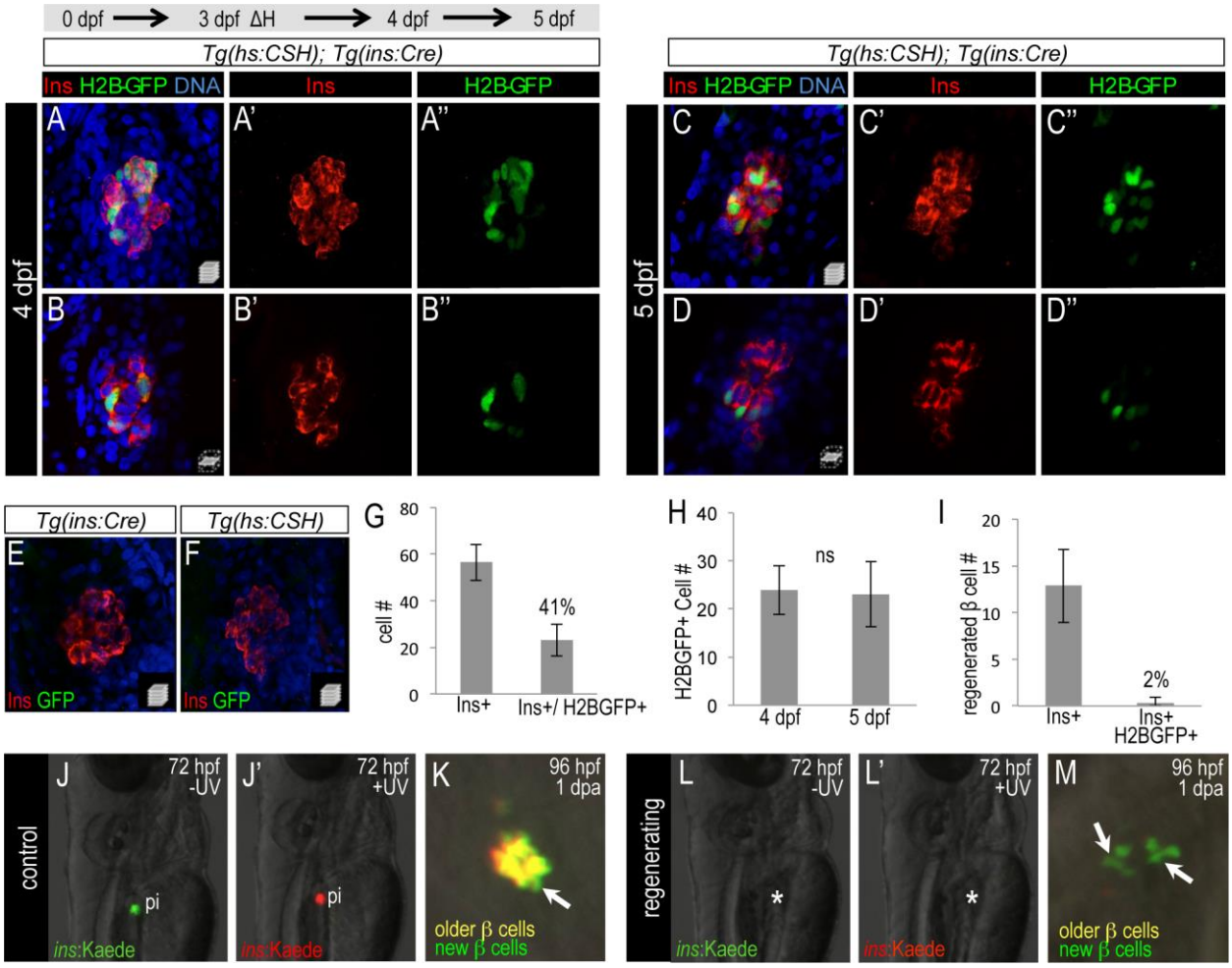

Figure S1. Pre-existing β cells do not contribute to β cell regeneration. (A-D) Confocal projections (A,C) and confocal planes (B,D) of *Tg(hs:CSH)*; *Tg(ins:cre)* islets at 4 dpf (A,B) and 5 dpf (C,D) that were heat shocked at 3 dpf and stained for insulin (red), GFP (green), and DNA (blue). Insulin^{+} cells were specifically labeled by H2B-GFP. (E, F) Confocal projections of 4 dpf *Tg(ins:cre)* (E) and *Tg(hs:CSH)* (F) islets showed no aberrant or leaky H2B-GFP fluorescence after heatshock. (G) Quantification of insulin^{+} and insulin^{+}/H2B-GFP^{+} cells at 4 dpf showed 41% of βcells were marked (*n*=11). (H) The number of labeled β cells remained constant from 4 to 5 dpf (*n*=7). (I) Quantification of total and H2B-GFP^{+} β cells in *the* regenerating islets represented in Fig 1I. Only ~2% of new βcells were labeled (*n*=13). (J-M) Epifluorescent images of *ins:Kaede; ins:CFP* larvae. (J) Green Kaede and (J')

converted red Kaede in the βcells of a 72 hpf larva. (K) A 96 hpf larva 24 hours after photo-conversion exhibited βcells present during UV exposure (yellow) as well as new βcells formed after the exposure (green only, arrow). (L) Neither green Kaede (L) nor converted red Kaede (L') are evident at 72 hpf after MTZ-ablation of βcells. (M) A 96 hpf photo-converted larvae 24 hours after photoconversion and cessation of MTZ treatment shows only new βcells (green, arrows).Student's t-test was used in H for statistical analysis.

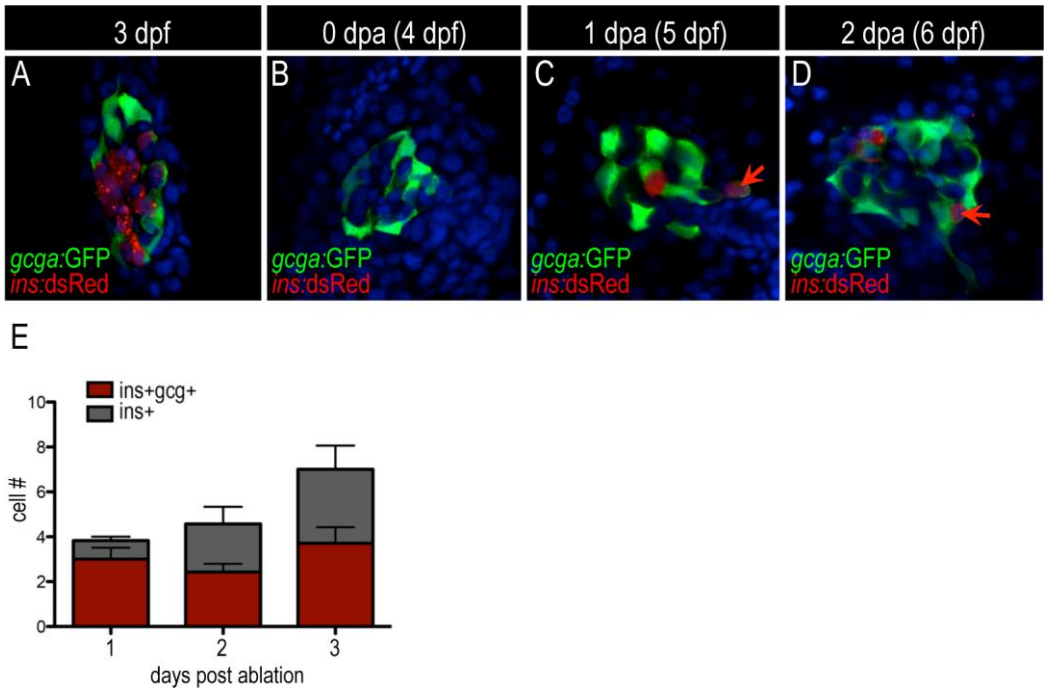

Figure S2. Emergence of *gcga:*GFP$^+$ *ins:*dsRed$^+$ dual hormone cells during regeneration. (A-D) Confocal planes of *Tg(gcga:GFP); Tg(ins:dsRed); Tg(ins:Flag-NTR)* islets in non-ablated 3 dpf (A), ablated 4 dpf (B), 1 dpa (C), and 2 dpa (D) larvae stained for DNA (blue). *gcga:*GFP$^+$ *ins:*dsRed$^+$ dual hormone expressing cells are marked by red arrows. (E) Quantification of *ins:*dsRed$^+$ single positive and *ins:*dsRed$^+$ *gcga:*GFP$^+$ dual positive cells in regenerating islets from 1 to 3 dpa (n≥3).

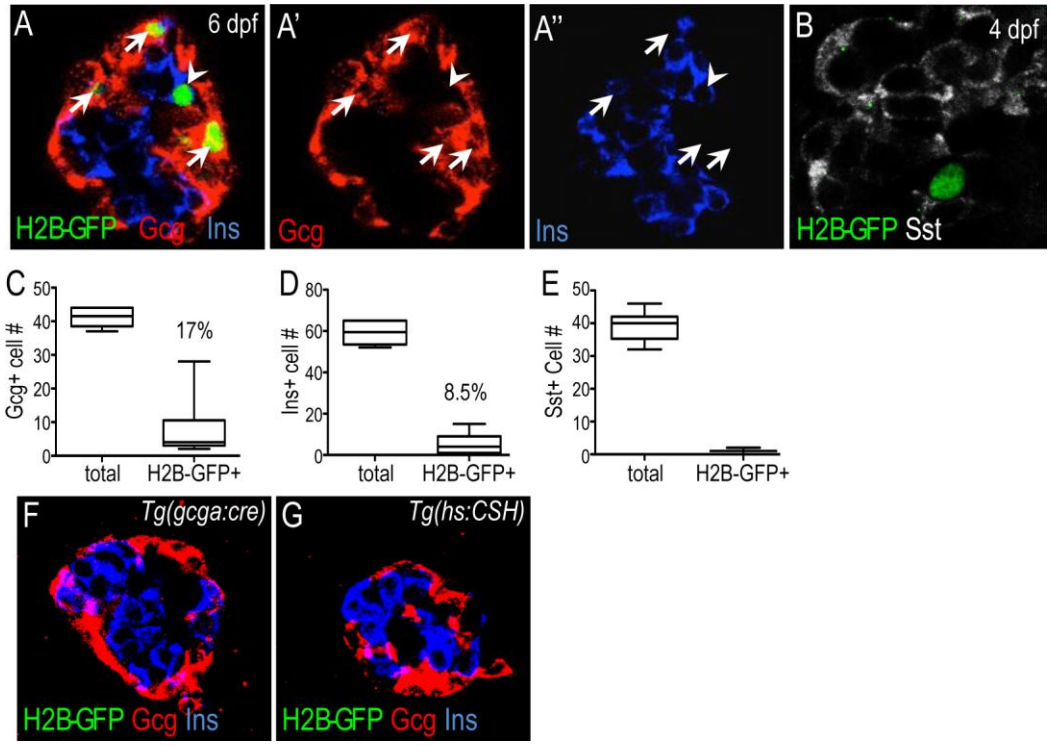

Figure S3. Characterization of *glucagon* promoter activity in *Tg(gcga:Cre)*. (A) Merged and single channel confocal planes of 6 dpf *Tg(gcga:cre);Tg(hs:CSH)* islets heat shocked at 3 dpf and stained for GFP (green), Insulin (blue) and glucagon (red). (B) Confocal plane of 4 dpf *Tg(gcga:cre);Tg(hs:CSH)* islet heat shocked at 3 dpf and stained for Somatostatin (white) and GFP (green). (C) Quantification of total glucagon^{+} and H2B-GFP^{+} glucagon^{+} cells in A shows that ~17% of αcells were marked (*n*=10). (D) Quantification of total Ins^{+} and H2B-GFP^{+} Ins^{+} cells in A shows that ~8.5% of βcells were marked (*n*=6). (E) Quantification of Sst+ and Sst+ H2B-GFP^{+} cells in B (*n*=11). (F-G) Confocal planes of 4 dpf *Tg(gcga:cre)* (F) and *Tg(hs:CSH)* (G) islets showed no aberrant or leaky H2B-GFP expression after heat shock.

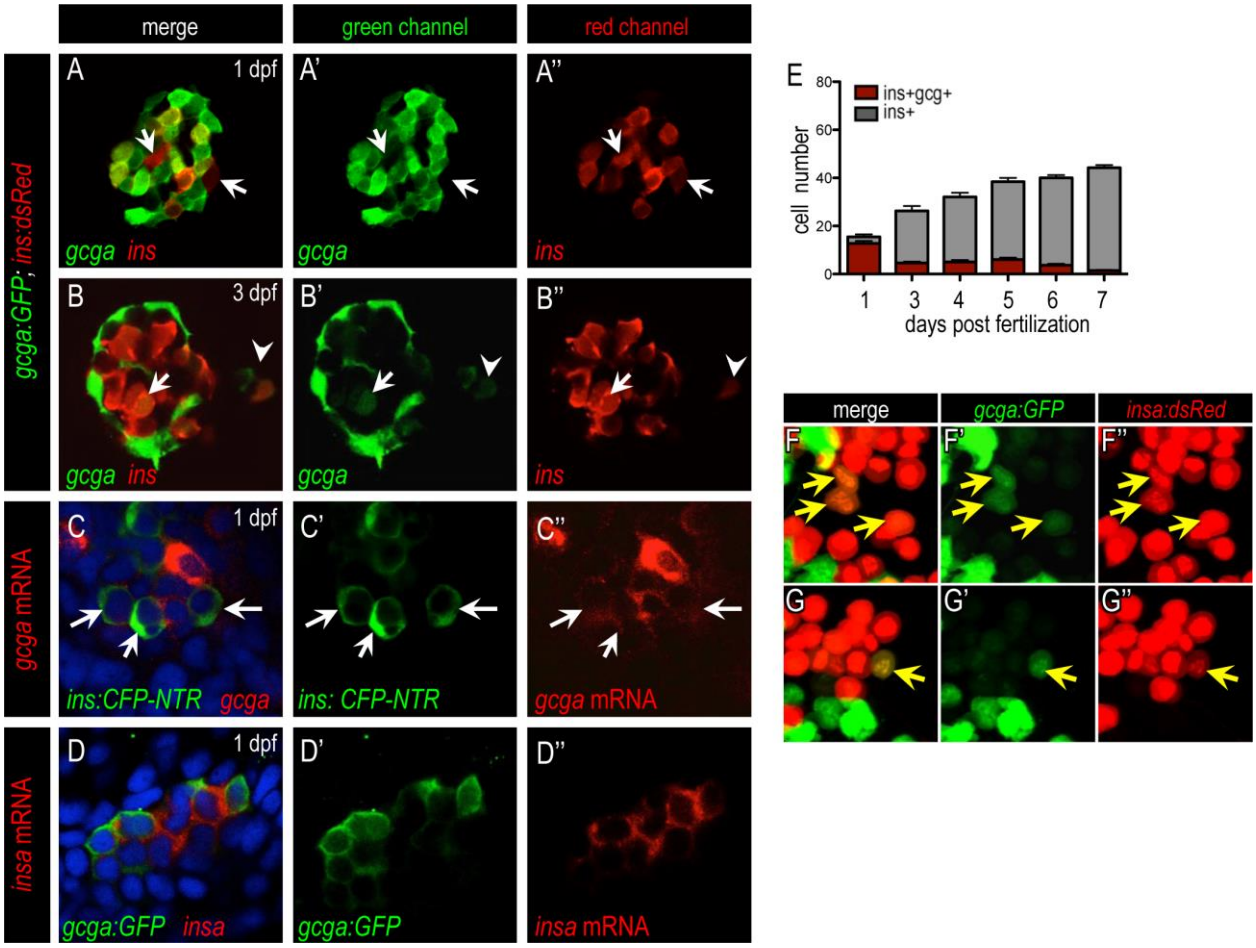

Figure S4. *ins+ gcga+* cells during early islet development. (A) Confocal planes of 1 dpf *Tg(gcga:GFP); Tg(ins:dsRed)* islet showed that the majority of *ins+* cells at 1 dpf are also *gcga+*. Single positive *insulin* expressing cells are indicated by arrows. (B) Confocal planes of 3 dpf *Tg(gcga:GFP); Tg(ins:dsRed)* islet showed *ins+ gcga+* cells in the principal islet (arrow) and *ins+ gcga+* newly formed βcells (arrowhead) in the extrapancreatic duct. (C-D) Merged and single channel confocal planes of fluorescent in situ hybridization (C) *proglucagon* (red) expressed in *ins+* cells (green) at 1 dpf in *Tg(ins:CFP-NTR)* islets immunostained for GFP (green). (D) *insulin* (red) expressed in *gcga+* cell(green) in 1 dpf *Tg(gcga:GFP)* islet immunostained for GFP (green). (E) Quantification of *ins+ gcga+* and *ins+* cells at 1 dpf through 7 dpf. *Tg(gcga:GFP); Tg(ins:dsRed)* islets showed a decrease of dual hormone-expressing cells with islet maturation.

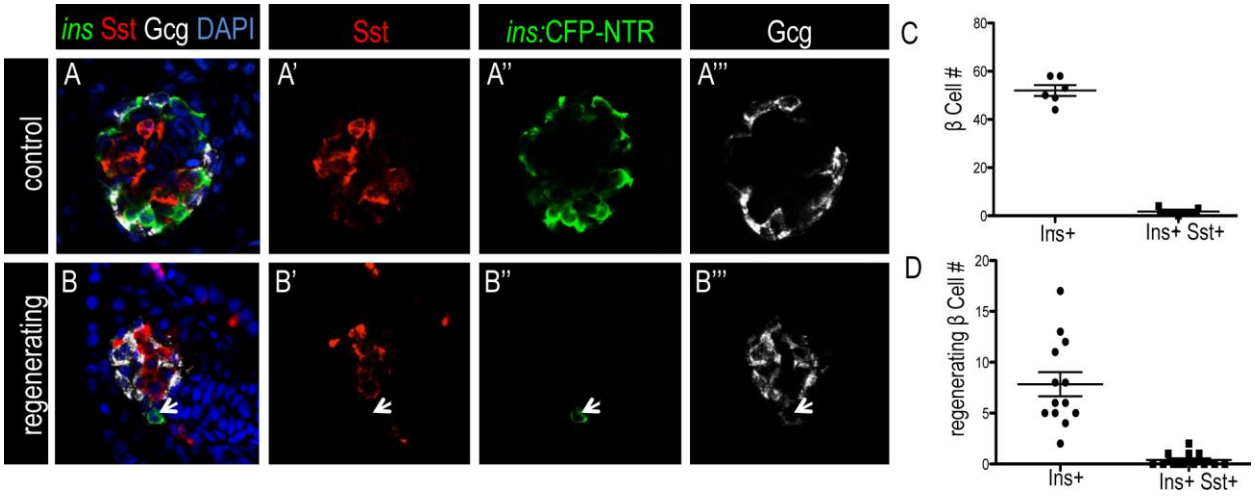

Figure S5. Insulin and somatostatin are rarely co-expressed in non-ablated or regenerating islets. (A-B’’’) Merged and single channel confocal images of 4 dpf *Tg(ins:CFP-NTR)* islets stained for CFP (green), somatostatin (red), and glucagon (white), that were not ablated (A), or ablated from 2-3 dpf (B). Regenerating *insulin*+ βcells (arrow) were rarely labeled. (C,D) Quantification of βcells and regenerated βcells that were *insulin*$^{+}$ or *insulin*$^{+}$ somatostatin^{+}.

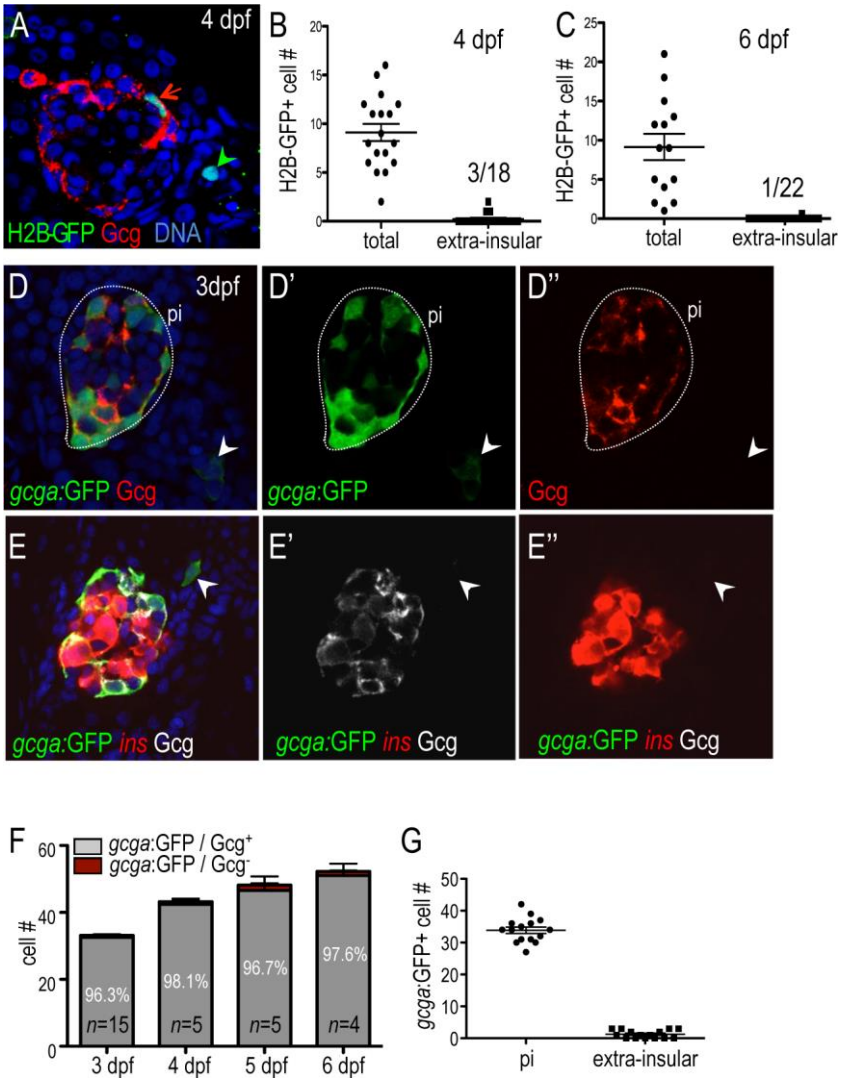

Figure S6. Characterization of *glucagon* promoter activity in extra-insular endocrine cells.

(A) Confocal Plane of 4 dpf *Tg(gcga:cre); Tg(hs:CSH)* islet heat shock at 3 dpf stained for Glucagon (red) and GFP (green). Green arrow head indicates Glucagon-H2B-GFP$^+$ Cells outside the islet. (B,C) Quantification of total and extra-insular H2B-GFP$^+$ cells in *Tg(gcga:cre);Tg(hs:CSH)* larvae heat shocked at 3 dpf. At 4 dpf (B), 3 out of 18 samples were found to have extra-insular H2B-GFP$^+$ cells. At 6 dpf (C), 1 out of 22 samples was found to have extra-insular H2B-GFP$^+$ cells. (D) Merged and single channel confocal planes of 3 dpf *Tg(gcga:GFP)* islets stained for GFP (green) and glucagon (red). Arrowhead indicates *gcga:GFP*$^+$ glucagon- cells in pancreatic duct region outside of principal islet. (E) Merged and single channel confocal planes of 3 dpf *Tg(gcga:GFP);Tg(ins:dsRed)* islets stained for GFP (green) ,dsRed (red) and Glucagon (white). Arrowhead indicates *gcga:GFP*$^+$ *ins*:dsRed$^-$Glucagon$^-$ cells in pancreatic duct region outside of principal islet. (F)

Quantification of *gcga:GFP*$^{+}$ glucagon^{-} and *gcga:GFP*$^{+}$ glucagon^{+} cell number in principal islet from 3 dpf through 6 dpf. The percent of *gcga:GFP*$^{+}$ glucagon^{-} cells is consistent but small. (G) Quantification of insular and extra-insular *gcga:GFP*+ cell numbers.

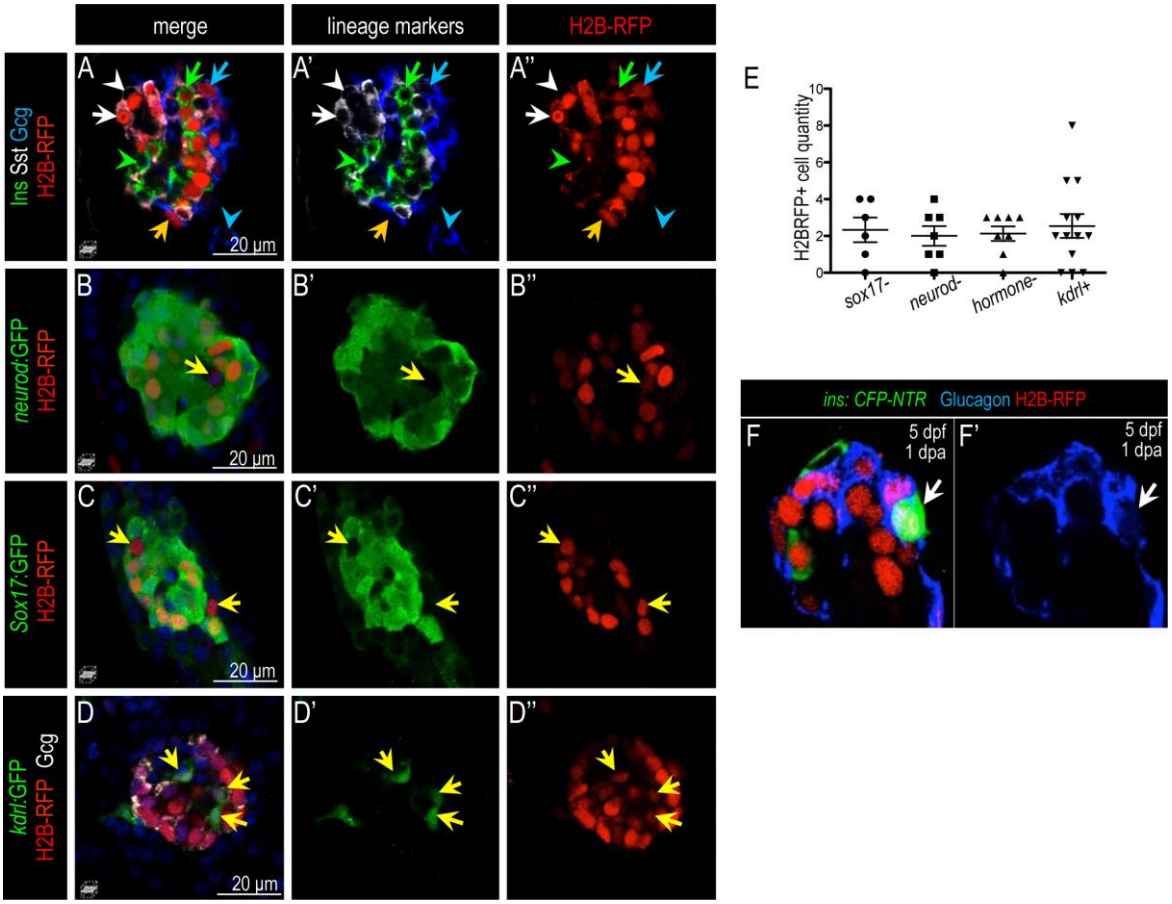

Figure S7. *H2B-RFP* label retaining assay marks early differentiated endocrine cells. (A-D) Confocal planes of islets labeled with H2B-RFP. (A) Wild type islet stained for Insulin (green), Somatostatin (white) and Glucagon (blue) at 5 dpf. There are 2 endocrine populations in the islet: H2B-RFP^{+} (arrows) and H2B-RFP^{-} (arrowheads). Yellow arrow indicates H2B-RFP^{+} cells in the islet lacking hormone staining. (B) *TgBAC(neurod1:*EGFP*)* islet stained for GFP (green) at 3 dpf. Yellow arrow indicates *neurod1*$^{-}$ H2B-RFP^{+} non-endocrine cells in the principal islet. (C) *Tg(sox17*:GFP*)* islet stained for GFP (green) at 3 dpf. Yellow arrows indicate *sox17*$^{-}$ H2B-RFP^{+} non-endodermal cells in the principal islet. (D) *Tg(kdrl:*GFP*)* islet region stained for GFP (green) and Glucagon (white) at 4 dpf. Yellow arrows indicate *kdrl:GFP*$^{+}$ H2B-RFP^{+} blood vessel cells in the principal islet. (E) Quantification of *sox17*$^{-}$ H2B-RFP^{+}, *neurod1*$^{-}$ H2B-RFP^{+}, and islet hormone (Ins/Gcg/Sst)$^{-}$ H2B-RFP^{+} cell quantities in the islet. (F) 1 dpa regenerating *Tg(ins:CFP-NTR)* islet stained for CFP (green) and glucagon (blue). The white arrow indicates a triply positive *ins*:CFP-NTR^{+} glucagon^{+} H2B-RFP^{+} βcell.

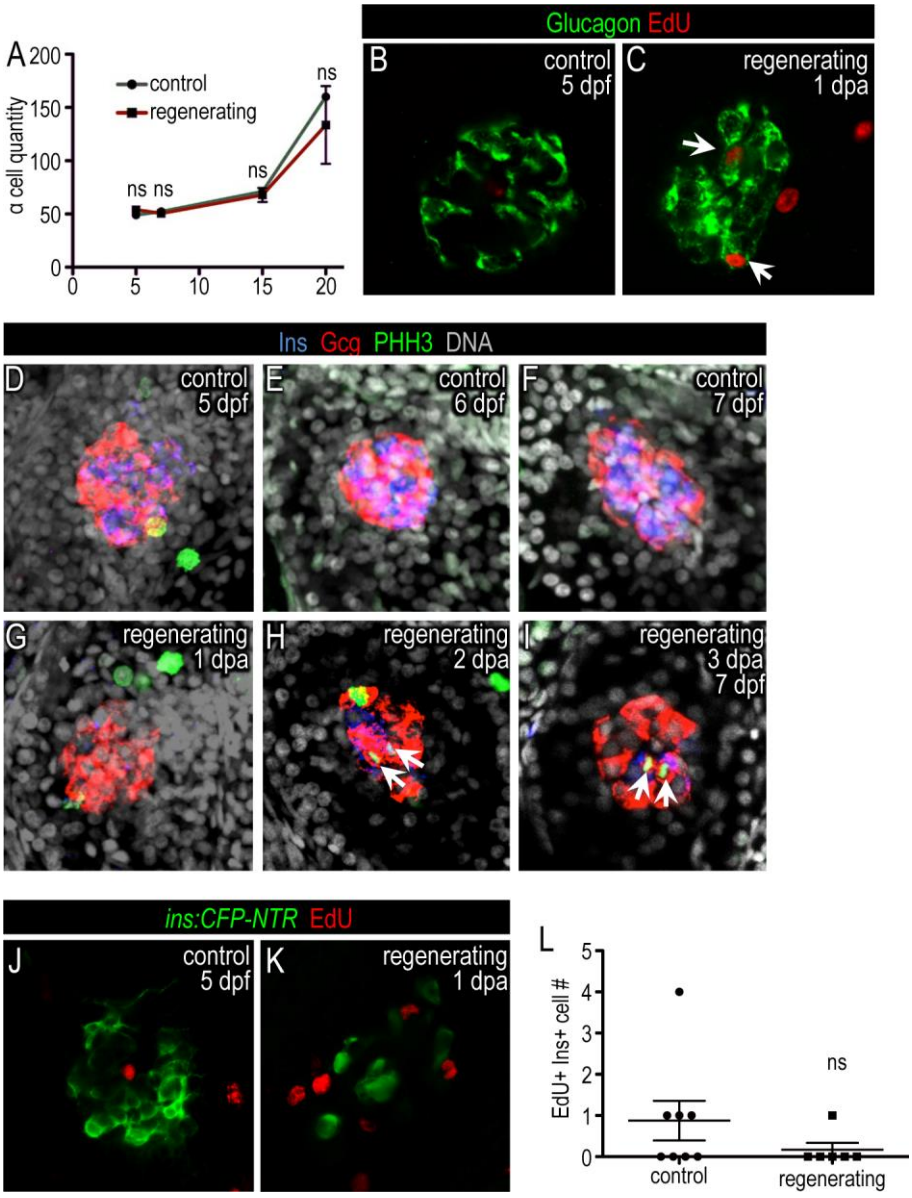

Figure S8. Proliferation of α cells but not β cells during β cell regeneration. (A) Quantification of αcell number between 5 dpf and 20 dpf in control (gray) and regenerating (red) islets ($n \geqq 3$). (B-C) Confocal planes of non-ablated (B) or 1 dpa (C) 5 dpf *Tg(ins:FlagNTR)* islets labeled with EdU (red) and stained for Glucagon (green). White arrows indicate Glucagon$^+$ EdU$^+$ cells in 1 dpa islet. (D-I) Confocal projections of *Tg(ins:CFP-NTR)* islets stained for phospho-histone H3 (green), glucagon (red), insulin (blue) and DNA (white) that were not ablated (D-F) or ablated from 3-4 dpf and regenerating (G-I). Islets were analyzed at 5 dpf (D,G), 6 dpf (E,H), and 7 dpf (F,I). No phospho-histone staining was observed in any non-ablated islet (n=21) and 17.6% of regenerating islets showed staining (n=17). (J-K) Confocal planes of non-ablated (J) or 1 dpa (K) 5 dpf

Tg(ins:CFP-NTR) islets labeled with EdU (red) and stained for GFP (green). (L) Quantification of $ins^{+}EdU^{+}$ cells in intact and ablated 5 dpf islets. Two-way ANOVA was used in A and Student's t-test was used in L for statistical analysis.

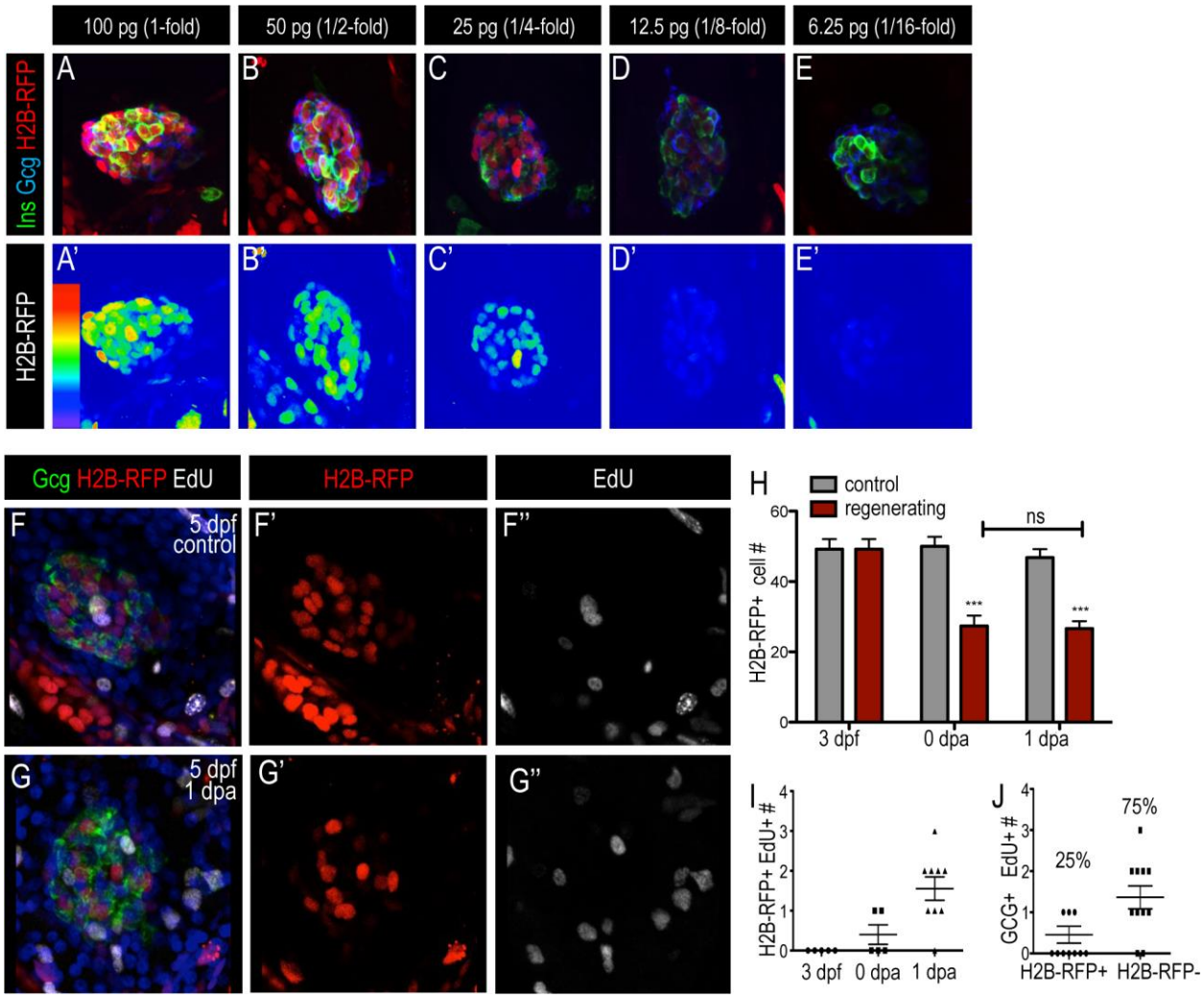

Figure S9. Proliferation of H2B-RFP$^+$ cells during regeneration does not dilute H2B-RFP mRNA. (A-E) Confocal projections of 5 dpf islets injected with decreasing amounts of *H2B-RFP* (red) mRNA that were immunostained for insulin (green) and glucagon (blue) to show endocrine differentiation. (A'-E') heat map representation of H2B-RFP fluorescence from confocal projections in A-E. Note that there is detectable H2B-RFP signal when mRNA was injected at the dose of 12.5 pg/embryo, which is an eightfold dilution. (F-G) Confocal projections of *H2B-RFP* mRNA injected 5 dpf control (F) and 1 dpa regenerating (G) islets that were labeled with EdU (white) for 1 hour, and stained for glucagon (green), RFP (red), and DNA (blue). (H) Quantification of total H2B-RFP$^+$ cells in islets from 3 dpf to 5 dpf in both intact islet (grey) and ablated islets (red). There is a significant decrease of total H2B-RFP$^+$ cell quantity in the islet during βcell ablation, but no difference of total H2B-RFP$^+$ cell number in the islet during regeneration between 0 dpa and 1 dpa. (I) Quantification of H2B-RFP$^+$ Edu$^+$ cell number during regeneration showed there are at most 2 Edu$^+$ H2B-

RFP^+ cells per islet during regeneration. (J) Quantification of H2B-RFP^+ and H2B-RFP^- Glucagon+ Edu^+ cell number in 1 dpa regenerating islets showed that 75% of proliferating α cells were H2B-RFP^-. Two-way ANOVA was used in H for statistical analysis.

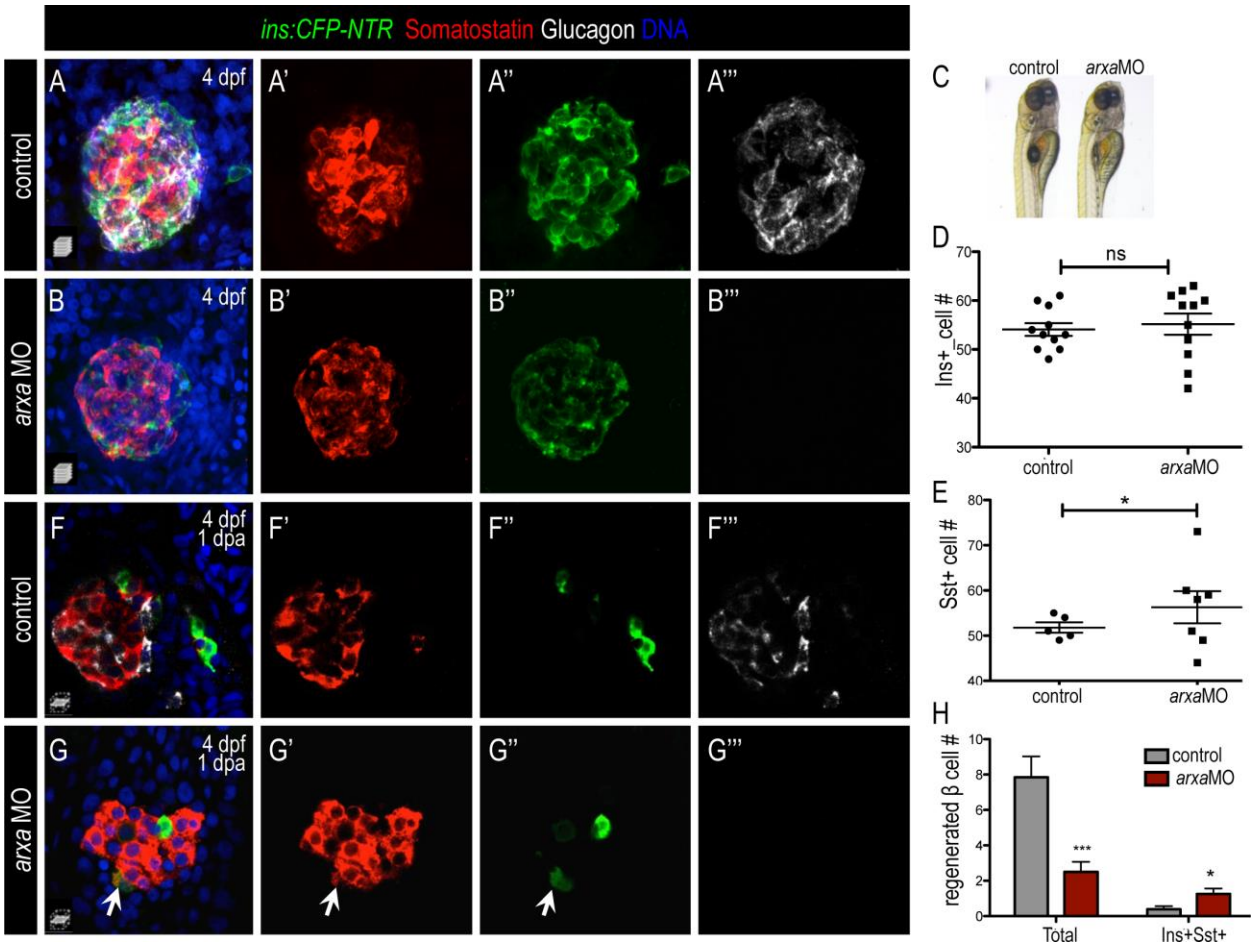

Figure S10. *arxa* knockdown induces the appearance of insulin/somatostatin co-expressing cells. (A-B) Confocal projections of 4 dpf control MO (A) and *arxa*MO- (B) injected *Tg(ins:CFP-NTR)* islets stained for somatostatin (red), glucagon (white) and GFP (green). (C) Control MO and *arxa*MO-injected 4 dpf larvae show no general developmental defects. (D-E) Quantification of total insulin$^+$ and somatostatin$^+$ cell number in 4 dpf control and *arxa*MO-injected larvae. (F-G) Confocal planes of 1 dpa control (F) and *arxa*MO-injected (G) *Tg(ins:CFP-NTR)* regenerating islets stained for somatostatin (red), glucagon (white) and GFP (green). White arrow in G indicates somatostatin$^+$ *insulin*$^+$ regenerating β cells in *arxa*MO-injected regenerating islet. (H) Quantification of total *insulin*$^+$ βcells and *insulin*$^+$ somatostatin$^+$ βcells in 1 dpa control and *arxa*MO-injected regenerating islets. Student's t-test was used in D,E, and H for statistical analysis.

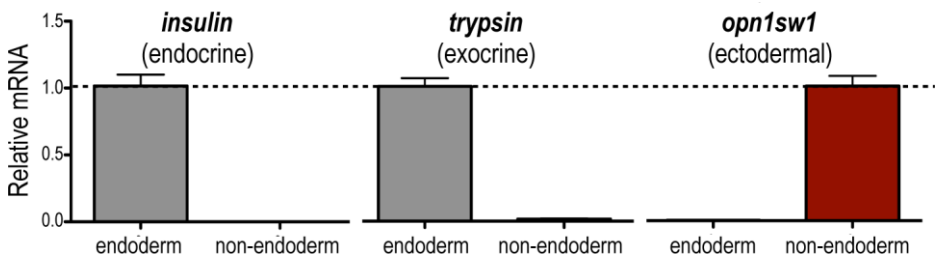

Figure S11. Endodermal organ cDNA preparations are enriched for endoderm. Quantitative PCR analysis on cDNA isolated from pools of dissected endodermal organs (pancreas, liver, intestine) and non-endodermal organs (the remainder of the larva). Preparations were tested for markers of the endocrine pancreas (*insa*), the exocrine pancreas (*trypsin*), and a non-endodermal opsin (*opn1sw1*). Virtually all of the *insa* and *trypsin* signal was limited to the endodermal fraction, while *opn1sw* was restricted to the non-endodermal fraction. *insa* and *trypsin* were normalized to the expression level in endoderm, while *opn1sw1* was normalized to non-endoderm. *n*=6 for all samples.

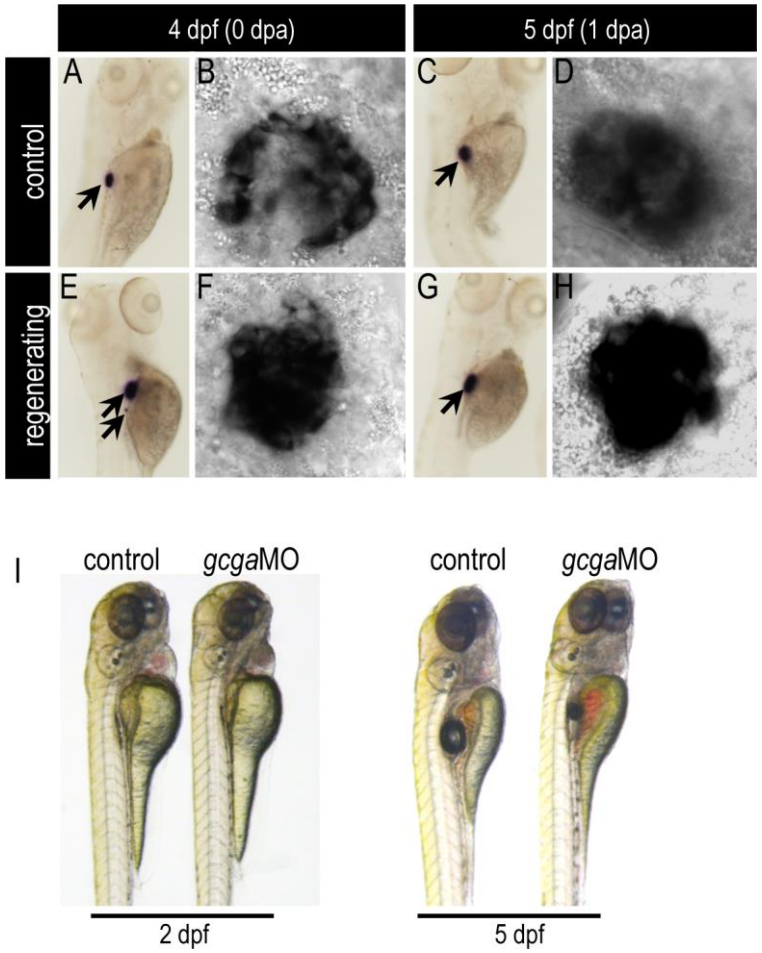

Figure S12. *glucagon* expression is increased in regenerating pancreata, but not is required for general embryonic development. Expression of *glucagon* revealed by *in situ* hybridization in non-ablated control (A-D), 0 dpa βcell-ablated (E,F), and 1 dpa regenerating (G,H) *Tg(ins:CFP-NTR)* islets at 4 dpf (A,B,E,F) and 5 dpf (C,D,G,H). (A,C,E,G) Arrows indicate *glucagon* expression in photomicrographs of whole larvae. (B,D,F,H) Confocal projections of islets. (I) 2 dpf embryos and 5 dpf larvae injected with control MO or *gcga* MO.

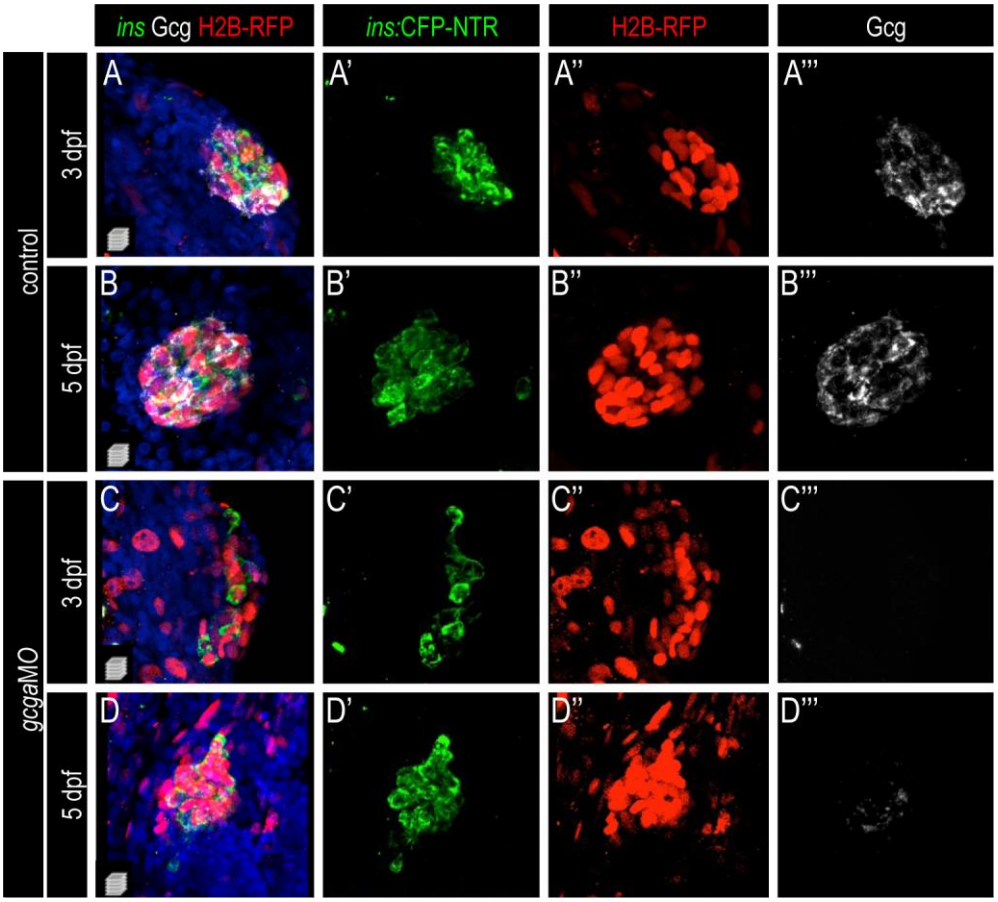

Figure S13. *gcga* knockdown decreases ventral pancreatic bud-derived β cell differentiation. (A-D’’’) Merged and single channel confocal projections of *Tg(ins:CFP-NTR)* islets injected with *H2B-RFP* mRNA alone (A-B’’’) or *H2B-RFP* mRNA + *gcga* MO that were stained for glucagon (white) ,CFP (green) and DNA (blue) at 3 dpf (A,C) and 5 dpf (B,D).

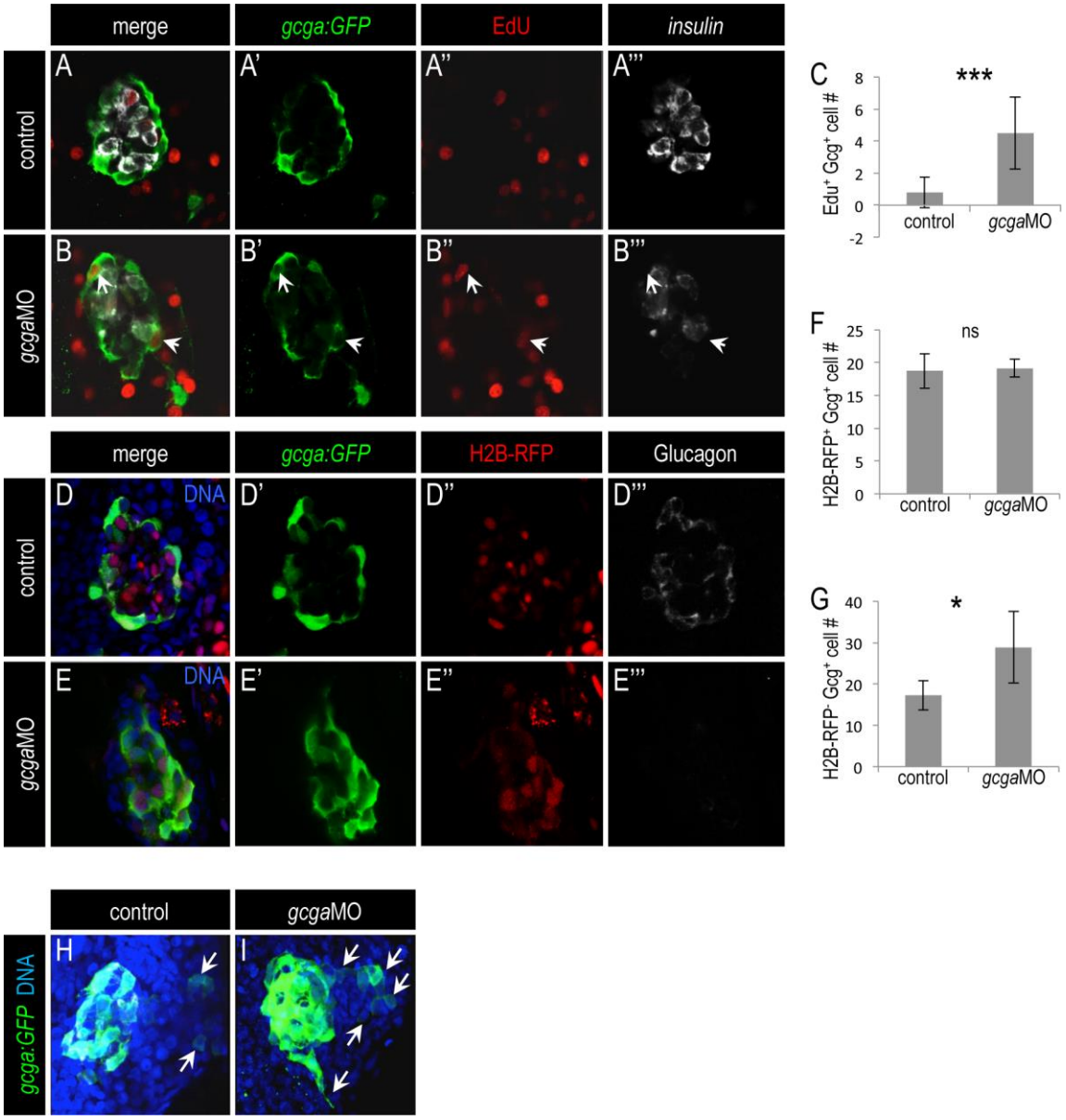

Figure S14. *gcga* knockdown increases α cell proliferation and neogenesis. (A-B) Merged and single channel confocal planes of 4 dpf control (A) and *gcga*MO-injected (B) *Tg(gcga:GFP)* islets labeled by insulin antibody (white) and EdU incorporation (red). (C) Quantification of EdU^{+} *gcga:GFP*$^{+}$ proliferating α cells in control (*n*=10) and *gcga*MO injected (*n*=12) islets. (D,E) Merged and single channel confocal planes of 3 dpf control (D) and *gcga*MO-injected (E) *Tg(gcga:GFP)* islets that were injected with H2B-RFP mRNA and labeled by Glucagon antibody (white). (F,G) Quantification of H2B-RFP^{+} *gcga:GFP*$^{+}$ dorsal bud-derived α cells (F) and H2B-RFP^{-} *gcga:GFP*$^{+}$ ventral bud-derived α cells (G) in control (*n*=7) and *gcga*MO (*n*=7) islets. (H-I) Confocal projections of 3 dpf control (H; *n*=7) and *gcga*MO-injected (I; *n*=7) *Tg(gcga:GFP)* islets. Arrows indicate newly forming *gcga:GFP*+ cells derived from pancreatic ducts.

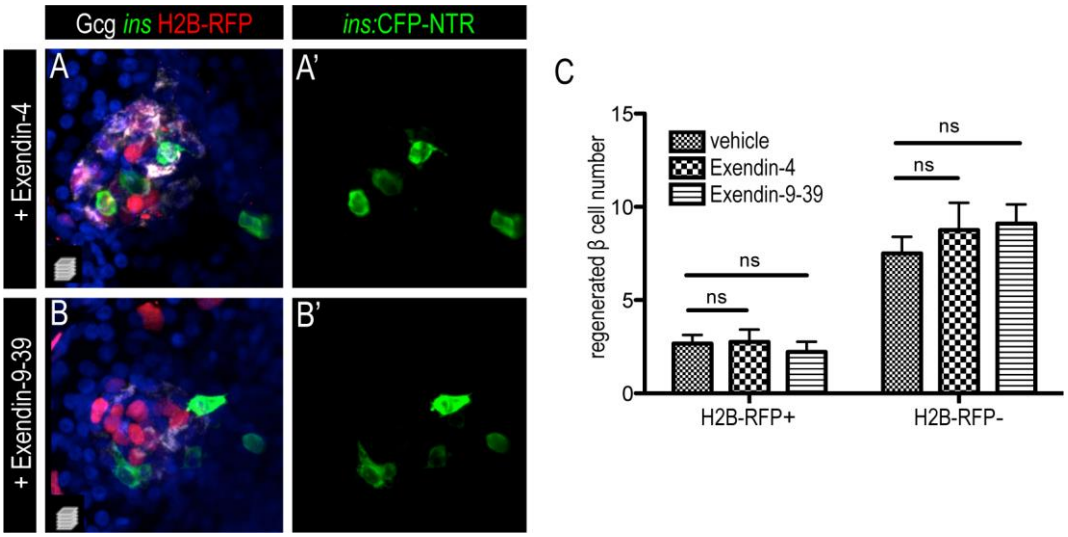

Figure S15. Glp-1 receptor agonist or antagonist treatment does not affect β cell regeneration. (A-B') Merged and single channel confocal projections of 5 dpf regenerating 1 dpa *H2B-RFP* mRNA injected islets that were treated with Exendin-4 (A) or Exendin-9-39 (B) during regeneration (comparable untreated control islets are shown in Fig. 4). (C) Quantification of H2B-RFP^{+} and H2B-RFP^{-} regenerated βcells in vehicle, Exendin-4, and Exendin-9-39 treated islets. Two-way ANOVA was used in C for statistical analysis.

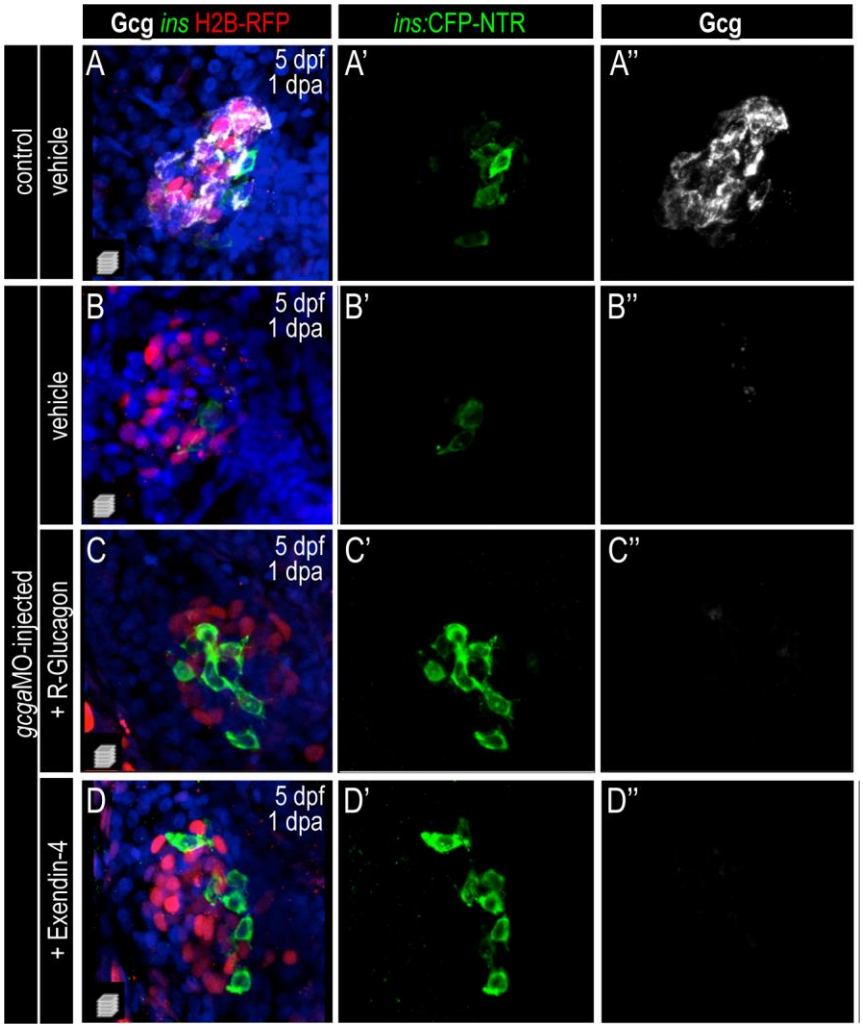

Figure S16. Glucagon or Exendin-4 injections rescue β cell regeneration in *gcga MO*-injected islets. (A-D'') Merged and single channel confocal projections of 5 dpf/1 dpa regenerating *H2B-RFP mRNA*-injected (red) islets that were not morpholino-injected (A), or injected with *gcgaMO* (B-D) and stained for CFP (*ins:CFP-NTR*; green), glucagon (white), and DNA (blue).

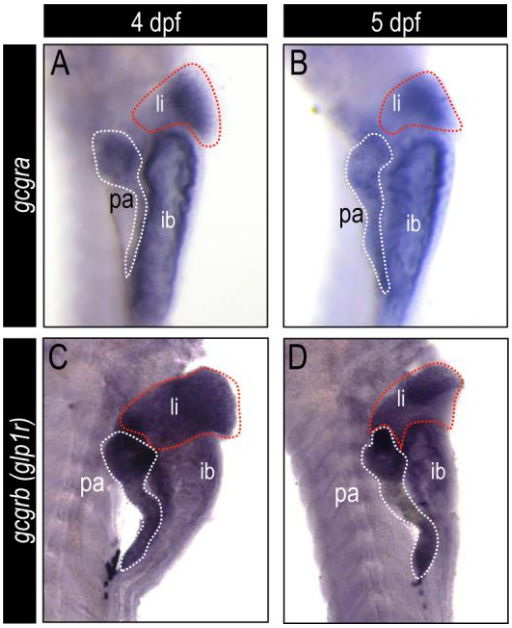

Figure S17. *glucagon receptor* and *glp-1 receptor* mRNAs are expressed in the larval pancreas. (A-D) Whole mount *in situ hybridization* showing regionally restricted expression of glucagon receptor (*gcgra*; A,B) and glp-1 receptor (*gcgrb/glp1r*; C,D) in the endodermal organs of 4 dpf (A,C) and 5 dpf (B,D) larvae. Note that both receptors are expressed throughout the pancreas, including exocrine and islet regions. Abbreviations: pa: pancreas; li: liver; ib: intestinal bulb.

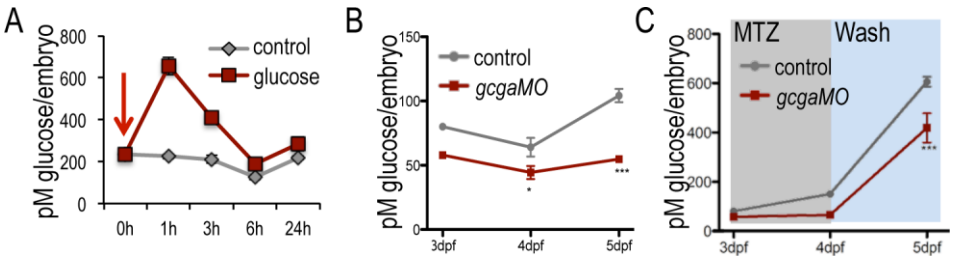

Figure S18. *glucagon* gene products regulate free glucose levels in zebrafish. (A) Free glucose levels measured periodically after mannose control (gray line) or glucose (red line) injection (arrow). (B) Free glucose measurement of control (gray line) and *gcga MO*-injected (red line) larvae at 3, 4 and 5 dpf. (C) Free glucose measurement in control uninjected and *gcgaMO*-injected *Tg(ins:CFP-NTR)* larvae in which βcells were ablated from 3 to 4 dpf. In both B and C, morpholino-injected zebrafish have diminished glucose levels. Two-way ANOVA was used in B and C for statistical analysis.

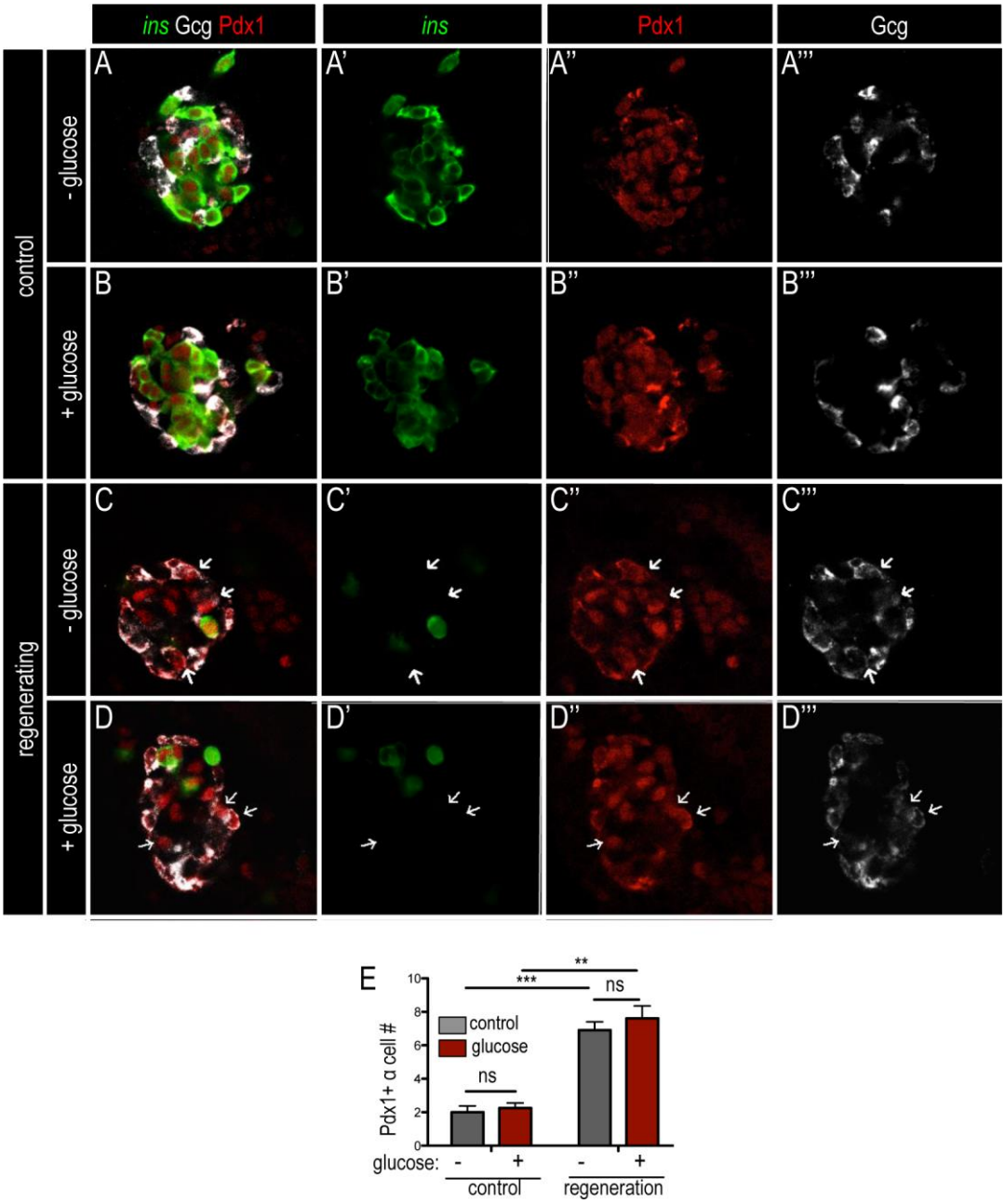

Figure S19. Quantity of Pdx1^{+} α cells is not affected by glucose injection. (A-D’’’) Merged and single channel confocal projections of 5 dpf non-ablated control (A-B’’’) and 1 dpa regenerating (C-D’’’) *Tg(ins:CFP-NTR)* islets that were not injected (A,C) or were injected with glucose (B,D) at 4 dpf. Islets were stained for Pdx1 (red), CFP (*ins:CFP-NTR*; green), and glucagon (white). Pdx1^{+} glucagon^{+} double positive cells are marked with white arrows in C,D. (E) Quantification of Pdx1 expression in αcells of intact and regenerating larvae was not altered by glucose injection; control group (*n=8*) and glucose treated group (*n=12*). Two-way ANOVA was used in E for statistical analysis.

Supplementary Table 1. Pancreatic gene expression in regenerating endoderm.

age		**gene**						
		insa	***gcga***	***arx***	***mafa***	***mafb***	***neurod1***	***ela3l***
0 dpa	**4 dpf**	-22.08**	+1.577**	-1.262	-1.335*	-1.801**	-1.189	-1.010
1 dpa	**5 dpf**	-39.43***	+2.00***	-1.317	+1.682***	-1.275	-1.324	+1.054
3 dpa	**7 dpf**	-10.45***	-1.15	-1.243	+1.686***	+1.022	+1.262*	-1.241*
Positive and negative value indicates fold increase or decrease of gene expression in regenerating endoderm compared with age matched un-ablated control. MTZ was added from 3 dpf to 4 dpf for ablation of βcells. *n*=3 for each group. Two way ANOVA analysis * p≤0.05, ** p≤0.01, *** p≤0.001								

Supplementary Table 2. Primers used for qPCR analyses.

gene	**strand**	**sequence**
lmnb1	sense	5’-ACCCGCGGCAAGAGAAAGCG
	antisense	5’-TCCTGCCATCGGCTGGTCCT
insa	sense	5’-TCTGCTTCGAGAACAGTGTG
	antisense	5’-GGAGAGCATTAAGGCCTGTG
gcga	sense	5’-AAGGCGACAGCACAAGCACA
	antisense	5’-GCCCTCTGCATGACGTTTGACA
arx	sense	5’-AAAAGCAAGTCGCCCACCGT
	antisense	5’-AATTTGGGCGGCAGGTGCATGT
mafa	sense	5’-ATTGTTCGCCGGGCTGTGTT
	antisense	5’-TGCTTTTGGCACAACCGGCA
mafb	sense	5’-CGCCAAACTGTGTTTGCGCTGA
	antisense	5’-AGGCGGCTTTAACGGGAGAAGT
neurod1	sense	5’-ACGCAGCGCTGTGATATACCGA
	antisense	5’-TCGCGTTCAACTGGGCGTTCAT
ela3l	sense	5’-GCTGAGCCTGTGACACTGAG
	antisense	5’-TCTCTGTGTGTTGGTTTTCTGG
trypsin	sense	5'-AGACCGTCTCTCTGCCTTCA
	antisense	5’- CAACACGCCATGATAACGAC
isl1	sense	5’-AGCAGCAGCAACCCAACGACAA
	antisense	5’-TGCACCTCCACTTGGTTTGCCT
opn1sw1	sense	5’-CCCAAATGGGCGTTCTACCT
	antisense	5’-CAAGGACCATCCCGTCACAA

Supplementary Table 3. Primers used for In Situ Hybridization.

glucagon	sense	5’-ATAAGCGAGGAGACGATCCA
	antisense	5’-GctaatacgactcactataggGCAATGAAGCCATCAGTTCTC
gcgr	sense	5’-GAGTGTCACCGCAGTTCAGT
	antisense	5’-GctaatacgactcactataggCTGTCCGTCTGCATCACACT
glp1r	sense	5’-CCGCTCATATTTGTGCTGCC
	antisense	5’-GctaatacgactcactataggAGCGGAGCCTTCATTGTTGA

Supplementary Table 4. Morpholino sequences

name	gene	morpholino sequence	dose
standard control MO	no target in *Danio rerio*	5'- CCTCT TACCT CAGTT ACAAT TTATA	4 or 8 ng
***arx*MO**	*arx* (NM_131384.1)	5'-TATCG TCGTC GTACT GACTG CTCAT	4 ng
***gcga*MO**	*gcga* (NM_001008595.3)	5'- GGCAA AATAC TGGAC GCCTT TCATT	8 ng

SUPPLEMENTARY MATERIALS AND METHODS

Zebrafish maintenance and strains: The following transgenic lines were used in the experiments: *TgBAC(neurod1:EGFP)* (Obholzer et al., 2008), *Tg(sox17:GFP)*^{s870} (Sakaguchi et al., 2006),*Tg(insa:Cre; cryaa:YFP)*^{s924} (Hesselson et al., 2009) ,*Tg(Ins:CFP-NTR)*^{s892} (Curado et al., 2007), *Tg(gcga:GFP)*^{ia1} (Pauls et al., 2007), *Tg(ins:dsRed)*^{m1018} (Anderson et al., 2009), *Tg(ins:Flag-NTR;cryaa:mCherry)*^{s950} (Andersson et al., 2012), and *Tg(hs:loxp-mCherry-STOP-loxp-H2BGFP)*^{s925} (Hesselson et al., 2009). *Tg(gcga:Cre; cryaa:YFP)*^{s962} and *Tg(sst2:Cre; cryaa:YFP)*^{s963} were constructed and generated by meganuclease transgenesis as described (Hesselson et al., 2009). To construct the *Cre* transgenes, *glucagon* promoter (gift of F. Argenton) or *sst2* promoter was subcloned into *ins:Cre; cryaa:Venus*. A 2 kb *sst2* promoter region was amplified from the CH211-232H16 zebrafish genomic clone (CHORI) using the oligos: 5'-GCATG AATTC AGCCT CTATG TCCTT CGTCT and 5'-GCATG GATCC TGCTG CTTCT TTAAC TCAG.

Detection of protein, mRNA, and cell proliferation: The following antibodies were used: chicken anti-GFP (1:500; Aves Labs #GFP-1020); guinea pig anti-insulin (1:100; Life Technologies #180067); mouse anti-glucagon (1:100; Sigma #G2654); rabbit anti-somatostatin (1:100 Serotec # 8330-0154); rabbit anti-dsRed (1:250; Clontech # 632496); mouse anti-PCNA (1:100; Abcam #PC10); rabbit anti-PHH3 (1:250; Cell Signaling #9701), guinea anti-Pdx1 (1:50 gift of Dr. C. Wright). Alexa Fluor-conjugated antibodies were used for visualization (1:500; Life Technologies). *glucagon* probe template was PCR amplified from cDNA with 5'-ATAAG CGAGG AGACG ATCCA and 5'-GCTAA TACGA CTCAC TATAG GGCAA TGAAG CCATC AGTT CTC primers. For quantitative PCR, we enriched endodermal organs by manual dissection of the digestive system with watchmakers forceps, which included pancreas, intestine, and liver from 4, 5, and 7 dpf larvae. 20-30 endoderm

preparations were pooled for each condition to minimize variability between dissections. mRNA was extracted with Trizol (Life Technologies) and reverse transcribed with iScript (BioRad). The Mastercycler Realplex PCR system (Eppendorf) was used with Sybr Green mix and Mytaq (BioLine) to generate Ct values. The relative expression of each sample was determined by normalizing to *lmnb1* using the relative standard curve method (Hesselson et al., 2009).

Microinjections: *H2B-RFP* mRNA was transcribed with SP6 mMessage machine kit (Invitrogen), and 100 pg were injected into zygotes. Anti-dsRed (Clontech) and Alexa568 antibodies (Life Technologies) were used to amplify the signal. The following antisense morpholinos (Gene Tools LLC) were injected into zygotes: control MO (4 or 8 ng), *gcga*MO (8 ng), and *arxa*MO (4 ng). Specificity of morpholino knockdown was addressed in multiple ways: First, significant off-target effects were unlikely in either *glucagon* or *arxa* morphants, as we observed no gross morphological defects, which are characteristic of such off-target and other non-specific toxic effects in morphants, (Figures S10C, S12I). Secondly, *gcga* and *arxa* morpholino phenotypes recapitulated phenotypes seen in mouse with knockdown of components of the glucagon signaling pathway (Hayashi et al., 2009; Vuguin and Charron, 2011) or Arx (Collombat et al., 2003). Thirdly, regeneration of beta cells in the *gcga* knockdown was rescued by injection of recombinant human Glucagon peptide or the Glp1 receptor agonist Exendin-4, but not the Glp1 receptor antagonist Exendin9-39 (Fig. 5A-G; S16). Finally, injection of a second *arxa* morpholino (*arxa*MO2 = 5'- ATGTT TGTAT CGTCC TCAGT CGTGC) produced identical phenotype in the islet (R.M.A., unpublished). To address morpholino efficacy, *gcga*MO eliminated glucagon protein in the islet (Fig. 4D'), and *arxa*MO specifically eliminated expression of an *arxa-GFP* DNA reporter construct that was co-injected into zygotes (R.M.A. unpublished).

Drug/chemical treatments: For βcell ablation, *Tg(ins:CFP-NTR)*^{s892} *or Tg(ins:Flag-NTR)*^{s950} animals were incubated in 0.1% DMSO (Sigma) ± 10 mM Metronidazole (MTZ, Sigma) in egg water. After ablation (generally 24 hrs), embryos were washed extensively with egg water, and recovered for 1-16 days. For peptide treatments, a mixture of KCl (0.2M), phenol red (0.1%), and either vehicle, recombinant human Glucagon (Sigma), Exendin-4 (Sigma), Exendin9-39 (Sigma), glucose, or mannose was injected into the pericardial sac of each embryo following ablation. The total mass of each drug injected was 20 pg. Glucose or mannose was injected with a final mass of 10 ng/larva. After injection, embryos recovered at 28˚C for 5 h before ablation.

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