1 **Pancreatic injury induces β–cell regeneration in axolotl**

2 **Running Title:** Axolotl Pancreatic Injury Response (33/40 characters)

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Abstract (194/200 characters)

Background: Diabetes is a condition characterized by a loss of pancreatic β–cell function which results in the dysregulation of insulin homeostasis. Using a partial pancreatectomy model in axolotl, we aimed to observe the pancreatic response to injury.

Results: Here we show a comprehensive histological assessment of pancreatic islets in axolotl. Following pancreatic injury, no apparent blastemal structure was observed. We found a significant, organ-wide increase in cellular proliferation post-resection in the pancreas compared to sham-operated controls. This proliferative response was most robust at the site of injury. We found that β–cells actively contributed to the increased rates of proliferation upon injury. β–cell proliferation manifested in increased β–cell mass in injured tissue at two weeks post injury. At four weeks post injury, we found organ-wide proliferation to be extinguished while proliferation at the injury site persisted, corresponding to pancreatic tissue recovery. Similarly, total β–cell mass was comparable to sham after four weeks.

Conclusions: Our findings suggest a non-blastema-mediated regeneration process takes place in the pancreas, by which pancreatic resection induces whole-organ β–cell proliferation without the formation of a blastemal structure. This process is analogous to other models of compensatory growth in axolotl, including liver regeneration.

Background (608 characters)

Diabetes is a condition characterized by a loss of pancreatic β–cell function which results in the body's inability to maintain insulin homeostasis. Treatment for diabetes can be, in most cases, lifelong, requiring administration of exogenous insulin or glycemic control agents, and glucose monitoring. Diabetes can cause complications such as heart failure, kidney disease, and tissue necrosis requiring amputation.[1,2,3] Several treatment strategies, such as cadaveric β–cell transplantation, polymer encapsulated stem cell-derived β–cell transplantation, transplantation of chemically induced pluripotent stem-cell-derived islets, and personalized endoderm stem cell-derived islet tissue, aimed at functional restoration, have been implemented to varying degrees of success.[4,5,6,7] These strategies, however, are invasive procedures that do not address the root cause of initial β–cell loss of function. A thorough understanding of genetic pathways that can promote pancreatic regeneration is important for developing therapeutic strategies for diabetes based on stimulating endogenous β-cell replacement.

Pancreatic regeneration has been reported in several animal models, such as mice and zebrafish, with strong conservation in injury responses.[8,9] Pancreatic regeneration studies typically focus on the endocrine pancreas i.e. β–cells due to their relevance to diabetes. β–cells are the principal insulin producing cells in the body, and characterizing their development, function, and fate has been the topic of research for decades. Partial pancreatectomy studies in rats and mice have reported proliferation of existing islet and exocrine cells, demonstrating substantial regenerative ability in these organisms.[8] Further studies in mice demonstrate β–cell regeneration to be a result of pre-existing β–cell differentiation in conjunction with transdifferentiation of other endocrine cell types.[4,10] Despite strong characterization of the pancreas in mammals, endogenous β–cell regeneration has not been accomplished in human patients.

The axolotl (*Ambystoma mexicanum*) is a highly regenerative species of salamander that can provide insights into how molecular mechanisms of regeneration might be harnessed therapeutically in humans. While the axolotl has been shown to regenerate several tissue

68 types such as limbs, lungs, and liver; few studies have investigated pancreatic

69 regenerative ability in axolotls.[11,12,13] Conservation of pancreatic regeneration in many

70 other organisms leads us to reason the axolotl may display a similar proficiency for

71 pancreatic response to injury.

72

73 Although the pancreas is well studied in humans and mice, only one study has reported

74 on the pancreas of axolotls.[14] This study, which established a diabetic axolotl model via

75 chemical ablation of β–cells using streptozotocin, demonstrated a return to normal

76 glycemic function after 84 days. These results indicated a restoration of β–cell function –

77 possibly due to regeneration. However, streptozotocin was reported to cause side effects

78 such as edema, lowered red blood cell count, and increased mortality.[14] These findings

79 introduced the axolotl as a promising animal model to study diabetes and illustrated the

80 need to develop a more consistent injury model to show whether axolotls exhibit robust

81 pancreatic regeneration. We sought to expand on the limited literature currently available

82 in regard to axolotl pancreas regeneration by studying the regenerative response to

83 partial pancreatectomy in axolotls.

84

85 Here, we characterize pancreatic tissue morphology and the pancreatic response to injury

86 in axolotls. We established a novel pancreatic resection surgery model to investigate how

87 animals respond to this injury over the course of 28 days. We identified key genes such

88 as *Pdx1*, *Ins*, *Tgf-β1*, *Ctrb2*, *Marco*, *Kazald2*, and *Cirbp* to be differentially regulated in

89 response to pancreatic injury.

90

Results (1335 characters)

A novel pancreatic resection model in the axolotl

We established a pancreatic resection surgery model with a sham-operated counterpart in order to investigate the injury response after a loss of pancreatic tissue mass (Figure 1A-B). Pancreatic resection was performed by surgical incision at a site adjacent to the base of the duodenum, resulting in removal of approximately twenty percent of the length of the organ. Animals with larger portions of their pancreas resected generally did not survive. Injury response was investigated at 14 and 28 days post injury (dpi). Ninety percent of animals survived the surgery, and death was non-specific to the sham or resection group. Mortality could have resulted from blood loss, infection, or other unknown causes. By 14 dpi, the abdominal incision wound had partially healed, while the pancreas appeared to have fully healed. The abdominal incision had fully healed by 28 dpi. No visible increase in tissue mass or blastema-like structure were observed at the site of pancreatic resection. Pancreatic resection studies in mice suggest that regeneration in pancreatic tissue can materialize in several forms, leading us to hypothesize that non-blastemal regeneration mechanisms such as transdifferentiation, endocrine cell-specific proliferation, or compensatory growth are possible and could have been utilized by the axolotl in response to injury.[15]
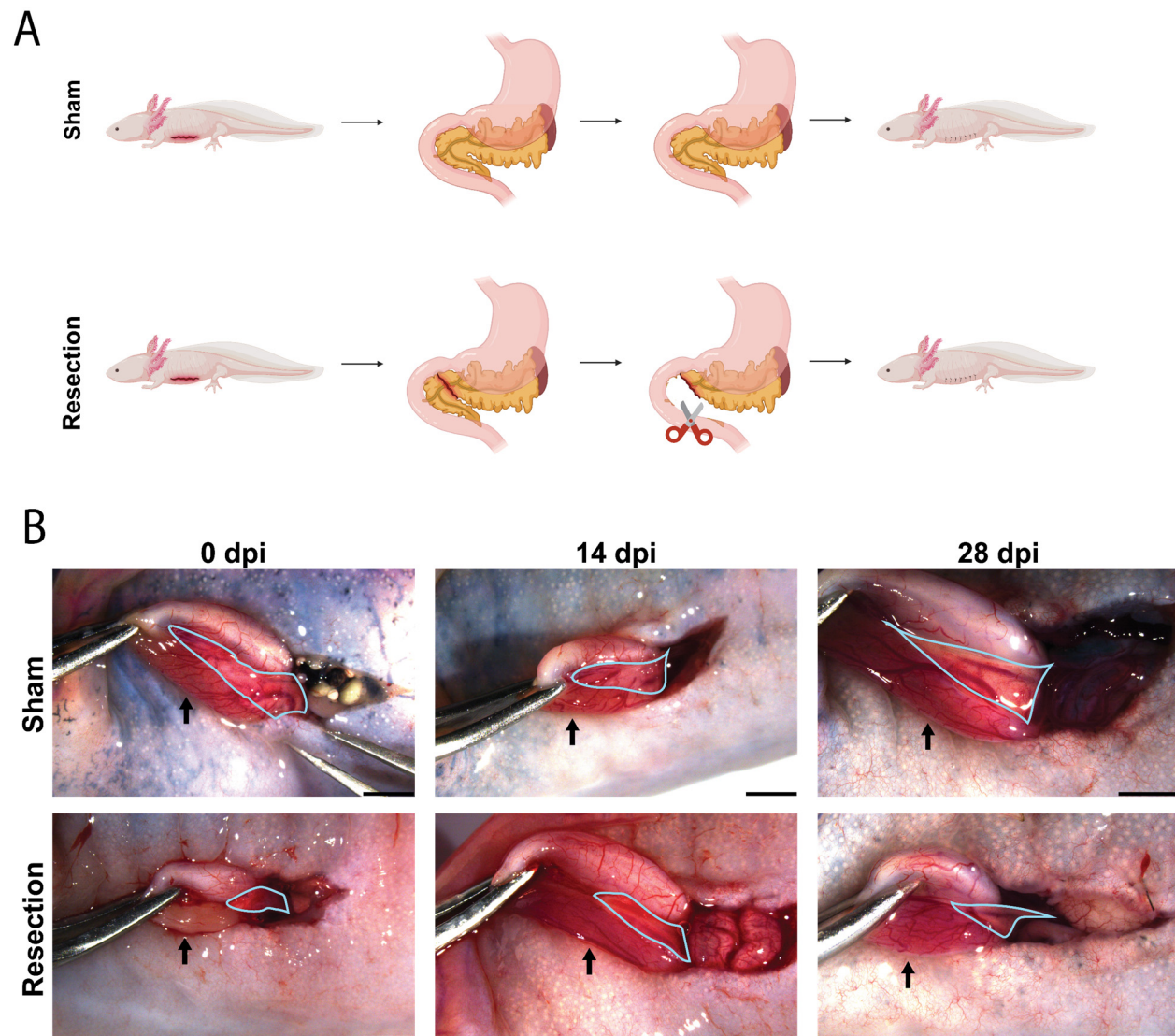
Figure 1. Pancreatic resection surgery model. A. Cartoon depicting sham and pancreatic resection surgery. **B.** Morphology of sham and pancreatic resection surgery at 0, 14 and 28 dpi. The pancreas is outlined in blue while black arrows point to the duodenum. 0 dpi image of the resection condition was taken immediately following 20% removal of pancreas by length. The majority of the pancreas extends into the abdominal cavity where it connects to the liver. In each image, the orientation from left to right follows a rostral to caudal direction. Black scale bars in the bottom right corner of each image are 2 mm.

Pancreatic resection leads to significant increases in cellular proliferation

To assess cellular proliferation, we used 5-ethynyl-2´-deoxyuridine (EdU) staining on coronal sections of pancreas (whole organ) from the sham and resection animal groups. We used a single pulse of EdU, followed by a short chase period, to identify all cells which have recently gone through S-phase of the cell cycle. Organ-wide proliferation was analyzed and compared to proliferation within a standardized area local to the site of injury (Figure 2I). We found a significant increase in organ-wide cellular proliferation at 14 dpi in resected pancreas samples compared to the sham samples (Figure 2A–B). This proliferative response was significantly increased local to the site of injury in comparison to organ-wide proliferation (Figure 2C). Additionally, proliferation local to the site of injury was significantly increased in comparison to an analogous area in sham samples (Figure 2D, 2I). These results suggest that at 14 dpi, the axolotl pancreas undergoes both an organ-wide proliferative response to resection and a proliferative response local to the site of injury.

At 28 dpi, we found that the organ-wide proliferative response was comparable to the sham-operated controls (Figure 2E-F). However, the proliferative response local to the site of injury remained significantly increased in comparison to the organ-wide response (Figure 2G). Likewise, the proliferative response local to the site of injury was still significantly increased in comparison to an analogous area in sham samples (Figure 2H-I). These results suggest that the whole-organ proliferative response had subsided at 28 dpi while the local proliferative response persisted.

We also compared the injury response between 14 and 28 dpi pancreas samples. Compared to 14 dpi, there was a significant decrease in proliferation both throughout the organ and local to the site of injury at 28 dpi (Figure 2J-K).
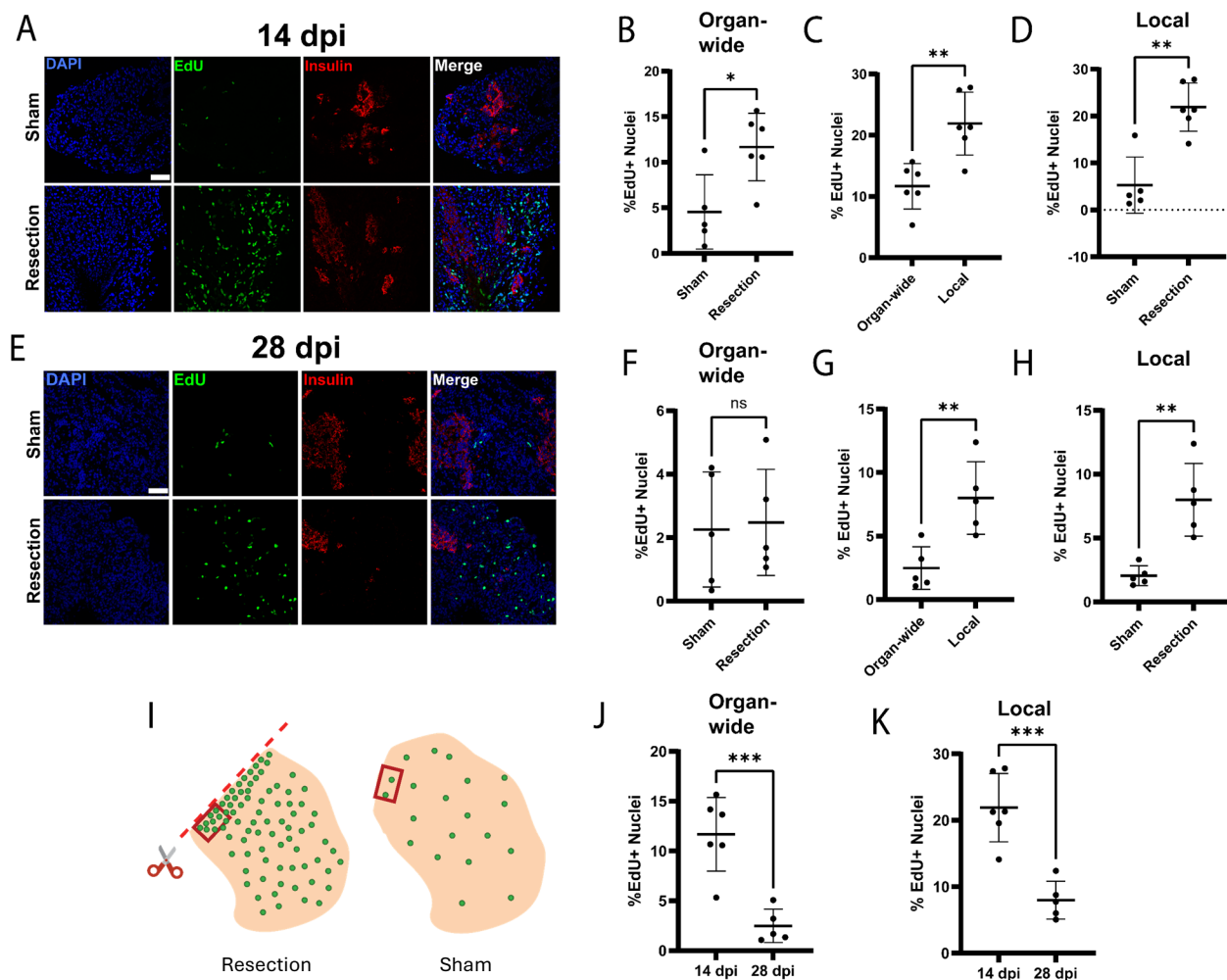
Figure 2. Pancreatic resection initially provokes substantial proliferative responses throughout the entire organ, but cell proliferation becomes restricted local to the site of injury later in the process. A. EdU and insulin (IHC) stain of pancreas samples at 14 dpi. **B.** Organ-wide proliferation is increased in resection samples at 14 dpi (p=0.0162) (sham n=5, resection n=6). **C.** Proliferation local to the site of injury is higher within resection samples at 14 dpi (p=0.0032). **D.** Proliferation local to the site of injury is increased at 14 dpi (p=0.0012). **E.** EdU and insulin stain of pancreas samples at 28 dpi (sham n=5, resection n=5). **F.** Organ-wide proliferation in resection samples is non-significant in comparison to sham at 28 dpi (p=0.8459). **G.** Proliferation local to the site of injury is higher within resection samples at 28 dpi (p=0.0085). **H.** Proliferation local to the site of injury is increased at 28 dpi (p=0.0079). **I.** Cartoon representation of how the area local to the site of injury was chosen. **J.** Organ-wide proliferation is decreased between

8

158   resection samples from 14 to 28 dpi (p=0.0009). **K.** Local proliferation is decreased

159   between resection samples from 14 to 28 dpi (p=0.0005). Scale bars are 100 μm.

160

161   **Pancreatic resection induces β–cell regeneration**

162   The identity of proliferating cells is important for understanding the regenerative

163   mechanisms at play, which will ultimately determine whether an organ will be capable of

164   proper function after regeneration occurs. We identified and quantified EdU positive cells

165   expressing insulin/ proinsulin protein (from here referred to as insulin positive) in order to

166   understand the injury response of β–cells (Figure 3A-D). We found that β–cells

167   proliferated at a significantly higher rate in resected samples when compared to the sham,

168   indicative of β–cell regeneration in response to injury (Figure 3E).[10] Additionally, at 14 dpi,

169   there was a significant increase in the number of insulin positive β–cells in the resection

170   condition; we refer to this as increased β–cell mass (Figure 3F). By 28 dpi, there was a

171   non-significant difference in the amount of insulin positive β–cells between the sham and
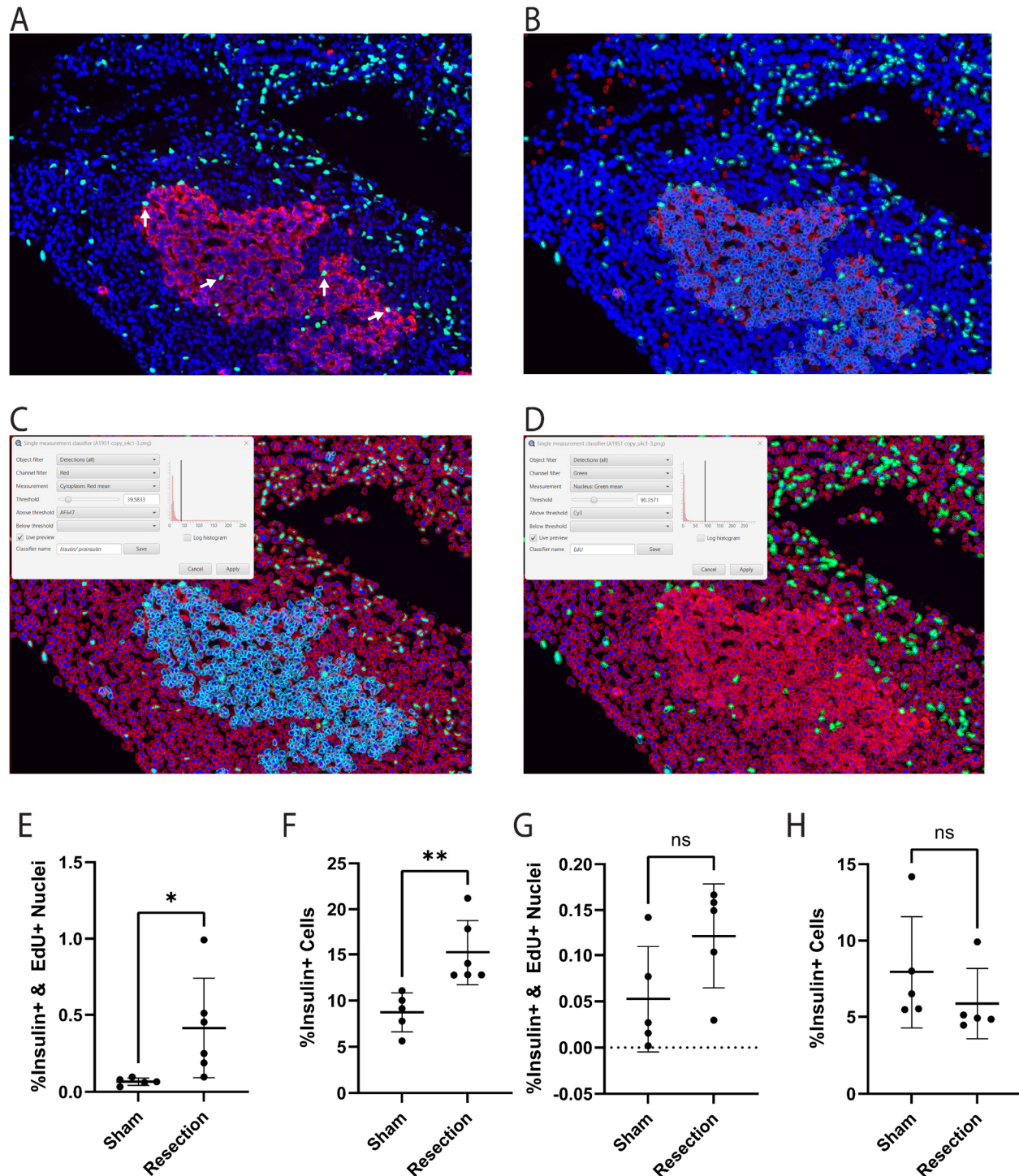
172   resected samples (Figure 3G-H).

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Figure 3. β–cell proliferation is observed at 14 days post injury but subsides by 28 days. A. Representative image of a coronal section of regenerating pancreatic tissue at 14 dpi. White arrows point to proliferating β–cells. **B.** Visualization of image quantification with QuPath Software. Cells were categorized by expression of insulin/ proinsulin

178 antibody and EdU stain, then counted for downstream statistical analysis. **C.** Settings
179 used to identify insulin/ proinsulin positive cells. Light blue outlines represent insulin/
180 proinsulin positive cells. Red outlines represent cells negative for insulin/ proinsulin. **D.**
181 Settings used to identify EdU positive cells. Green outlines represent EdU positive cells.
182 Red outlines represent EdU negative cells. **E.** A significant increase in cells positive for
183 both EdU and insulin antibody was observed at 14 dpi (p = 0.0463) (sham n=5, resection
184 n=6). **F.** A significant increase in insulin positive cells was observed at 14 dpi (p = 0.005)
185 (Sham n=5, Resection n=6). **G.** No significant difference in cells positive for both EdU and
186 insulin antibody was observed at 28 dpi (p = 0.0936) (Sham n=5, Resection n=5). **H.** No
187 significant difference in insulin positive cells was observed at 28 dpi (p = 0.3181) (Sham
188 n=5, Resection n=5).

189

190 **Axolotl pancreatic resection induces transcriptional changes in well-studied injury**
191 **response pathways**

192 To identify changes in gene expression associated with pancreatic resection, we
193 generated cDNA libraries (n=4 sham, n=4 resection) with polyadenylated RNA extracted
194 from whole-organ pancreas tissue at 14 dpi. cDNA was sequenced using the Oxford
195 Nanopore Technologies (ONT) PromethION sequencing platform. Sequencing returned
196 160.34M reads, of which 138.6M reads received a qscore greater than 8. After filtering by
197 quality, 97.79% of reads were successfully demultiplexed by their respective barcode
198 (Figure 4A). Classified reads were aligned to the axolotl transcriptome with a median read
199 alignment identity of 98.41% and alignment N50 of 526.48 bp (Figure 4B).[16] Subsequent
200 analyses of the sequencing data showed 1618 differentially expressed genes between
201 the sham and resection groups. A heatmap and volcano plot were created in order to
202 show several of the most significant transcriptional changes between conditions (Figure
203 4C-D). KEGG analysis revealed several categories to which these genes can be
204 attributed, such as cell cycle markers, regeneration markers, antitumor markers,
205 transcription factors, metabolic pathways, and protein transporters.

206

207 Many of these genes have been implicated in axolotl limb regeneration such as *Kazald2*,
208 which was found to be differentially upregulated.[17] We also found that genes *Cirbp* and
209 *Marco* were differentially upregulated, which we validated via HCR-FISH (Figure 5A-B).
210 Interestingly, *Kazald2* and *Cirbp* are regeneration markers in other contexts such as
211 axolotl limb regeneration.[17] *Marco* is a macrophage marker, suggesting an immune
212 response is provoked by pancreatic resection, which parallels the macrophage-mediated
213 immune response necessary in axolotl limb regeneration and in a variety of other
214 organ/appendage regeneration contexts in other species.[18,19,20] These findings suggest
215 that pancreatic response to injury shares characteristics with other regenerative tissues
216 and organs in the axolotl.[17,19] Additionally, we found *Tgf-β1* to be differentially upregulated.
217 *Tgf-β1* is a key regulator of wound epithelium formation, blood clot formation, and
218 inflammation in mammals, all of which are essential for regeneration to take place in
219 axolotls.[21] Further, Levesque *et al.* functionally demonstrated *Tgf-β1* to be essential in the
220 initiation and control of the axolotl limb regeneration process.[21] Parallels in axolotl limb
221 and pancreas regeneration may reveal conserved regeneration pathways that are present
222 in all axolotl injury responses.

223

224 We found several pancreatic development and maintenance genes to be differentially
225 expressed such as *Ctrb2, Ins,* and *Pdx1*. *Ctrb2* is a gene encoding chymotrypsin, an
226 essential molecule produced by the exocrine pancreas to aid in digestion. We use *Ctrb2*
227 to visualize the distinction between the exocrine and endocrine pancreas (Figure 5A).
228 Interestingly, both *Pdx1* and *Ins* were found to be downregulated at 14 dpi. *Ins* is the
229 transcript responsible for insulin production by β–cells. *Pdx1* is a master regulator of
230 pancreatic development and function. *Pdx1* is expressed throughout the gastrointestinal
231 tract and central nervous system during development; however, it is primarily expressed
232 in β–cells in early development and persists into maturity.[22] Downregulation of these
233 transcripts supports our hypothesis that β–cell regeneration has exceeded the β–cell
234 mass needed for homeostatic function, leading to extinguished expression of transcripts
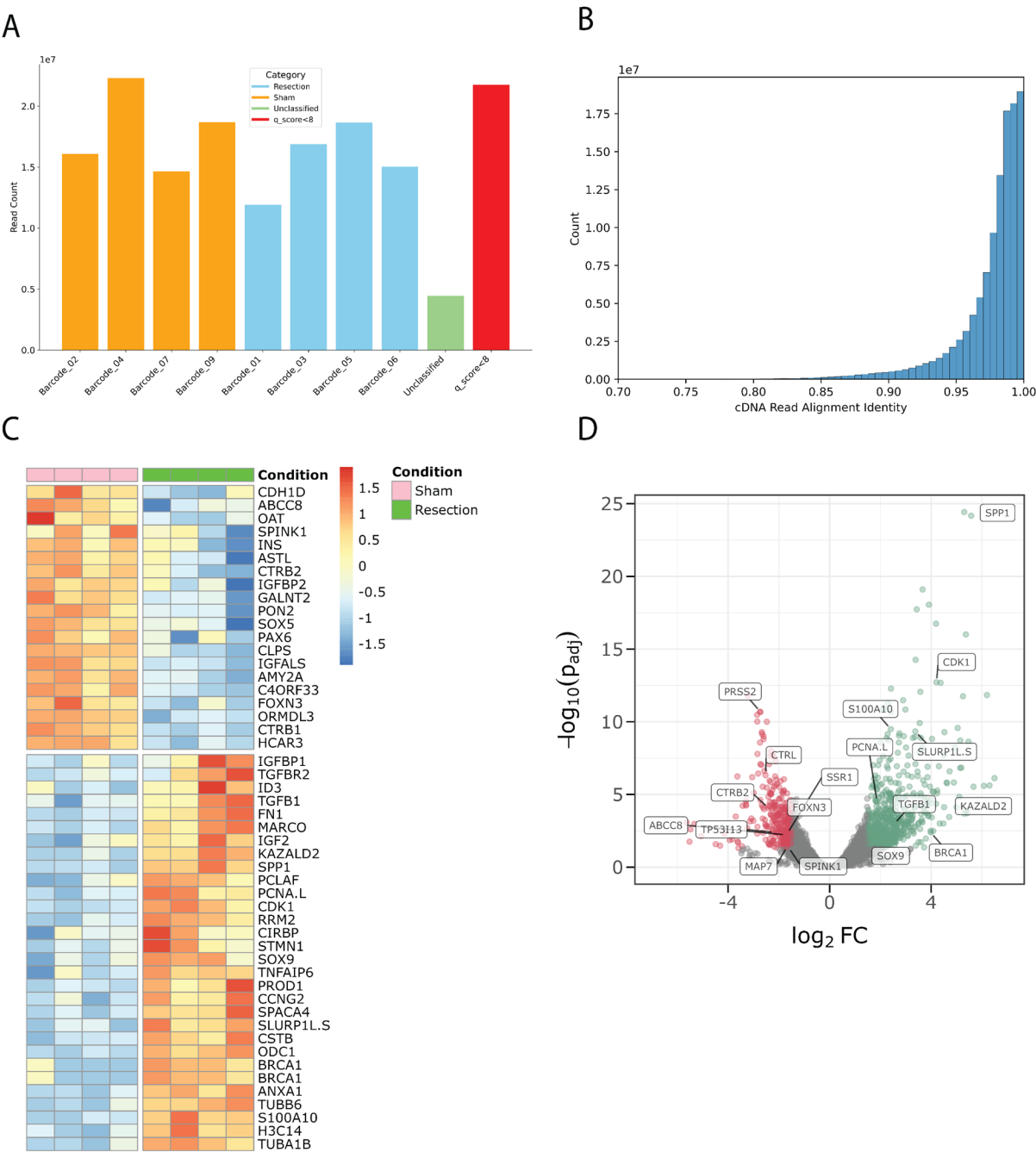235 responsible for β–cell development and insulin production.

Figure 4. Nanopore sequencing data reveals transcriptomic changes in response to injury. A. Bar plot detailing reads per sample after demultiplexing. Unclassified reads and reads with a qscore less than 8 were filtered out. **B.** Histogram detailing aligned read identity vs total read counts. **C.** Heatmap of differentially expressed genes at 14 dpi **D.** Volcano plot of differentially expressed genes at 14 dpi.
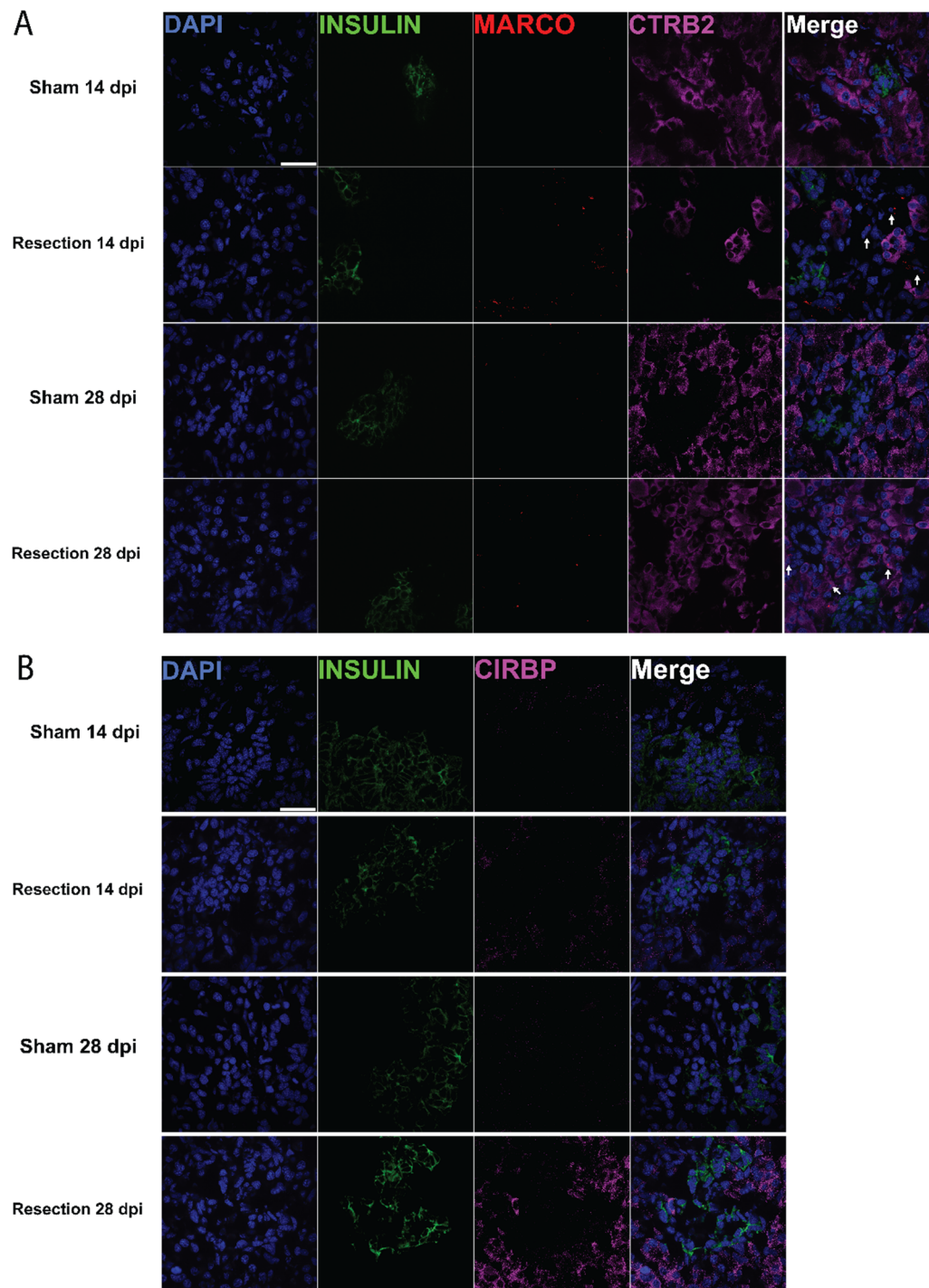
Figure 5: Genes previously implicated in regeneration exhibit upregulation in axolotl pancreatic injury model. A. HCR-FISH showing differential expression of insulin and *Marco* transcripts. *Ctrb2* was not found to be differentially expressed; however, it provides structural context for exocrine tissue. *Marco*+ cells shown with arrows. **B.** HCR-

14

248   FISH showing differential expression of insulin and *Cirbp* transcripts. Scale bars are

249   50 μm.

250

251   **Axolotl pancreatic tissue morphology**

252   The axolotl pancreas is a thin, triangular tissue connected directly to the base of the

253   duodenum, with connections to the liver (Figure 1B). It has been reported that the axolotl

254   pancreas exhibits protein level insulin expression similar to the mammalian pancreas.[23]

255   However, the expression of other common endocrine and exocrine pancreatic markers in

256   axolotl is not well documented. We utilized hybridized chain reaction-fluorescent in situ

257   hybridization (HCR-FISH) to show spatial transcript level expression of several pancreatic

258   markers. This staining showed the presence of *Ins-* (insulin) and *Nkx6-1-* expressing

259   β–cells, *Gcg-* (glucagon) expressing α-cells, *Sst-* (somatostatin) expressing δ-cells,

260   (Figure 6A-B). *Pdx1*, a critical gene in pancreatic development and function, was found

261   to be expressed in both endocrine and exocrine cells (Figure 6B).[22] These results

262   demonstrate that axolotl pancreatic tissue morphology shares similarities with both mice

263   and humans, suggesting that pancreas studies in axolotl could have functional

264   therapeutic relevance to mammalian research (Figure 6C).[8,24]

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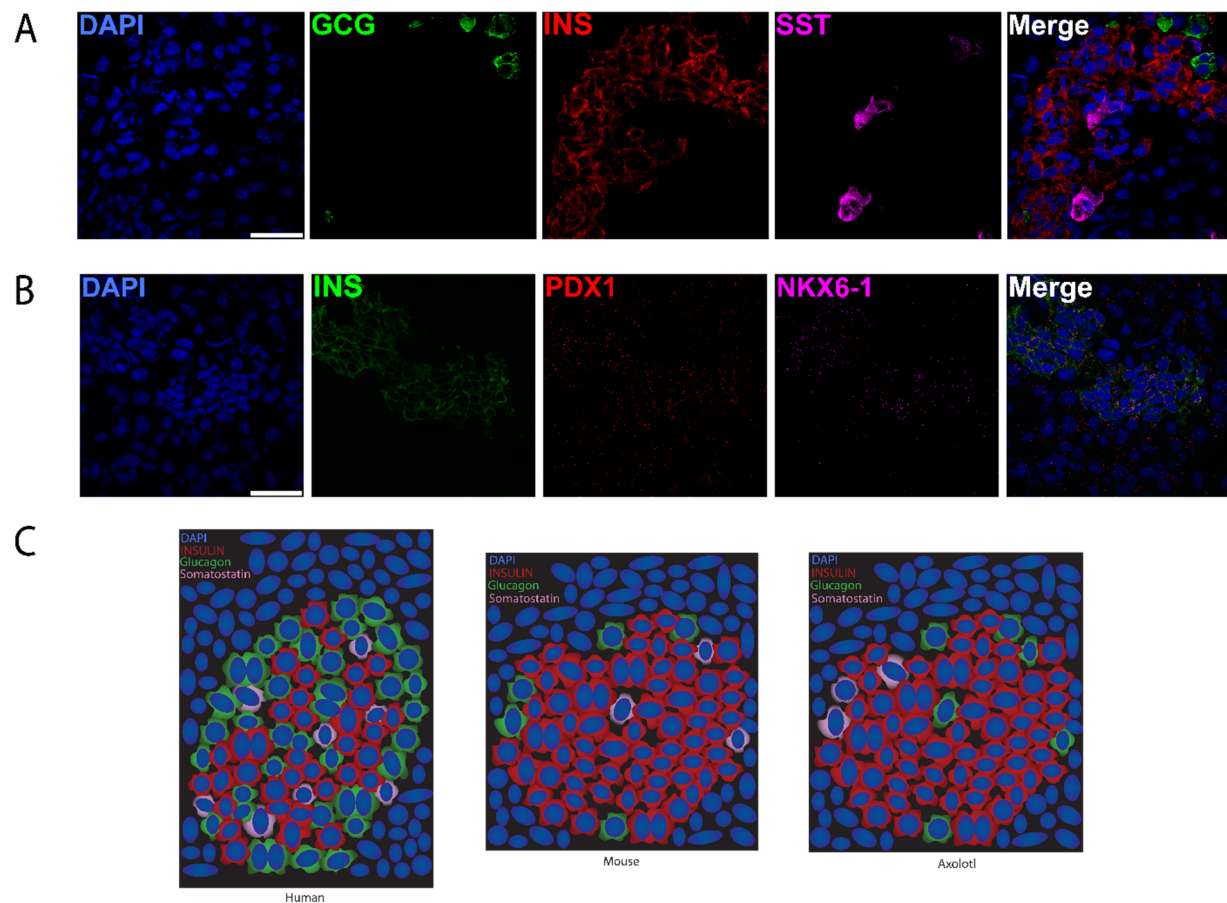
Figure 6. Transcript level pancreatic islet morphology in axolotl A. HCR-FISH of glucagon (*Gcg*), insulin (*Ins*), and somatostatin (*Sst*) transcripts. **B.** HCR-FISH of insulin (*Ins*), *Pdx1*, and *Nkx6-1* transcripts. **C.** Cartoon representation comparing axolotl islets to mice and humans. Mouse and human islet cartoons based on data from Abdulreda *et al*[25]. Scale bars are 50 μm.

16

Axolotl pancreas morphology

Here, we show transcript-level expression of evolutionarily-conserved pancreatic hormones such as insulin, glucagon and somatostatin in the axolotl via HCR-FISH. Additionally, key developmental markers such as *Nkx6-1* and *Pdx1* are shown to be conserved in this context. These transcriptional characteristics establish the axolotl as a comparable model of pancreatic development and function to mice and humans.[8]

Future studies should aim to investigate the functional role of notable genes in promoting or preventing pancreatic cell function and proliferation in the context of injury. The animals used for this study were too small to tolerate repeated blood draws of the quantity needed for ELISA assays. As such, our findings would benefit from future studies that identify whether the injury-induced increase in β–cell mass in axolotl allows for the maintenance of functional insulin homeostasis. Future work should also characterize the origin of new β–cells. There is significant debate in the field as to whether pancreatic stem cells exist in mammals.[10] Axolotl salamanders have been demonstrated to use several stem-cell-mediated mechanisms for regeneration.[20] Lineage tracing should be performed in axolotl pancreatic injury models to ascertain whether new β–cells originate from existing β–cells or from pancreatic stem cells.

We conclude that axolotl pancreatic islets closely resemble those in mice and humans, demonstrated by the conservation of several key pancreatic gene markers (Figure 6A-B). Further, a significant increase in β–cell mass is a direct parallel between mouse and axolotl pancreatic regeneration (Figure 3F).[10] Although mouse models are very well characterized in the context of pancreatic regeneration, translating these insights into natural β–cell regeneration has not yet manifested in humans. We assert that the axolotl is an important animal model to study because its highly regenerative nature could reveal additional factors required to improve regenerative outcomes in humans.

Experimental Procedures

<u>Pancreas resection surgery</u>

All animal experimentation was approved by and conducted in accordance with Harvard University's Institutional Animal Care and Use Committee (Protocol # 19-02-346). Adult wild type axolotl salamanders, 16 cm in length, were anesthetized in 0.1% tricaine solution and subjected to either a pancreatic resection surgery or a sham control surgery. The sham surgery consisted of a 2-inch lateral incision of the abdomen followed by gentle perturbation of the pancreas. The abdominal incision was closed with two horizontal mattress stitches using 6-0 nylon sutures. The resection surgery consisted of a similar 2-inch lateral incision of the abdomen followed by an approximate 20% resection of the pancreas starting from the base of the duodenum. Substantial bleeding was observed. Sterile wooden cotton tipped applicators were used to successfully clot the bleeding in all cases. The abdominal incision was closed with two horizontal mattress stitches using 6-0 nylon sutures. The animals were allowed to respond to this injury for 14 days, or 28 days post-incision, after which the whole pancreas was collected and used for histology/ sequencing.

<u>Tissue collection and cryosectioning</u>

Upon collection of the whole pancreas at 14 days, or 28 days post incision, animals were perfused with diethyl pyrocarbonate phosphate buffered saline (DEPC PBS) via aortic injection in order to clear tissue of blood and digestive enzymes while preserving RNA integrity. Tissue samples were subsequently preserved using various methods based on the intended analysis of that tissue:

Pancreas tissue samples intended for use in histology were transferred to a 4% paraformaldehyde in DEPC PBS solution upon collection. Samples were rocked overnight at 4°C. The following morning, samples were washed with DEPC PBS and transferred to a 30% sucrose in DEPC PBS solution. Samples were rocked overnight at 4°C. The

19

361 following morning, samples were transferred to an OCT -30% sucrose mixture for 1 hour

362 at room temperature. Subsequently, samples were embedded in 100% OCT on dry ice.

363 Samples were stored at -80°C until further use. Tissue samples embedded in OCT were

364 cryosectioned on the Leica cm 1950 cryostat at -22°C into 10 μm sections and mounted

365 on Fisher Brand Superfrost Plus microscope slides. Samples were stored at -80°C.

366

367 Pancreas tissue samples intended for bulk RNA-seq were subject to a modified protocol

368 based on Jun et al. which required samples to be injected with RNAlater upon collection

369 and minced into small pieces to maximize tissue surface area contact with RNAlater[27].

370 Samples were subsequently transferred into 500μL of RNAlater and snap frozen on dry

371 ice. Samples were stored at -80°C until further use. RNA extraction was completed using

372 a phenol/chloroform phase separation with subsequent clean up using the Zymo Clean &

373 Concentrator 5 kit.

374

375 <u>EdU incorporation</u>

376 A stock 5-ethynyl-2′-deoxyuridine (EdU) solution was made by dissolving EdU in

377 dimethylsulfoxide (DMSO) at a concentration of 5μg/μL. This stock solution was diluted

378 to 0.1μg/μL in 0.7× PBS for injection. Animals were anesthetized in 0.1% tricaine solution

379 for 30 minutes prior to injection. EdU solution was injected intraperitoneally with care not

380 to puncture internal organs. Each animal received 20μL per gram body weight of the

381 injection solution. Animals were returned to normal housing conditions to incorporate with

382 EdU for 18 hours post injection. Tissue collection immediately followed.

383

384 <u>EdU and immunohistochemistry</u>

385 Slides were rehydrated in 1× PBS and permeabilized with 0.5% Triton-X PBS (TX-PBS)

386 for 30 minutes. EdU reaction was completely using copper sulfate ($CuSO_4$), ascorbic acid

387 ($C_6H_8O_6$) and sulfo-Cy3 azide. Slides were blocked with 2% BSA in 0.1% TX-PBS for 30

388 minutes followed by addition of primary antibody (1:200 in blocking solution) against

389   insulin/ proinsulin overnight (D3E7 (5B6/6), ThermoFisher Scientific, cat no. MA1-83256).

390   Secondary antibody was added for 2 hours (AlexaFluor Donkey Anti-Mouse 647,

391   ThermoFisher Scientific, cat no. A-31571).

392

393   HCR FISH

394   Oligonucleotides were designed using a probe generator model developed by the

395   Monaghan Lab at Northeastern University with transcripts identified in our sequencing

396   data through alignment to the Nowoshilow transcriptome[16]. HCR stain was completed as

397   described by Lovely *et al.*[28].

398

399   Imaging and quantification

400   Histological imaging was performed using the Nikon Spinning Disk Microscope, Zeiss

401   Axioscan Microscope, and Zeiss LSM 900 Microscope. EdU & insulin/ proinsulin stains

402   were quantified using Cell Profiler and Qupath. Technical replicates were quantified

403   individually and then averaged into a single biological replicate. For EdU+ nuclei, all EdU+

404   nuclei in a section were counted and normalized to the total number of nuclei in that

405   section. This was expressed as %EdU+ nuclei, calculated as (EdU+/total nuclei) * 100.

406   Qupath used a spatial categorization method to count insulin/ proinsulin & EdU+ nuclei.

407   Similarly, the proportion of insulin/proinsulin and EdU+ nuclei was determined by counting

408   these nuclei in a section and normalizing them to the total number of nuclei in the section,

409   expressed as %insulin & EdU+ nuclei = (insulin & EdU+/total nuclei) × 100. Generally,

410   tissue sections were composed of 5-10 thousand total nuclei. Biological replicates at 14

411   dpi: Sham n=5; Resection n=6. Biological replicates at 28 dpi: Sham n=5; Resection n=5.

412

413   For selection of area local to the site of resection, a 350 x 500 μm control area was

414   selected randomly along the plane of resection in histology images. Nuclei within the

415   control area were analyzed as described above. A comparable area in sham samples was

416   selected by utilizing a 350 x 500 μm control area placed along the edge of the tissue

417   section. Nuclei in the control area were analyzed as described above. Randomized
418   repetitions of control area placement yielded comparable results.

419   <u>RNA extraction</u>

420   Whole pancreas tissue from the sham and resection groups (n=4) was transferred to
421   500mL of Trizol and homogenized for 30-seconds with the Bio-Gen PRO200 Hand-Held
422   homogenizer. A phenol chloroform phase separation was performed, and the aqueous
423   layer was transferred to Zymo spin columns. The RNA was cleaned up following the
424   protocol for the Zymo Clean & Concentrator 5 kit. RNA quality was determined by
425   Nanodrop spectrophotometer, Tapestation, and Qubit.

426

427   <u>Nanopore cDNA Sequencing</u>

428   High quality RNA from the previous step was library prepped for sequencing according to
429   protocol for the ONT PCR cDNA kit with barcode capability (SQK-PCB111.24). Each
430   sample was barcoded, the libraries were combined and sequenced simultaneously on
431   two PromethION flow cells.

432

433   Sequencing data was analyzed using a custom in-house Nextflow pipeline. This pipeline
434   utilized Dorado to basecall, Minimap2 to align reads to the axolotl transcriptome
435   (Nowoshilow et al., 2018), and featureCounts to quantify the data. Differential gene
436   expression was calculated using DESeq2.

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