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## Centroacinar Cells Are Progenitors That Contribute to Endocrine Pancreas Regeneration

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**Diabetes is associated with a paucity of insulin-producing  $\beta$ -cells. With the goal of finding therapeutic routes to treat diabetes, we aim to find molecular and cellular mechanisms involved in  $\beta$ -cell neogenesis and regeneration. To facilitate discovery of such mechanisms, we use a vertebrate organism where pancreatic cells readily regenerate. The larval zebrafish pancreas contains Notch-responsive progenitors that during development give rise to adult ductal, endocrine, and centroacinar cells (CACs). Adult CACs are also Notch responsive and are morphologically similar to their larval predecessors. To test our hypothesis that adult CACs are also progenitors, we took two complementary approaches: 1) We established the transcriptome for adult CACs. Using gene ontology, transgenic lines, and in situ hybridization, we found that the CAC transcriptome is enriched for progenitor markers. 2) Using lineage tracing, we demonstrated that CACs do form new endocrine cells after  $\beta$ -cell ablation or partial pancreatectomy. We concluded that CACs and their larval predecessors are the same cell type and represent an opportune model to study both  $\beta$ -cell neogenesis and  $\beta$ -cell regeneration. Furthermore, we show that in *cfr* loss-of-function mutants, there is a deficiency of larval CACs, providing a possible explanation for pancreatic complications associated with cystic fibrosis.**

The zebrafish has a remarkable regenerative capacity, including an ability to regenerate pancreatic  $\beta$ -cells (1,2). The regenerative capacity of the fish has already revealed new avenues by which therapies may be developed to replace

tissues in patients that otherwise would not normally heal (3). Humans and other mammals can also undergo somewhat limited  $\beta$ -cell regeneration (4–6), yet whether such regeneration includes  $\beta$ -cell neogenesis is still under debate (7–9). Investigation into whether  $\beta$ -cell neogenesis occurs in mammals has likely been hindered by the more limited regenerative capacity of those models. Knowledge of the mechanisms used in the adult zebrafish pancreas to regenerate endocrine tissue has the potential to reveal routes by which missing  $\beta$ -cells could be replaced in diabetic patients.

Pancreata from zebrafish and mammals share considerable similarity in terms of morphology and gene expression. The larval zebrafish pancreas contains a single principal islet and, starting at 5 days postfertilization, secondary islets that form throughout the pancreas parenchyma (10). The adult zebrafish pancreas consists of four lobes: gallbladder-spleen, middle, left, and ventral.  $\beta$ -Cells are arranged as isolated single cells and in islets along with other endocrine cells. The principal islet is normally located in the gallbladder-spleen lobe (11).

During zebrafish development, pancreatic Notch-responsive cells (PNCs) give rise to endocrine, ductal, and centroacinar (CACs) cells (10,12). CACs are defined as specialized ductal epithelial cells located at the ends of ducts within the acinar lumen. Both PNCs and CACs are ductal cells that share the following characteristics: 1) possession of long-cytoplasmic extensions (13), 2) distinctive ultrastructure (14), and 3) responsiveness to Notch signaling (10,15,16). CACs have been proposed to be adult multipotent progenitors in rodents (15–19). Although the

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progenitor status of mammalian CACs remains controversial (20–24), the idea is supported by the observation that mammalian CACs proliferate in response to different injuries, including streptozotocin-induced destruction of  $\beta$ -cells (25), partial pancreatectomy (26), and acute or chronic administration of caerulein (27).

To characterize CACs in more detail, we used RNA-Seq to define their transcriptome. Gene ontology analysis revealed that CACs are enriched for genes important in progenitor biology. To explore whether CACs are involved in  $\beta$ -cell regeneration, we have used two injury models in zebrafish: 1) a transgenic method to specifically ablate  $\beta$ -cells and 2) partial pancreatectomy (PPx), a surgical procedure that removes the left lobe of the pancreas. Using inducible genetic lineage tracing, we find that CACs do indeed contribute to  $\beta$ -cell regeneration. Our discovery that CACs act as endocrine progenitors in a rapidly regenerating, genetically tractable model can reveal mechanisms important in  $\beta$ -cell neogenesis that have been difficult to study in mammals and that may ultimately be applied to improve regenerative capacity in diabetic patients to restore  $\beta$ -cell mass.

## RESEARCH DESIGN AND METHODS

### Transgenic Lines

Tg(*HS4-sst2:CFP;ins:PhiYFP-m-dest1-2TA-nfsB*)<sup>lmc009</sup> (abbreviated *ins:NTR*) carries a transgene containing 1) insulin promoter driving a zebrafish codon-optimized NTR-T2A–yellow fluorescent protein (YFP) open-reading frame and 2) somatostatin2 promoter/enhancer driving *CFP* transcription (Fig. 4A). Other lines are listed in Supplementary Table 2.

### CAC Isolation

Three-month postfertilization adult *Tp1:GFP* (method 1) and *Tp1:gfp;flt1:DsRed* (method 2) fish were killed and pancreata dissected in 1 $\times$  Hanks' balanced salt solution (HBSS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>. Pancreata were dissociated in 0.7 mg/mL Collagenase P (Roche) (37°C, 20 min), followed by addition of FBS (Gibco) to a 5% final concentration on ice. Cells were collected by 3  $\times$  5 min centrifugation (700g, 4°C) with resuspension in 1 $\times$  HBSS with 5% FBS. Cells were then resuspended in 1 $\times$  HBSS, 0.01% Trypsin (Gibco) (37°C, 5 min), followed by 3  $\times$  5 min centrifugation (700g, 4°C) with resuspension in 1 $\times$  HBSS and 5% FBS and filtration through 70  $\mu$ mol/L and 40  $\mu$ mol/L nylon filters (BD Falcon). For method 1, allophycocyanin-conjugated anti-CD-105 (60039AD; STEMCELL Technologies) was used to label endothelial cells. Cells were sorted on a BD Biosciences FACSaria III cell sorter.

### RNA Sequencing

Total RNA was isolated (RNeasy Mini, Qiagen) and RNA-Seq libraries were prepared (version 2; TruSeq RNA Sample Preparation kit, Illumina). Fifty-cycle single-end reads were collected (Genome Analyzer II, Illumina). Reads were processed and mapped to *Zv9/danRer7* using RSEM (28). EBSeq (29) was used to determine differential expression and significance values. Data files have been submitted to GEO (<http://www.ncbi.nlm.nih.gov/geo/>).

### Quantitative PCR

cDNAs were made (SuperScript III First-Strand Synthesis kit, Invitrogen). Quantitative PCR (qPCR) was performed on a Bio-Rad C1000 Bio-Rad thermal cycler and analyzed using the CFX Manager software (Bio-Rad). qPCR primers are listed in Supplementary Table 3.

### Drug Treatments

For ablation of  $\beta$ -cells, 30 mmol/L metronidazole (MTZ) (M3761; Sigma) in PBS was intracoelomically (i.c.) injected into *ins:NTR* fish at a dose of 0.25 g/kg body wt. Control fish were injected with PBS. For activation of CreERT2, 20  $\mu$ L of 2  $\mu$ mol/L 4-hydroxytamoxifen (4OHT) (T176; Sigma) (12) was i.c. injected daily for 3 days. For induction of 2° islets, 50  $\mu$ mol/L N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) in E3 medium (30) was applied to larvae from 3–5 dpf (10).

### Partial Pancreatectomy

Fish were anesthetized in 0.168 mg/mL tricane (Sigma) (30). An incision was made on the left flank, and most of the left pancreatic lobe was removed. Sham surgeries were performed as controls. Fish were kept in still water (28°C) that was changed after daily feeding. For the first 3 days after surgery, EdU was added to the water (2.5  $\mu$ mol/L). After PPx plus 3 days, EdU was delivered by i.c. injection of 20  $\mu$ L of 25  $\mu$ mol/L 5-ethynyl-2'-deoxyuridine (EdU).

### Immunohistochemistry, In Situ Hybridization, and Microscopy

Five dpf larvae were fixed in 4% paraformaldehyde (4°C overnight). Antibody staining and in situ hybridization were performed as previously described (12,31). Primers for making riboprobes are listed in Supplementary Table 3. For adult pancreata, 3-month postfertilization zebrafish were fixed overnight in 10% formalin at 4°C. Viscera were then dissected, embedded in paraffin blocks, and processed using standard procedures (Abcam). Antibodies are listed in Supplementary Table 4. Images were collected using a Nikon AZ100 microscope or a Nikon A1-si Laser Scanning Confocal microscope. For adult quantification, at least five sections of each individual islet >1,000  $\mu$ m were counted per fish.

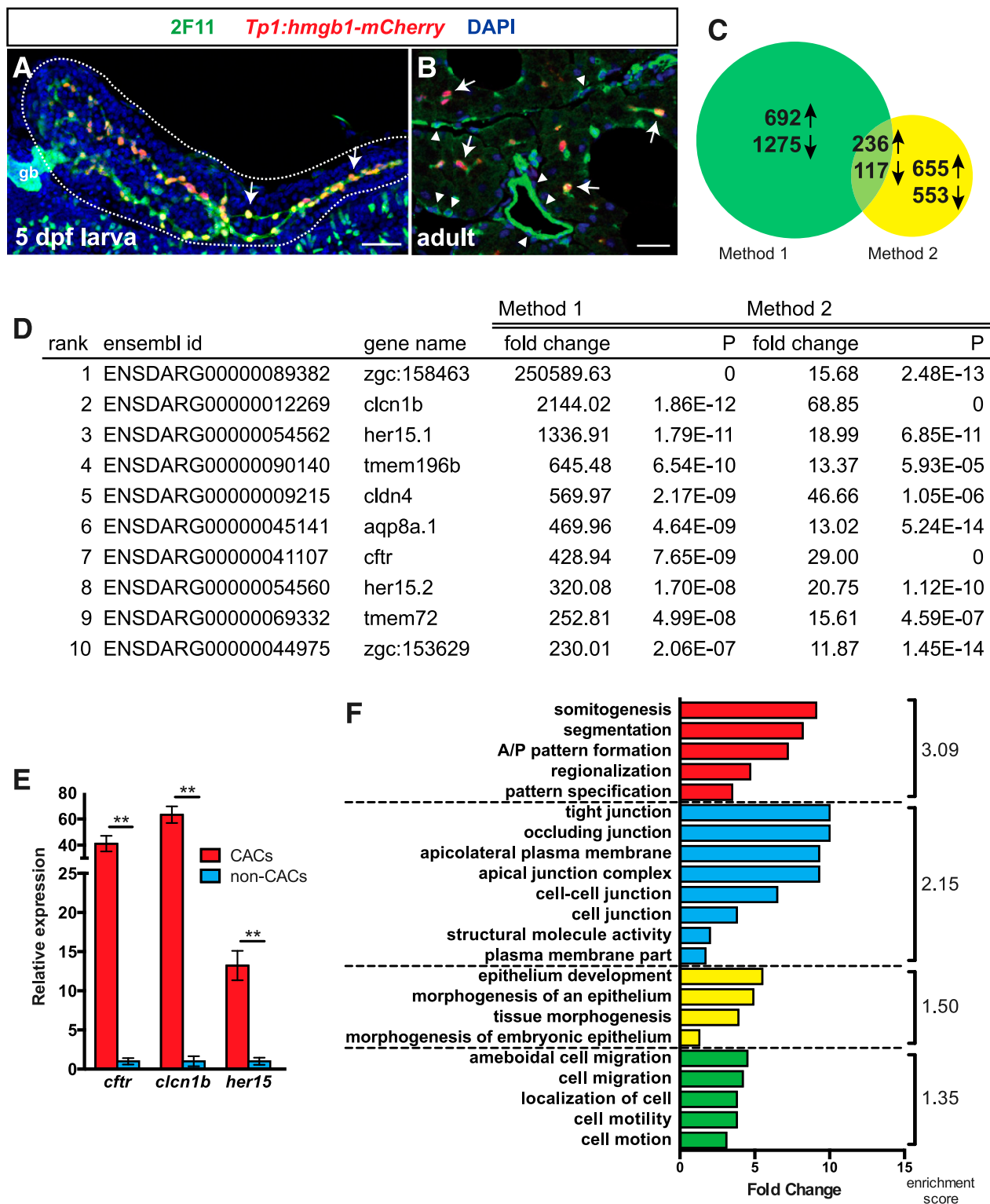
### Glucose Assays

Adult fish were fasted (24 h) and killed, and blood glucose was measured using a OneTouch Ultra (LifeScan) glucose meter (32). Larval glucose levels were determined in 5 dpf larvae using a glucose assay kit (BioVision). The *cfr*<sup>pd1049</sup> mutant larvae were genotyped by observing Kupffer vesicle in 10-somite stage embryos (33). As a positive control, *ins:nfsb-mCherry*<sup>jh4</sup> larvae were treated with 10 mmol/L MTZ (Sigma) or vehicle from 3 to 5 dpf (2).

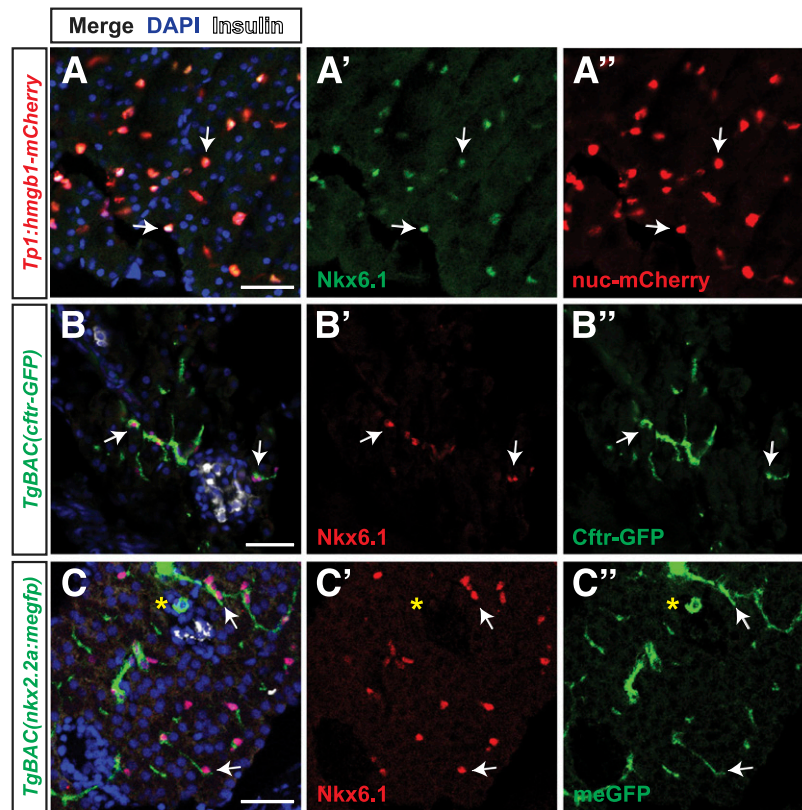
## RESULTS

### Transcriptome Analysis of CACs

During development, endocrine cells, ductal cells, and CACs originate from ductal PNCs, which are the only Notch-responsive cells in the larval pancreas (Fig. 1A)



**Figure 1**—RNA-Seq of the CAC transcriptome. **A:** PNCs (arrows) form the entire pancreatic duct (green) in 5 dpf larvae. gb, gallbladder. Scale bar = 50  $\mu$ m. **B:** In the adult pancreas, CACs (arrows) represent a subset of the ductal epithelium (green), which contains mostly non-Notch-responsive cells (arrowheads). CACs are the only epithelial pancreas cells that remain Notch responsive. Scale bar = 20  $\mu$ m. Anti-2F11 (green), *Tp1:hmgbl-mCherry* (red), DAPI (blue). **C:** Differential gene expression (fold change >1.5) between CACs and non-CACs from two separate preparations. Expression of 236 genes was enriched in CACs in both preparations. **D:** Genes most highly enriched in CACs vs. non-CACs. P, probability of a gene being equally expressed in CAC and non-CAC samples. **E:** qPCR for *cfr*, *clcn1b*, and *her15* from an independent cell preparation confirms their enrichment in CACs (\*\* $P \leq 0.005$ , *t* test). **F:** Functional annotation clustering of genes upregulated in CACs. DAVID analysis of biological process, cellular component, and molecular function gene ontology terms reveals significant enrichment (enrichment score >1.3) of four clusters of terms associated with the identified 236 CAC markers.



**Figure 2**—CAC-enriched gene expression in adult pancreas. **A**: Nkx6.1 (green) is expressed in CACs visualized using the Notch reporter *Tp1:hmgbl-mCherry* (red). **B**: Endogenous expression pattern and subcellular location of a *Cftr* in *TgBAC(cftr-GFP)* fish. *Cftr*-GFP fusion protein (green) is expressed in cell extensions of Nkx6.1-expressing cells (red). **C**: Endogenous expression pattern of *nkx2.2a* in *TgBAC(nkx2.2a:meGFP)* fish; membrane GFP (green) is expressed in Nkx6.1-expressing cells (red). Membrane GFP expression is occasionally observed in non-CACs cells within islets (yellow asterisk). CACs (arrows), scale bars = 20  $\mu$ m.

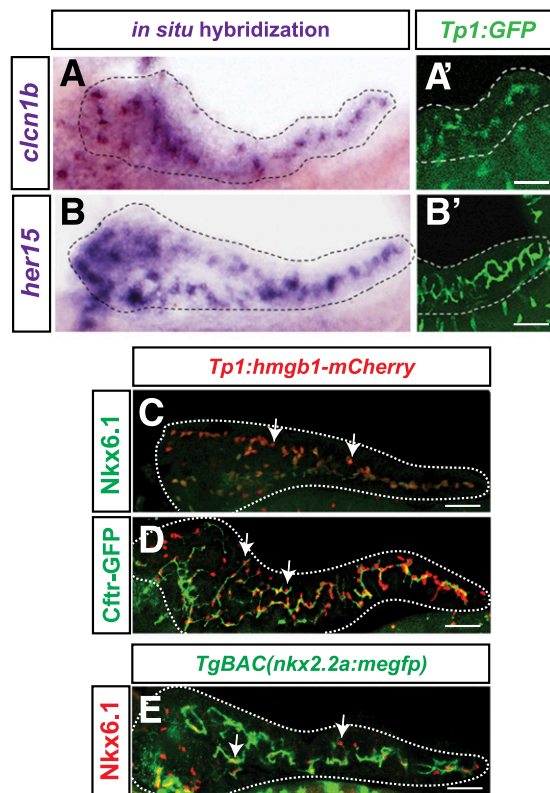
(10,12). CACs are the only PNC-derived cells that remain Notch-responsive in adults (12) (Fig. 1B); hence, we hypothesized that CACs may function as adult progenitors. To explore this possibility, we sought to characterize the CAC transcriptome using RNA-seq. To flow sort adult CACs, we dissociated pancreata from *Tp1:GFP* adult fish and used two methods to remove contaminating Notch-responsive endothelial cells: a CD105 allophycocyanin-conjugated antibody to label endothelial cells red (method 1) and a second transgene, *fli:dsRed*, which drives red fluorescent protein expression in endothelial cells (method 2) (Supplementary Fig. 1A). Using either red fluorescent blood vessel marker (bv), we collected four populations from dissociated adult pancreata: green fluorescent protein (GFP)+/bv−, GFP+/bv+, GFP−/bv+, and GFP−/bv− (Supplementary Fig. 1B). As expected, qPCR revealed high expression of the CAC marker *sox9b* in GFP+ populations and of the vascular marker *cdh5* in bv+ populations (Supplementary Fig. 1C).

Using each method, we sequenced RNA from GFP+/bv− (CAC) populations and GFP−/bv− (non-CAC) populations. Considering the intersection of both sequencing experiments, we identified 353 genes that were differentially expressed with >1.5-fold change difference in CACs versus

non-CACs, including 236 upregulated genes (Fig. 1C and D and Supplementary Table 1). As expected for an epithelial cell type, these genes included a number of ion channels (*clcn1b*, *cftr*) but also transcription factors important in regulating pancreas progenitors such as the Notch-downstream targets *her15.1/her15.2* (34). Using method 2, we generated another independent preparation of RNA and quantified expression of *cftr*, *clcn1b*, and *her15* by qPCR. These genes were significantly upregulated in CACs versus non-CACs (Fig. 1E), confirming that our catalog is enriched for CAC markers.

Using the functional annotation-clustering algorithm in DAVID (35), we investigated whether our 236 CAC markers were enriched for particular gene ontology terms. This analysis identified four biological clusters with a statistically significant enrichment score >1.3 (Fig. 1F): 1) developmental programming, 2) epithelial cell biology, 3) epithelial development, and 4) cell motility/cytoskeletal organization (Supplementary Fig. 2). This analysis confirms that CACs express a genetic program consistent with an epithelial progenitor cell population, supporting our hypothesis that CACs function as an adult progenitor cell pool.

We next investigated the expression pattern of three CAC markers in adult pancreas: Nkx6.1, *Cftr*, and Nkx2.2a.



**Figure 3**—PNCs express CAC markers. *A* and *B*: Whole mount in situ hybridization for *clcn1b* and *her15* mRNA (purple) in *Tp1:GFP* (green) pancreata at 5 dpf. *C* and *D*: *Nkx6.1* and *TgBAC(cfr-GFP)* expression (green) in whole mount *Tp1:hmgbl1-mCherry* (red nuclei) pancreata at 5 dpf. *E*: *TgBAC(nkx2.2a:meGFP)* expression in *Nkx6.1*-expressing cells in 5 dpf pancreas. Scale bars = 50  $\mu\text{m}$ .

*Nkx6.1* is important for endocrine differentiation and is a known marker of PNCs (36). Using the *Tp1:hmgbl1-mCherry* reporter line, in which all Notch-responsive cells express nuclear mCherry, we observed *Nkx6.1* expression in all CACs (Fig. 2*A*). Using *TgBAC(cfr-gfp)* fish, we observed the endogenous expression pattern of a *Cfr*-GFP fusion protein (33), which was localized to the thin cellular extensions of *Nkx6.1*-expressing cells (Fig. 2*B*). Without the advantage of using CAC or cell polarity markers, others have concluded that *Cfr* marks the apical surface of ductal cells (37). In fact, the protein is restricted to the thin extensions of CACs that cover the inner lumen of the pancreatic duct. (See also Fig. 3*D*.) Lastly, we used *TgBAC(nkx2.2a:meGFP)* [membrane GFP] fish to examine the endogenous expression pattern of *nkx2.2a*, a transcription factor important in endocrine differentiation (38). In these fish, *Nkx6.1*-expressing CACs expressed meGFP (Fig. 2*C*). Overall, our analysis establishes a new set of markers that are consistent with the predicted role of CACs as progenitor cells.

#### Larval Notch-Responsive Progenitors Express CAC Markers

Our transcriptome analysis suggested that CACs are adult progenitors, so we next hypothesized that CACs and PNCs may represent a single cell type. Thus, we used in situ

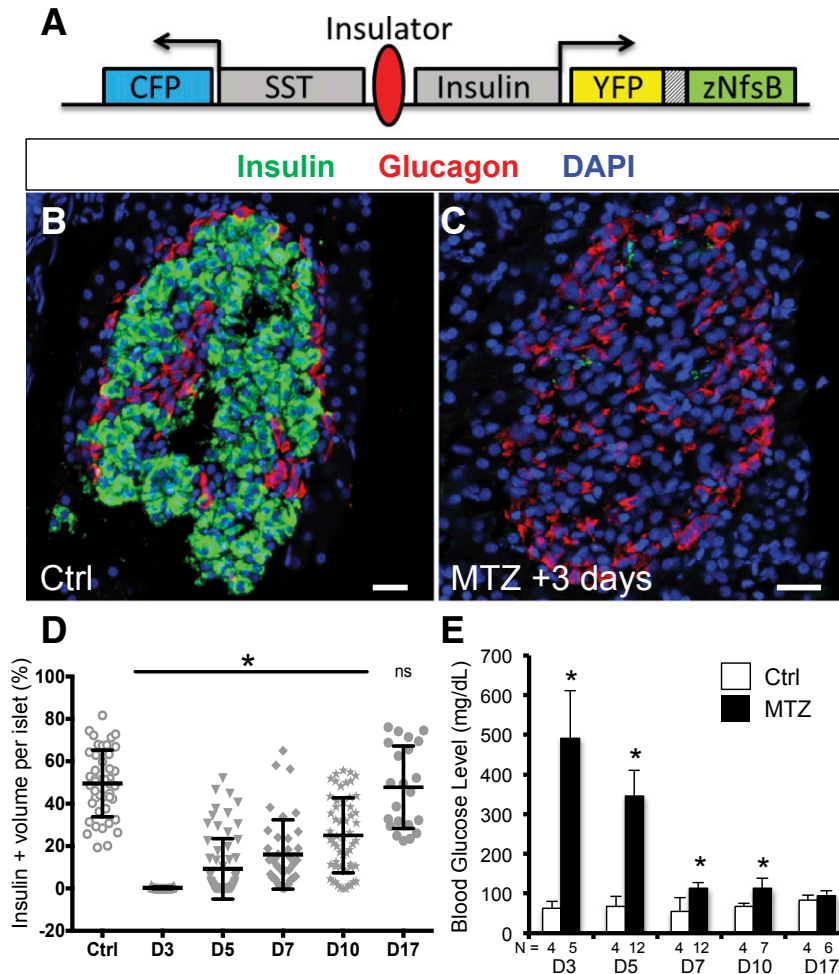
hybridization and the above-mentioned transgenic lines to examine the expression pattern of several of our new CAC markers in larval pancreata. To visualize PNCs, we used our Notch-responsive reporter lines, *Tp1:GFP* or *Tp1:hmgbl1-mCherry*. In 5 dpf fish, we observed *clcn1b*, *her15*, *Nkx6.1*, *Cfr-GFP*, and *nkx2.2a:meGFP* expression in PNCs (5 of 5 genes examined) (Fig. 3*A–E*). As PNCs are progenitors for adult CACs (12) and share marker gene expression, cell morphology, and ultrastructure with them (10), we conclude that PNCs are in fact an early population of CACs. Henceforth, we will refer to PNCs as larval CACs.

#### CACs Proliferate and Are More Proximal to Islets During $\beta$ -Cell Regeneration

Larval CACs contribute to endocrine and ductal cell populations during development (12). Thus, we next set out to discover whether adult CACs play a role in  $\beta$ -cell regeneration. To ablate  $\beta$ -cells, we used *HS4-sst2:CFP;ins:PhiYFP-m-dest1-2TA-nfsB<sup>lmc009</sup>* zebrafish (abbreviated *ins:NTR*). In these animals,  $\beta$ -cells express destabilized YFP and nitroreductase, which converts MTZ to a cytotoxin (Fig. 4*A*). Fish were initially injected with MTZ (MTZ +0 days) or vehicle and then were killed at MTZ +3, +5, +7, +10, and +17 days. We then analyzed blood glucose levels and pancreas morphology. At MTZ +3 days, we observed near total  $\beta$ -cell ablation (99.7%) and an unaffected  $\alpha$ -cell population (Fig. 4*B–D* and Supplementary Fig. 3*A* and *B*). These fish were severely hyperglycemic but returned to euglycemia after 2 weeks of recovery (Fig. 4*E*), confirming previous studies (1). By MTZ +5 to +7 days, the number of single  $\beta$ -cells and  $\beta$ -cell clusters (small groups of  $\beta$ -cells not containing  $\alpha$ -cells) initially increased and then resolved between MTZ +10 and +17 days (Supplementary Fig. 3*C* and *D*). This observation is consistent with a model of  $\beta$ -cell neogenesis, where single cells differentiate, proliferate, and form islets (25).

To visualize CACs after  $\beta$ -cell ablation, we used the Notch reporter *Tp1:GFP*. Single  $\beta$ -cells were often seen proximal to or in contact with CACs in controls (Fig. 5*A*). By measuring the distance between single  $\beta$ -cell nuclei and the nuclei of the two nearest GFP-positive cells, we determined that at MTZ +5 and +7 days, CACs were significantly closer to single  $\beta$ -cells ( $14.35 \pm 0.6 \mu\text{m}$  and  $14.74 \pm 0.7 \mu\text{m}$ , respectively) than in controls ( $22.81 \pm 1.56 \mu\text{m}$ ,  $P < 0.0001$ ) (Fig. 5*A–D*). Additionally, adjacent to CACs we occasionally saw single insulin-expressing cells that were GFP positive and lacked extensions (6 of 19 fish at MTZ +5 and +7 days) (Fig. 5*C*). These results are consistent with a cell-fate transition from CAC to single  $\beta$ -cell via a double-positive stage.

Large islets were associated with CACs in regenerating and control pancreata (Fig. 5*E*). During regeneration, the distance between large islets and CACs significantly decreased ( $10.84 \pm 0.6 \mu\text{m}$  in controls vs.  $6.27^{+/-} 0.4 \mu\text{m}$  at MTZ +17,  $P < 0.0001$ ) (Fig. 5*H*). GFP-positive cells, many of which coexpressed insulin (59.1% at MTZ +10 days, 7 of 7 fish), could even be found inside regenerating islets (Fig. 5*F*, *G*, and *I*). Together, these results are



**Figure 4**— $\beta$ -Cell ablation and regeneration. *A*: *ins:NTR* transgene (see RESEARCH DESIGN AND METHODS). SST, *somatostatin2*. *B* and *C*: Three days following treatment, control transgenic fish (*B*) compared with MTZ-treated fish (*C*). Insulin-expressing  $\beta$ -cells (green) were nearly absent from islets. Anti-insulin (green), anti-glucagon (red), DAPI (blue). Scale bars = 50  $\mu$ m. *D*: The percentage of  $\beta$ -cells per islet returns to control levels by MTZ +17 days. *E*: Blood glucose levels in MTZ- and vehicle-treated fish.  $\beta$ -Cell ablation results in hyperglycemia, which returns to control levels by MTZ +17 days. Ctrl, control; D, day; N, number of fish analyzed. \* $P < 0.05$ ; ns, not significant, *t* test compared with control.

consistent with CAC migration during regeneration—a hypothesis supported by similar observations in rodents (39).

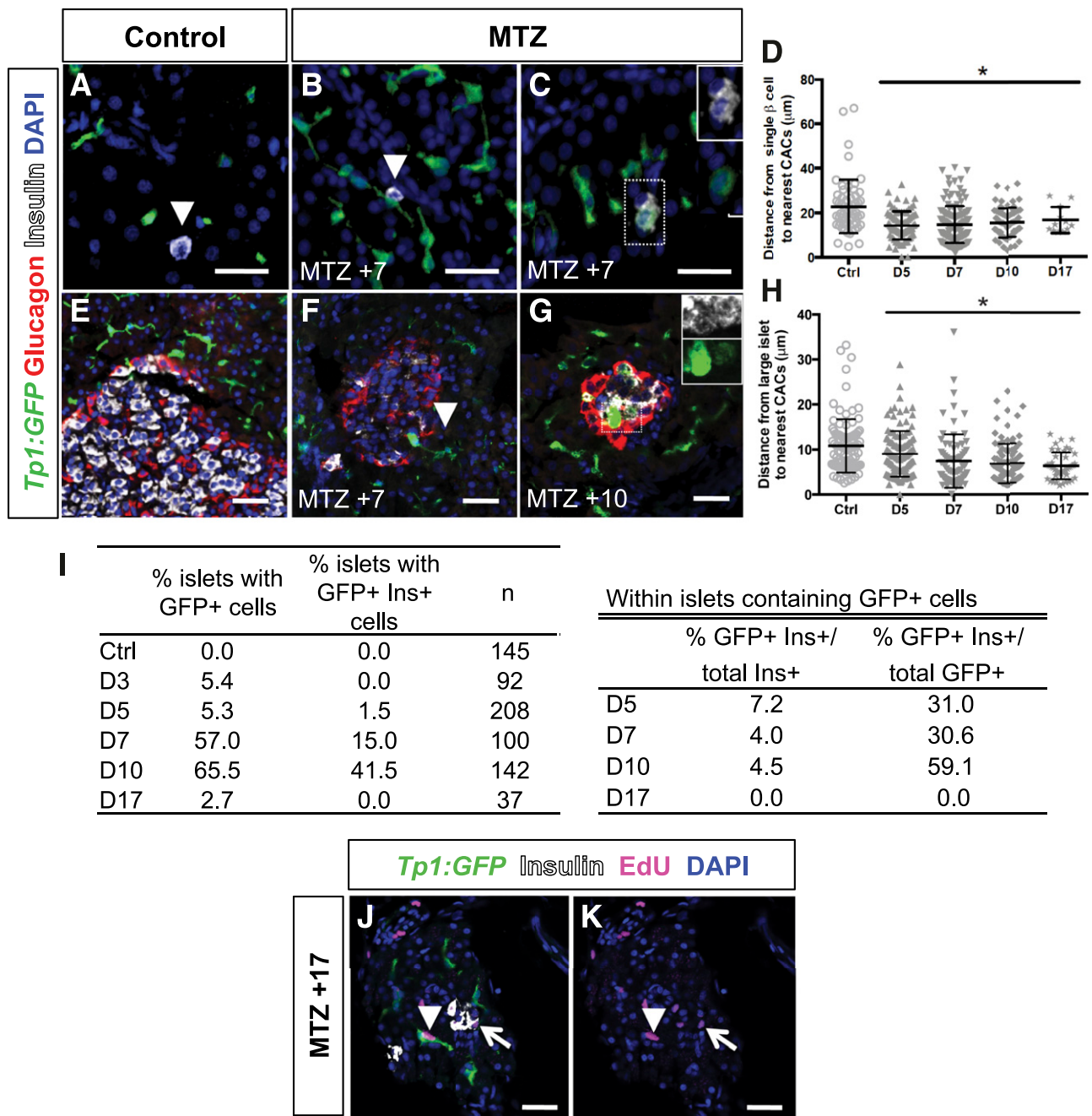
We also observed cell proliferation during  $\beta$ -cell regeneration using EdU incorporation (40). Fish were coinjected with EdU on MTZ +0 days and every other day thereafter until sacrifice. In contrast to controls, by MTZ +17 days many  $\beta$ -cells were labeled with EdU (4% vs. 50%,  $n = 4$  and 6 fish, respectively), and CACs peripheral to islets were also EdU labeled (6% vs. 30%, respectively) (Fig. 5J and K). Thus, proliferation of both  $\beta$ -cells and CACs had occurred during regeneration after cell-specific ablation. Altogether, our observations led us to hypothesize that CACs play an active role in  $\beta$ -cell regeneration.

#### CACs Directly Contribute to $\beta$ -Cell Regeneration

To directly test whether CACs are a bona fide adult progenitor population, we used our cre-based strategy to lineage trace Notch-responsive cells (12,41). This system uses two transgenes: 1) a Notch-responsive cre driver, *Tp1:CreERT2*, and 2) the cre responder, *βactin:lox-stop-*

*lox-hmgb1-mCherry*. Zebrafish carrying both transgenes are called lineage-tracing fish (LT). Addition of 4OHT to LT fish indelibly labels Notch-responsive cells with nuclear-mCherry (nuc-mCherry). To quantify the efficiency of 4OHT-dependent labeling in adults, we used LT fish that also carried the Notch-responsive reporter, *Tp1:GFP*. After injection of adult LT; *Tp1:GFP* fish with 4OHT daily for 3 days, 75% of GFP-positive cells were labeled with nuc-mCherry ( $n = 5$ ) (Fig. 6A–C).

LT; *ins:NTR* triple-transgenic zebrafish were injected daily for 3 days with 4OHT to label CACs and on the next day were injected with MTZ to induce  $\beta$ -cell ablation. Control fish received identical 4OHT injections followed by vehicle injection. In control pancreata, nuc-mCherry label was seen in CACs but never in insulin-expressing cells ( $n = 1,191$  cells from 5 fish). During early regeneration at MTZ +3 days,  $\beta$ -cells were ablated and only CACs were nuc-mCherry labeled, as in controls. However, by MTZ +7 days, in addition to CACs, cells within recovering islets were

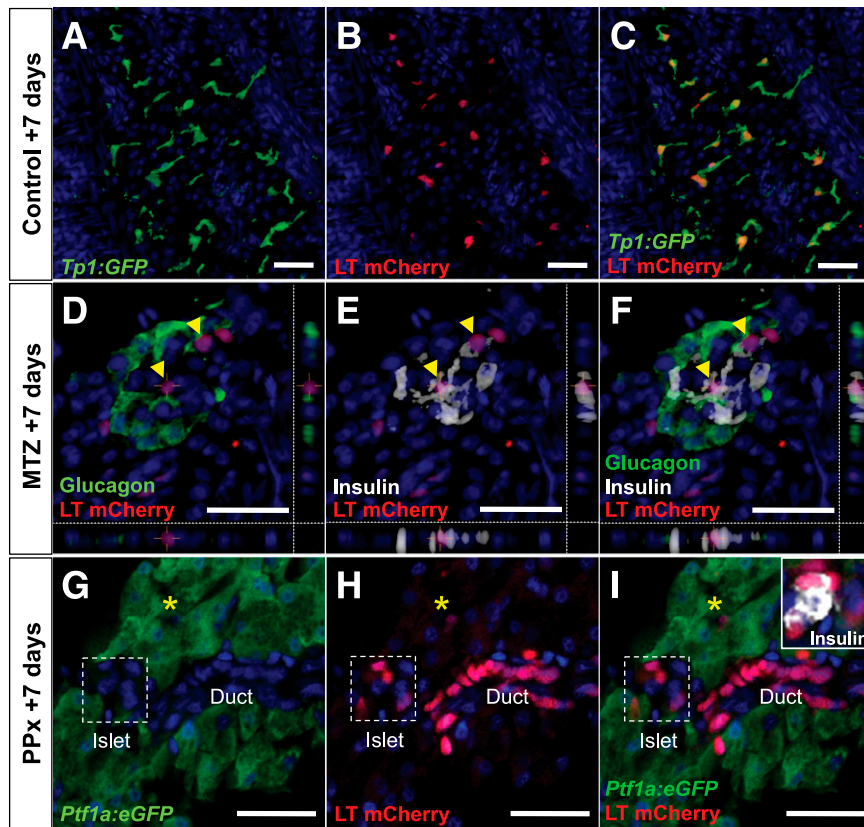


**Figure 5**—CACs respond dynamically and proliferate during β-cell regeneration. *A–C*: *ins:NTR* fish contain single β-cells (arrowheads) (*A*), which become more closely associated with CACs by MTZ +7 days (*B*). GFP/insulin double-positive single cells are seen at this time (*C*). *D*: Quantification of the distance between single β-cells and the nearest two CACs. *E*: Large islets are also associated with CACs. *F*: By MTZ +7 days, CACs (arrowhead) are also found within islets. *G*: GFP/insulin double-positive cells are observed in islets at MTZ +10 days (inset). *H*: Quantification of the distance between islets and the nearest two CACs. *I*: Quantification of the percentage of islets containing GFP+ cells and the percentage of islets containing GFP/insulin (Ins) double-positive cells. Within those islets containing GFP+ cells, quantification of GFP+ β-cells and insulin+ CACs. *J* and *K*: At MTZ +17 days, β-cells (open arrow) and CACs (arrowhead) have incorporated EdU. *Tp1:GFP* (green), anti-glucagon (red), anti-insulin (white), anti-EdU (magenta), DAPI (blue). Scale bars = 50 μm. Ctrl, control; D, day. \**P* < 0.05, *t* test compared with control.

also labeled. At MTZ +10 days, 43% of insulin-expressing cells were nuc-mCherry labeled (*n* = 320 cells from 4 fish) (Fig. 6*D–F*). Hence, we concluded that CACs are progenitors that contribute to β-cell neogenesis.

**CACs Contribute to Ductal Cells and Endocrine Cells After Partial Pancreatectomy**

We predicted that adult CACs represent a progenitor source for other pancreatic cell types. To test this capacity



**Figure 6**—CACs are progenitors of endocrine and ductal cell types. A–C: 4OHT injection labels 75% of Notch-responsive cells (green) with nuc-mCherry (red nuclei) in *LT; Tp1:GFP* fish. D–F: *LT; ins:NTR* regenerating islets at MTZ +7 days. nuc-mCherry labels insulin-expressing CAC progeny (yellow arrowheads). G–I: *LT; Ptf1a:gfp* fish at PPx +7 days. nuc-mCherry (red nuclei) labels regenerating duct, CACs (yellow asterisks), and insulin-expressing islet cells (white) but not eGFP-positive acinar cells (green). Anti-insulin (white), anti-glucagon (green in B), anti-mCherry (red), DAPI (blue). Scale bars = 25  $\mu$ m.

of CACs during regeneration, we next used PPx in *LT; Ptf1a:eGFP* double-transgenic fish, which express enhanced GFP (eGFP) in all acinar cells. We injected fish with 4OHT and EdU as previously before surgically removing the left pancreatic lobe and treating with EdU for the following 7 days. By PPx +7 days, the operated pancreata appeared indistinguishable from sham controls and 27.6% of the cells in the regenerating lobe had incorporated EdU (vs. 1% in sham controls,  $n = 5$  and 5, respectively), demonstrating that proliferation had occurred (Supplementary Fig. 4). We detected nuc-mCherry-labeled endocrine cells, ductal cells, and CACs in the regenerated lobe but did not observe nuc-mCherry label in eGFP-expressing acinar cells (Fig. 6G–I). Accordingly, we concluded that CACs represent a progenitor pool for both endocrine and ductal cells during regeneration, as do larval CACs during development (12).

#### ***cftr* Is Necessary for Pancreas Development**

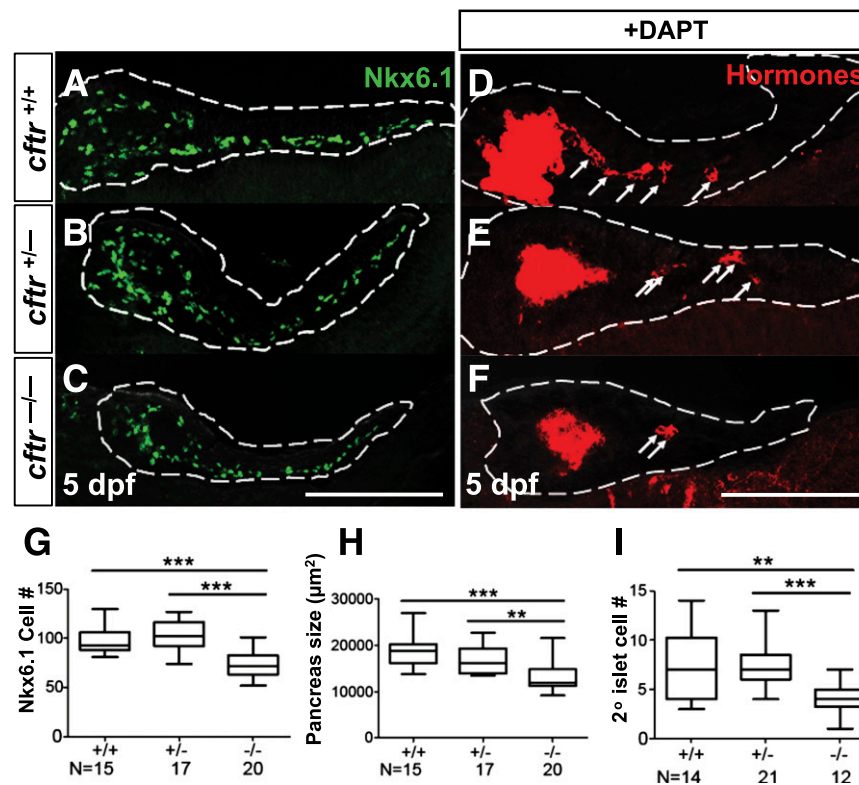
Having established that CACs are endocrine and ductal progenitors during development and regeneration, we next became interested in whether any of the CAC markers that we identified were important for pancreas development. The expression of *cftr* in CACs is of particular interest, as loss of function of this gene in humans

leads to cystic fibrosis (CF), a debilitating disorder associated in 90% of cases with pancreatic insufficiency, pancreatic ductal blockage, and CF-related diabetes (CFRD) (42). Analysis of the pancreata from *cftr* mutant larvae showed that by 5 dpf, there was a small but significant deficit in pancreas size and fewer Nkx6.1-expressing larval CACs (Fig. 7A–C, G, and H). Consequently, the potential of *cftr*<sup>−/−</sup> larvae to produce secondary islets was also significantly reduced (Fig. 7D–F and I), although this reduction did not affect glycemia (Supplementary Fig. 5). Taken together, our results suggest that *Cftr* plays a role in zebrafish CAC development and function. Ultimately, *cftr* mutant fish may serve as a critically important CFRD disease model.

#### **DISCUSSION**

Previously, we described a pancreatic Notch-responsive population that resides in the larval ducts. These progenitor cells differentiate and give rise to at least three kinds of cells in the adult: endocrine cells, ductal cells, and CACs. Two pieces of data suggested that CACs may also be progenitors: 1) as shown previously by us and others, CACs closely resemble their larval precursors in terms of





**Figure 7**—Cfr plays an important role in the pancreas. *A–F*: Five dpf *cfr*<sup>-/-</sup> pancreata have fewer CACs (anti-Nkx6.1, green) (*A–C*), and secondary islet cells induced by DAPT treatment (anti-insulin plus anti-glucagon plus anti-somatostatin, arrows, red) (*D–F*) compared with *cfr*<sup>+/-</sup> and wild-type pancreata. Scale bar = 100 µm. *G–I*: Quantification of the number (#) of CACs (*G*), surface area (*H*), and number of induced secondary islet cells (*I*) in wild-type, *cfr*<sup>+/-</sup>, and *cfr*<sup>-/-</sup> 5 dpf pancreata. N, number of pancreata quantified. \*\**P* < 0.05. \*\*\**P* < 0.001, *t* test.

morphology and ultrastructure (10,13,39), and 2) as reported here, our newly discovered markers for adult CACs are also expressed in the larval progenitors. As we are interested in the cellular origins of  $\beta$ -cell regeneration, we decided that CACs were a promising candidate and set out to see whether these cells were involved in pancreas recovery after tissue damage.

Using a Notch-responsive reporter, we observed CACs physically responding to the regeneration process, even appearing to infiltrate damaged islets. However, the formal possibility existed that non-CAC cells of unknown origin became Notch responsive during the regenerative response. Therefore, we used genetic lineage tracing of Notch-responsive cells in our *LT* fish to label CACs and their progeny before tissue damage. The presence of labeled cells near the periphery of islets suggests that lineage-traced CACs migrate from their centroacinar position toward damaged islets. Interestingly, our functional annotation analysis revealed that the CAC transcriptome is significantly enriched for genes important for cell migration in other contexts (e.g., *cxcl12*, *npr2a*). Investigation into the role these genes play during  $\beta$ -cell regeneration may reveal the mechanism behind this remarkable movement.

Clearly,  $\beta$ -cells can regenerate via neogenesis from CACs in the adult zebrafish pancreas in a manner analogous to endocrine formation during development.

However, the precise extent to which CACs contribute to  $\beta$ -cell neogenesis cannot be reliably quantified using our methods. For reasons discussed below, our data (and those of other lineage tracing experiments) should not be over-interpreted. Additionally, we appreciate that other cell types also contribute to pancreas regeneration, such as  $\alpha$ - and  $\delta$ -cells that have been shown to transdifferentiate in murine and zebrafish models of  $\beta$ -cell loss (43–45).

Hepatocytes in the liver of zebrafish can regenerate from biliary epithelial cells (BECs) in larvae and adults (41). Like CACs, BECs are Notch-responsive ductal cells. Hepatocyte regeneration is dependent on the function of the transcription factor *Sox9b*, which is upregulated as biliary epithelial cells dedifferentiate (41). *Sox9b* function is also required for the differentiation of pancreas progenitors toward an endocrine fate (31). In contrast to BECs, we observe that CACs maintain developmental programs, as evidenced by their continued expression of *sox9b* and *nkx6.1*. Indeed, the master regulators of  $\beta$ -cell differentiation *nkx6.1* and *nkx2.2a* are expressed in fish CACs but not in mammalian CACs (34,38). This difference may underlie the enhanced ability of the zebrafish pancreas to regenerate after  $\beta$ -cell loss, as maintenance of developmental programs in fish CACs may allow them to be continually available for differentiation once the need for new  $\beta$ -cells arises.

The notion that CACs and/or ductal cell types contribute to adult  $\beta$ -cell neogenesis in mammals has been the focus of intense scrutiny. A number of studies have used various loci to lineage trace CACs/ductal cells during murine pancreas development and regeneration including *CAII* (20), *Hnf1 $\beta$*  (21), *Muc1* (22), *Hes1* (23), and *Sox9* (24). While all of the labeled cell populations contributed to the endocrine compartment during embryogenesis, most of these studies were unable to demonstrate that adult pancreatic ductal cells contribute to  $\beta$ -cell neogenesis after either pancreatic ductal ligation or  $\beta$ -cell-specific ablation in adults (21–24). However, the results of these studies come with several caveats. If genes important in maintaining progenitor status (thus blocking differentiation) are used to drive lineage tracing, the highest creER activity will likely occur in cells that are the least expected to differentiate. As such, unless 100% of a particular cell population can be reliably labeled, one cannot definitively rule out the contribution of ductal cells to  $\beta$ -cell regeneration in mammals. Therefore, the contribution of zebrafish CACs to  $\beta$ -cell regeneration presents an intriguing and potentially critical model of  $\beta$ -cell neogenesis from endogenous progenitors.

Despite conflicting evidence for CAC contribution to mammalian pancreas regeneration, injury does seem to reactivate developmental pathways in the adult pancreas. After pancreatic ductal ligation, Sox9-expressing ductal cells give rise to a few cells expressing Ngn3 (24), a gene that encodes a master regulator of embryonic endocrine differentiation. In another study, lineage tracing of Ngn3-expressing cells demonstrated that some of these cells do indeed complete differentiation and become  $\beta$ -cells (46). Although this latter result clearly demonstrates that neogenesis can occur in the mammalian pancreas, this form of regeneration is very limited and dependent on the severity of the injury. Interestingly, isolated aldehyde dehydrogenase isoform 1 (ALDH1)+ murine CACs/terminal ductal cells can produce endocrine cells in both pancreatosphere and dorsal bud explant cultures (47). However, whether these ALDH1+ cells are in fact CACs is unclear, as they do not highly express Hes1, a downstream target of canonical Notch signaling and a hallmark of CACs (10,15,16). In the fish, Aldh1-expressing cells have recently been described in association with, but separate from, larval and juvenile CACs (48). Whether CACs represent a genetically uniform or heterogeneous population in either fish or mammals remains to be determined. Regardless, all together these observations suggest that it may be possible to manipulate developmental pathways in the adult in order to improve the regenerative capacity of the mammalian pancreas.

Knowing that CACs are adult progenitors in the pancreas will facilitate future studies into other human diseases. As an example, we decided to study the CF transmembrane conductance regulator (*cftr*), transcripts for which are highly enriched in zebrafish CACs. Abnormal CFTR channel function causes CF, a debilitating disease often associated with CFRD. CFRD is characterized by diminished  $\beta$ -cell mass and insulin dependence (42).

Our examination of *cftr* mutant larvae (5 dpf) revealed that these fish had a small but significant reduction in pancreas size. Such early pancreatic phenotypic consequences of *Cftr* loss of function were not observed by Navis and Bagnat (37). There are numerous reasons for this apparent discrepancy between studies: 1) the well-documented occurrence of phenotypic variation in CF due to modifiers in the genetic background (49), 2) confocal microscopy on microdissected pancreata providing better resolution, and 3) potential differences in animal husbandry, which is known to affect phenotype in other CF animal models (50). Using our Notch-responsive reporters and microdissection to facilitate careful observation, we detect fewer CAC progenitors in *cftr* mutant larvae. Possibly due to having fewer progenitors, these fish develop fewer secondary islets. As patients with CFRD have a diminished  $\beta$ -cell mass, these observations in mutant fish are intriguing and warrant further investigation.

In summary, regeneration occurs readily in the adult zebrafish pancreas, and we believe that studies with this model organism will continue to shed light on mechanisms that can be exploited to improve regeneration in diabetic patients. Furthermore, our new insights into the importance of *cftr* in pancreas development suggest that the role of CFTR in the human pancreas should be reevaluated.

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## References

1. Moss JB, Koustubhan P, Greenman M, Parsons MJ, Walter I, Moss LG. Regeneration of the pancreas in adult zebrafish. *Diabetes* 2009;58:1844–1851
2. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. *Mech Dev* 2007;124:218–229
3. Poss KD. Advances in understanding tissue regenerative capacity and mechanisms in animals. *Nat Rev Genet* 2010;11:710–722
4. Faustman DL, Tran SD, Kodama S, et al. Comment on papers by Chong et al., Nishio et al., and Suri et al. on diabetes reversal in NOD mice. *Science* 2006;314:1243
5. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia* 2005;48:2221–2228

6. Suri A, Calderon B, Esparza TJ, Frederick K, Bittner P, Unanue ER. Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. *Science* 2006;311:1778–1780
7. Bonner-Weir S, Li WC, Ouziel-Yahalom L, Guo L, Weir GC, Sharma A. Beta-cell growth and regeneration: replication is only part of the story. *Diabetes* 2010;59:2340–2348
8. Kopp JL, Dubois CL, Hao E, Thorel F, Herrera PL, Sander M. Progenitor cell domains in the developing and adult pancreas. *Cell Cycle* 2011;10:1921–1927
9. Xiao X, Chen Z, Shiota C, et al. No evidence for  $\beta$  cell neogenesis in murine adult pancreas. *J Clin Invest* 2013;123:2207–2217
10. Parsons MJ, Pisharath H, Yusuff S, et al. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. *Mech Dev* 2009;126:898–912
11. Chen S, Li C, Yuan G, Xie F. Anatomical and histological observation on the pancreas in adult zebrafish. *Pancreas* 2007;34:120–125
12. Wang Y, Rovira M, Yusuff S, Parsons MJ. Genetic inducible fate mapping in larval zebrafish reveals origins of adult insulin-producing  $\beta$ -cells. *Development* 2011;138:609–617
13. Leeson TS, Leeson R. Close association of centroacinar/ductular and insular cells in the rat pancreas. *Histol Histopathol* 1986;1:33–42
14. Ekholm R, Zelder T, Edlund Y. The ultrastructural organization of the rat exocrine pancreas. II. Centroacinar cells, intercalary and intralobular ducts. *J Ultrastruct Res* 1962;7:73–83
15. Miyamoto Y, Maitra A, Ghosh B, et al. Notch mediates TGF  $\alpha$ -induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* 2003;3:565–576
16. Stanger BZ, Stiles B, Lauwers GY, et al. Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell* 2005;8:185–195
17. Gasslander T, Ihse I, Smeds S. The importance of the centroacinar region in cerulein-induced mouse pancreatic growth. *Scand J Gastroenterol* 1992;27:564–570
18. Hayashi K, Takahashi T, Kakita A, Yamashina S. Regional differences in the cellular proliferation activity of the regenerating rat pancreas after partial pancreatectomy. *Arch Histol Cytol* 1999;62:337–346
19. Seymour PA, Freude KK, Tran MN, et al. SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A* 2007;104:1865–1870
20. Inada A, Nienaber C, Katsuta H, et al. Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proc Natl Acad Sci U S A* 2008;105:19915–19919
21. Solar M, Cardalda C, Houbracken I, et al. Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. *Dev Cell* 2009;17:849–860
22. Kopinke D, Murtaugh LC. Exocrine-to-endocrine differentiation is detectable only prior to birth in the uninjured mouse pancreas. *BMC Dev Biol* 2010;10:38
23. Kopinke D, Brailsford M, Shea JE, Leavitt R, Scaife CL, Murtaugh LC. Lineage tracing reveals the dynamic contribution of Hes1+ cells to the developing and adult pancreas. *Development* 2011;138:431–441
24. Kopp JL, Dubois CL, Schaffer AE, et al. Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. *Development* 2011;138:653–665
25. Nagasao J, Yoshioka K, Amasaki H, et al. Morphological changes in the rat endocrine pancreas within 12 h of intravenous streptozotocin administration. *Anat Histol Embryol* 2005;34:42–47
26. Hayashi KY, Tamaki H, Handa K, Takahashi T, Kakita A, Yamashina S. Differentiation and proliferation of endocrine cells in the regenerating rat pancreas after 90% pancreatectomy. *Arch Histol Cytol* 2003;66:163–174
27. Gasslander T, Smeds S, Blomqvist L, Ihse I. Proliferative response of different exocrine pancreatic cell types to hormonal stimuli. I. Effects of long-term cerulein administration. *Scand J Gastroenterol* 1990;25:1103–1110
28. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 2011;12:323
29. Leng N, Dawson JA, Thomson JA, et al. EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics* 2013;29:1035–1043
30. Westerfield M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR, University of Oregon Press, 2000
31. Manfroid I, Ghaye A, Naye F, et al. Zebrafish sox9b is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration. *Dev Biol* 2012;366:268–278
32. Eames SC, Philipson LH, Prince VE, Kinkel MD. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. *Zebrafish* 2010;7:205–213
33. Navis A, Marjoram L, Bagnat M. Cftr controls lumen expansion and function of Kupffer's vesicle in zebrafish. *Development* 2013;140:1703–1712
34. Schaffer AE, Taylor BL, Benthuyzen JR, et al. Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. *PLoS Genet* 2013;9:e1003274
35. Huang DW, Sherman BT, Tan Q, et al. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol* 2007;8:R183
36. Huang W, Wang G, Delaspre F, Vitery MdeIC, Beer RL, Parsons MJ. Retinoic acid plays an evolutionarily conserved and biphasic role in pancreas development. *Dev Biol* 2014;394:83–93
37. Navis A, Bagnat M. Loss of cftr function leads to pancreatic destruction in larval zebrafish. *Dev Biol* 2015;399:237–248
38. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, et al. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* 1998;125:2213–2221
39. Pour PM. Pancreatic centroacinar cells. The regulator of both exocrine and endocrine function. *Int J Pancreatol* 1994;15:51–64
40. Chehrehasa F, Meedeniya AC, Dwyer P, Abrahamsen G, Mackay-Sim A. EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J Neurosci Methods* 2009;177:122–130
41. He J, Lu H, Zou Q, Luo L. Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. *Gastroenterology* 2014;146:789–800.e8
42. Hameed S, Jaffé A, Verge CF. Cystic fibrosis related diabetes (CFRD)—the end stage of progressive insulin deficiency. *Pediatr Pulmonol* 2011;46:747–760
43. Thorel F, Népote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature* 2010;464:1149–1154
44. Chera S, Baronnier D, Ghila L, et al. Diabetes recovery by age-dependent conversion of pancreatic  $\delta$ -cells into insulin producers. *Nature* 2014;514:503–507
45. Ye L, Robertson MA, Hesselton D, Stainier DY, Anderson RM. Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. *Development* 2015;142:1407–1417
46. Van de Casteele M, Leuckx G, Cai Y, et al. Partial duct ligation:  $\beta$ -cell proliferation and beyond. *Diabetes* 2014;63:2567–2577
47. Rovira M, Scott SG, Liss AS, Jensen J, Thayer SP, Leach SD. Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. *Proc Natl Acad Sci U S A* 2010;107:75–80
48. Matsuda H, Parsons MJ, Leach SD. Aldh1-expressing endocrine progenitor cells regulate secondary islet formation in larval zebrafish pancreas. *PLoS One* 2013;8:e74350
49. Blackman SM, Commander CW, Watson C, et al. Genetic modifiers of cystic fibrosis-related diabetes. *Diabetes* 2013;62:3627–3635
50. Wilke M, Buijs-Offerman RM, Aarbiou J, et al. Mouse models of cystic fibrosis: phenotypic analysis and research applications. *J Cyst Fibros* 2011;10(Suppl. 2):S152–S171