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Suppression of Ptf1a activity induces acinar to endocrine conversion

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Summary

Pluripotent embryonic cells become progressively lineage-restricted during development in a process that culminates in the differentiation of stable organ specific cell types that perform specialized functions. Terminally-differentiated pancreatic acinar cells do not have the innate capacity to contribute to the endocrine β-cell lineage, which is destroyed in individuals with autoimmune diabetes [1]. Some cell types can be reprogrammed using a single factor [2, 3], whereas other cell types require continuous activity of transcriptional regulators to repress alternate cell fates [4–6]. Thus, we hypothesized that a transcriptional network continuously maintains the pancreatic acinar cell fate. We found that post-embryonic antagonism of Ptf1a, a master regulator of pancreatic development [7] and acinar cell fate specification [8, 9], induced the expression of endocrine genes including *insulin* in the exocrine compartment. Using a genetic lineage tracing approach, we show that the induced *insulin*+ cells are derived from acinar cells. Cellular reprogramming occurred under homeostatic conditions, suggesting that the pancreatic micro-environment is sufficient to promote endocrine differentiation. Thus, severe experimental manipulations [10, 11] may not be required to potentiate pancreatic transdifferentiation. These data indicate that targeted post-embryonic disruption of the acinar cell fate can restore the developmental plasticity that is lost during development.

Keywords

pancreas; acinar; beta cells; endocrine; zebrafish; diabetes; lineage tracing; ptf1a

Results and Discussion

The pancreas is derived from a dorsal and a ventral bud of endodermal tissue, which together generate endocrine and exocrine cell types [12]. In zebrafish, the acinar cells are exclusively derived from the ventral pancreatic bud [13]. To analyze the temporal differentiation of acinar cells, we examined pancreatic markers at multiple time-points in *Tg(ptf1a:*eGFP) [14] zebrafish. The *Tg(ptf1a:eGFP*) BAC reporter marks the ventral pancreatic bud by 32 hpf [8, 15]. At 36 hpf, *Tg(ptf1a:*eGFP*)*-expressing cells formed a homogeneous field, and co-expressed the homeodomain transcription factors Prox1 and Nkx6.1 (Fig. 1A–B′; [8]). Co-expression of these pancreatic progenitor markers in the *ptf1a* expression domain suggests that the pancreatic primordium remains multi-potent at 36 hpf.

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By 60 hpf, Nkx6.1 and *Tg(ptf1a:*eGFP) were expressed in mutually exclusive domains (Fig. 1C,C′), and Prox1 was no longer detected in the pancreas (Fig. 1D,D′). However, *Tg(ptf1a:*eGFP) expressing cells were not fully differentiated by 60 hpf since they did not co-express the acinar cell marker Elastase (Fig. 1E,E′). Zebrafish embryonic development (0–72 hpf) is fueled by the absorption of the maternally deposited yolk. After the embryonic to larval transition at 72 hpf, a functional digestive system becomes necessary to process external nutrient sources [16]. Consistent with this requirement, Elastase was cytoplasmically localized (Fig. 1F,F′) and packaged into secretory granules on the luminal surface of *Tg(ptf1a:*eGFP) cells (Fig. 1F′ inset) by 84 hpf. Our results are consistent with ultrastructural studies that have shown that acinar cell cytodifferentiation after 72 hpf coincides with the expression of acinar cell markers [17]. Since Ptf1a expression becomes restricted to acinar cells in the developing mouse pancreas [18], we predicted that co-expression of *Tg(ptf1a:*eGFP) and Elastase would coincide with cell fate commitment of the pancreatic progenitor pool. Consistent with this prediction, *Tg(ptf1a:*eGFP) expression was excluded from the Nkx6.1 positive intra-pancreatic ducts (Fig. 1G,G′), and the Islet-1 positive endocrine compartment (Fig. 1H,H′) at 84 hpf. We conclude that the multi-potent pancreatic primordium gives rise to terminally differentiated exocrine and endocrine cell types by 84 hpf.

During embryogenesis (0–72 hpf), the level of Ptf1a activity influences the fate of pancreatic cells; high levels of Ptf1a activity promote exocrine cell fates, while low levels of Ptf1a activity are compatible with endocrine differentiation [8]. Ptf1a activity requires assembly of the tripartite PTF1 complex, which consists of Ptf1a, an E protein and RBPJ(L), and which directly activates exocrine target gene expression [19]. In addition, the PTF1 complex autoregulates the *Ptf1a* promoter resulting in sustained expression of high levels of Ptf1a in acinar cells [20]. To further address the function of PTF1, we generated an Engrailed-Ptf1a fusion protein, in which we fused the Engrailed transcriptional repressor domain to the amino-terminus of the full length Ptf1a protein. The Engrailed repressor domain recruits chromatin-modifying complexes to repress target gene transcription [21]. To explore the regulation of PTF1 targets, we generated a stable cell line that expresses Luciferase under the control of a tandem array of PTF1 binding sites in human embryonic kidney 293 cells, which require exogenous Ptf1a to form a PTF1 complex [22] (Fig. 2A). We found that Engrailed -Ptf1a repressed the transcriptional activity of wild-type Ptf1a in a dose dependent manner (Fig. 2B). Our results suggest that overexpression of Engrailed-Ptf1a antagonizes transcription initiation at Ptf1a binding sites, and perm its only low level transcription of PTF1 target genes.

To test whether PTF1 activity is required to maintain acinar cell fate *in vivo*, we employed the Heat-Inducible overexpression of transgenes in a cre restricted domain (HOTcre) system [15] to express the Engrailed-Ptf1a fusion protein specifically in acinar tissue (Fig. 3A). We placed the Cre recombinase under the control of the *elastase3l* promoter to provide spatial control over gene expression. We used the *hsp70l* promoter to control the temporal expression of the Engrailed-Ptf1a fusion protein in cells that had undergone a Cre mediated excision event. For simplicity, we will refer to this double transgenic line as *elastase:cre-Ptf1aEN* (Fig. 3A).

To determine whether *Tg(ela3l:cre)* specifically marked differentiated acinar cells, we heat-shocked *Tg(ela3l:cre); Tg(hsp70l:loxP:mCherrySTOP:loxP:H2B-GFP)* double transgenic embryos, which induced H2B-GFP expression in cells that had undergone Cre mediated recombination. When the double transgenic embryos were heat-shocked at 60 hpf, a time-point before the onset of *elastase* expression (Fig. 1E′), H2B-GFP expression was not detected in the pancreas at 84 hpf (Fig. 3B,B′). However, when the double transgenics were heat-shocked during larval development at 4.5 dpf, at least 24 h after the onset of Elastase

expression (Fig. 1F′), broad expression of H2B-GFP was observed specifically in the pancreatic domain at 5.5 dpf (Fig. 3C,C′). Heat-shock induced expression of *Tg(hsp70l:loxP:*m Cherry*STOP:loxP:H2B-GFP)* was observed throughout the exocrine compartment in *Tg(ela3l:cre)* negative controls (Fig. S1). The cells that expressed H2B-GFP after heat-induction at 4.5 dpf co-expressed Elastase (Fig. 3D,D′, arrow, n=10 animals) but did not co-express the *β*-cell marker Insulin (Fig. 3E,E′, n=10 animals) at 5.5 dpf. We conclude that *Tg(ela3l:cre)* specifically marks differentiated acinar cells.

Next, we asked whether over-expression of Engrailed-Ptf1a impacted the gene expression profile of differentiated acinar cells. We crossed *elastase:cre-Ptf1aEN* to reporter fish for both the acinar cell marker *Tg(ela3l:*eGFP) [23] and the *β*-cell marker *Tg(insulin:*d sRED*)* [24]. Heat-shock induction of *elastase:cre-Ptf1aEN* at 4.5 dpf resulted in mosaic expression of *Tg(ela3l:*eGFP*)* at 6.5 dpf (Fig. 3F′ inset). Because the half-life of eGFP in cells is ~24h [25], *Tg(ela3l:*eGFP*)* expression was likely down-regulated shortly after *elastase:cre-Ptf1aEN* induction. This observation is consistent with the dominant-negative activity exhibited by Engrailed-Ptf1a *in vitro* (Fig. 2B). In contrast, *Tg(ela3l:*eGFP*)* expression was uniform in *Tg(ela3l:cre)* negative controls (Fig. 3G). Strikingly, a subset of cells (3–10 per animal, n=20) that exhibited low levels of *Tg(ela3l:*eGFP*)* expression also expressed *Tg(insulin:*dsRED*)* (Fig. 3F, inset); the origin of these insulin+ cells will be discussed below. *Tg(insulin:*d sRED*)* expression was not observed in cells that expressed high levels of *Tg(ela3l:*eGFP*)* (Fig. 3F) or in *Tg(ela3l:cre)* negative controls (Fig. 3G′). To determine whether acinar cells might have acquired a pluripotent endocrine progenitor fate prior to activating *Tg(insulin:*dsRED*)*, we analyzed the expression of the *Tg(pax6b:*eGFP*)* pan-endocrine progenitor reporter [26] one day after *elastase:cre-Ptf1aEN* heat-shock induction (Fig. 3H–I′). *elastase:cre-Ptf1aEN* larvae exhibited broad low-level expression of *Tg(pax6b:*eGFP*)* throughout the pancreas (com pare Fig. 3H′, I′). In addition, scattered cells outside of the islet expressed high levels of *Tg(pax6b:*eGFP*)* (Fig, 3H,H′ arrow s). In contrast, *Tg(pax6b:*eGFP*)* expression was only observed in the extra-pancreatic duct and principal islet in *Tg(ela3l:cre)* negative controls (Fig. 3I–I′, arrow heads). To further investigate the fate of cells that expressed *Tg(pax6b:*eGFP*)*, we analyzed *glucagon* expression using *Tg(gcga:*GFP) [27], a marker of α-cell fate, after *elastase:cre-Ptf1aEN* induction. As with the expression of *Tg(insulin:*d sRED*)* (Fig. 3F), we observed a small number of *glucagon*+ cells in the exocrine compartment of animals that expressed *elastase:cre-Ptf1aEN* (Fig. 3J,J′; 4–12 cells per animal, n=20), and we did not observe *glucagon*+ cells outside of the principal islet in *Tg(ela3l:cre)* negative controls (Fig. 3K,K′). Thus, *elastase:cre-Ptf1aEN* expression altered the gene expression profile of many exocrine cells but only a subset of these cells initiated an endocrine gene expression program.

There are at least two possible origins for the ectopic *insulin*+ cells (Fig. 3F). A form al possibility is that *elastase:cre-Ptf1aEN* acted cell non-autonomously to induce partial differentiation towards both acinar and endocrine cell fates in a small population of pancreatic progenitors. A second possibility is that acinar cells down-regulated *Tg(ela3l:*eGFP) and up-regulated *Tg(insulin:*d sRED) expression. To distinguish between these possibilities, we developed a genetic lineage tracing approach to mark acinar cells that activated *insulin* expression. We generated animals that expressed *Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP)* to specifically label with H2B-GFP *insulin* + cells that derived from a lineage that expressed *Tg(ela3l:*cre*)* (Fig. 4A). *insulin*+ cells derived from lineages that did not express *Tg(ela3l:*cre*)* will exhibit mCherry expression. In *Tg(ela3l:cre); Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP)* animals, *β*-cells in the principal islet exhibited mCherry expression at 6.5 dpf (Fig. 4B,B″), whereas H2B-GFP expression was not observed (Fig. 4B,B′, n>20 animals). Therefore, in wild-type animals, *β*-cells did not derive from acinar cells. However, after *elastase:cre-Ptf1aEN* induction at 4.5 dpf, H2B-GFP expression was detected in cells scattered throughout the exocrine

compartment of the pancreas (4–10 cells per animal, n=20, Fig. 4C–C″, arrow s). H2B-GFP expression was not observed in *Ptf1aEN* negative controls (Fig. 4D–D″, n>20 animals). We conclude that *elastase:cre-Ptf1aEN* can cell-autonomously induce the conversion of acinar cells into an *insulin*+ cell type.

To determine whether acinar cell maturation affects lineage plasticity, we repeated the lineage tracing experiments in older animals. A less intense heat-shock induction protocol was used because older animals were more heat - sensitive (see Experimental Procedures). Following *elastase:cre-Ptf1aEN* induction at 14 dpf, H2B-GFP was detected in the exocrine compartment in most animals two days later (0–2 cells per animal, n=10, Fig. 4E–E″, arrow), whereas H2B-GFP expression was not observed in *Ptf1aEN* negative controls (data nor show n). By 21 dpf, acinar cells appeared to be resistant to Ptf1aEN mediated cell type conversion (data not shown). Thus, higher levels of Ptf1aEN might be required after 14 dpf to disrupt acinar cell fate or maturing acinar cells might activate additional maintenance mechanisms.

It has been shown that inflammation in response to adenoviral packaging proteins is essential for cellular reprogramming of liver cells to pancreatic endocrine fates [28]. It is unclear whether inflammation is broadly required for *in vivo* reprogramming of other cell types. By the onset of larval development (72 hpf), the zebrafish innate immune system can infiltrate tissues in response to injury and infection [29]. We predicted that genetically encoded *elastase:cre-Ptf1aEN* expression did not initiate an immune response. However, it is possible that antagonism of normal acinar cell function or the endogenous heat-shock response could result in pancreatic injury and inflammation due to the inappropriate release of digestive enzymes. To address this possibility, we induced *elastase:cre-Ptf1aEN* expression in a *Tg(mpx:GFP)* background, which marks granulocytes during larval development [30]. In response to physical injury, zebrafish granulocytes immediately exhibit chemotaxis towards the wound and accumulate within the target tissue by 2 h post injury [31]. We analyzed pancreatic granulocytes at 2 h (Fig. S2A,B) and 24 h post heat-shock (data not shown) to determine whether heat-shock treatment or *elastase:cre-Ptf1aEN* induction triggered an inflammatory response. The number of pancreatic granulocytes was unchanged at both time-points in *elastase:cre-Ptf1aEN* animals compared to *Tg(ela3l:cre)* negative controls (Fig. S2C) suggesting that Engrailed-Ptf1a induced cell fate conversion does not involve an inflammatory response.

In conclusion, we have identified a novel mechanism to induce conversion of acinar cells to endocrine fates under homeostatic conditions. Our data support a model in which cues from the pancreatic micro-environment can reprogram acinar cells with low levels of Ptf1a activity into hormone expressing cells. Thus, if targeted knockdown of Ptf1a phenocopies Ptf1a antagonism, emerging methods for tissue-specific delivery of siRNAs [32, 33] could potentiate β-cell regeneration therapy.

Experimental Procedures

Zebrafish strains and lines

Zebrafish were raised under standard laboratory conditions at 28°C. Heat-shocks before 5 dpf were performed at 39.5°C for 25 minutes. Heat-shocks after 5 dpf were performed at 38.5°C for 25 minutes. We used the following lines: *Tg(ptf1a:eGFP)*^{jh1} [14], *Tg(ela3l:Cre; cryaa:Venus)*^{s932}*; Tg(hsp70l:loxP-EBFP2-STOP-loxP-Ptf1aEN ; cryaa:Cerulean)*^{s933} (defined here as "*elastase:cre-Ptf1aEN* "), *Tg(hsp70l:loxP-mCherry-STOP-loxP-H2B-GFP; cryaa:Cerulean)*^{s923} [15], *Tg(ela3l:GFP)*^{gz2} [23], *Tg(ins:dsRed)*^{m1018} [24], *Tg(P0-pax6b:GFP)*^{ulg515} [26], *Tg(gcga:GFP)*^{ia1} [27], *Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP; cryaa:Cerulean)*^{s934}, and *Tg(mpx:GFP)*^{i114} [30].

DNA Constructs and Transgenic lines

ela3l:Cre; cryaa:Venus was generated by placing the *Cre* coding sequence downstream of 2.9kb of the proxim al *ela3l* promoter. The *cryaa:Venus* cassette was inserted downstream of *Cre* in the reverse orientation using 0.54kb of the *cryaa* promoter [34]. *hsp70l:loxP-EBFP2-STOP-loxP-Ptf1aEN ; cryaa:Cerulean* was constructed by sequentially replacing *mCherry* and *H2B-GFP* fragments in *hsp70l:loxP-mCherry-STOP-loxP-H2B-GFP; cryaa:Cerulean* [15] with *EBFP2-STOP* and *Ptf1aEN* respectively. *insulin:loxP:mCherrySTOP:loxP:H2B-GFP; cryaa:Cerulean* was constructed by replacing the *hsp70l* promoter in *hsp70l:loxP-mCherry-STOP-loxP-H2B-GFP; cryaa:Cerulean* with 1.5kb of the proxim al *insulin* promoter. All constructs were generated in a pBluescript backbone that contains I-SceI meganuclease sites. Multiple transgenic lines were established for each construct using the I-SceI meganuclease method as described [35]. A single representative transgenic line for each construct was used for all experiments.

Immunofluorescence and confocal microscopy

Antibody staining was performed as described [36] using the following antibodies: Islet-1 (1:100, DSH B clone 39.4D5), Nkx6.1 (1:100, DSH B clone F55A10), Prox1 (1:1000, Millipore AB5474), Elastase (1:200, Millipore AB1216), and Alexa secondary antibodies (1:500, Invitrogen). Cell nuclei were visualized with TOPRO3 (1:2000, Invitrogen T3605). All confocal sections and z-stacks were acquired on a Zeiss LSM5 Pascal microscope and processed in ImageJ.

Generation of 293-3xRBPJ-Luc cell line

A fragment containing three 32-bp PTF1 binding sites from the mouse *Rbpjl* promoter upstream of a minim al *Ela1* promoter (−92 to +8) [22] was subcloned into the luciferase reporter vector pGL4.17 (Promega). Following transfection, 293 cells that contained stable integrations were selected using 800 μg/m L G418. A single representative line that exhibited low levels of basal luciferase expression was used for all experiments.

Luciferase Assay

293-3xRBPJ-Luc cells were plated at 80% confluence in 96-well plates and transfected with 40 ng total DNA/well and 1 μL Lipofectamine transfection reagent (Invitrogen) in 75 μL serum free DMEM. After 4 h the media was replaced with DMEM containing 10% FBS. Each transfection contained 10 ng renilla (pRL-TK, Promega) and variable amounts of mouse Ptf1a (pcDNA3.1/Ptf1a) and / or mouse Ptf1a-Engrailed (pcDNA3.1/Ptf1aEN) expression plasmids. An empty expression vector (pcDNA3.1, Invitrogen) was used to normalize the total amount of DNA/well. Two days post-transfection, cells were analyzed for luciferase and renilla activity using the Dual-Glo reporter assay system (Promega) per manufacturer's protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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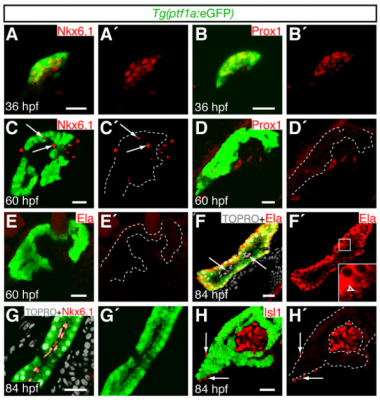

Figure 1. *pt f1a* expression becomes restricted to differentiated acinar cells during early larval development

Embryos express *Tg(ptf1a:*eGFP*)* in the pancreatic domain. Expression of pancreatic markers was analyzed by immuno-fluorescence. Scale bars = 20μm. (A–B′) Confocal projections of pancreatic tissue at 36 hpf. (C–H′) Confocal sections of pancreatic tissue at 60 (C–E′) and 84 hpf (F–H′). Dotted lines delineate *Tg(ptf1a:*eGFP*)* expressing tissue. (A–B′) *Tg(ptf1a:*eGFP*)* expressing cells initially co-express Nkx6.1 and Prox1. (C–D′) By 60 hpf, N kx6.1 (C,C′, arrow s) and Prox1 (D,D′) expression becomes excluded from the *Tg(ptf1a:*eGFP*)* expression domain. (E–F′) Co-expression of *Tg(ptf1a:*eGFP*)* with the acinar cell marker Elastase (Ela) is detected by 84 hpf. Elastase positive granules are detected on the apical surface of acinar cells (F′, arrow head), adjacent to *Tg(ptf1a:*eGFP*)* negative intra-pancreatic cells (F, arrow s). (G–H′) The duct marker Nkx6.1 (G,G′) and the endocrine marker Isl1 (H,H′) appear to be restricted to *Tg(ptf1a:*eGFP*)* negative ductal and islet cells respectively by 84 hpf. Isl1 is also expressed in the pancreatic mesenchyme (H,H′, arrows).

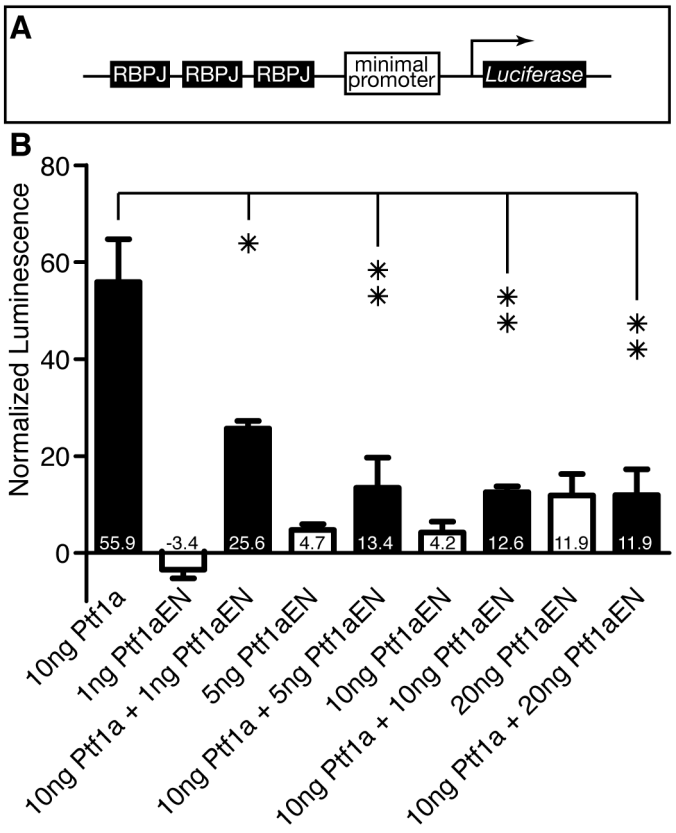

Figure 2. Engrailed-Ptf1a exhibits dose-dependent antagonism of wild-type Ptf1a activity

(A) A stable cell line (293-3xRBPJ-Luc) was generated in which Luciferase was placed under the control of RBPJ binding sites. (B) Normalized luminescence of 293-3xRBPJ-Luc cells that were transfected with Ptf1a and / or Ptf1aEN (mean +/− SD). *p<0.05; **p<0.01.

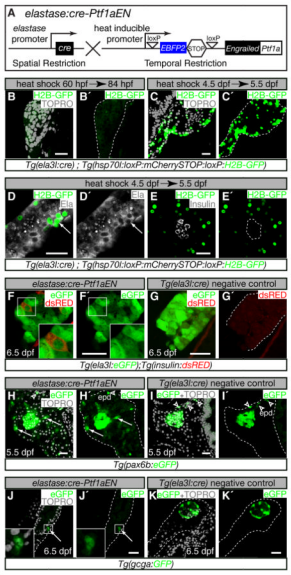

Figure 3. Inhibition of Ptf1a activity induces ectopic endocrine gene expression

(A) *elastase:cre-Ptf1aEN*, heat-inducible overexpression of Engrailed-Ptf1a in an *elastase3l*:Cre restricted domain. Expression of Cre in acinar cells excises the EBFP2-STOP cassette and perm its heat-inducible expression of Engrailed -Ptf1a. (B–K′) Confocal sections through pancreata marked with immuno-fluorescence (D–E′) or fluorescent transgenes (F–K′). Scale bars = 20μm. (B–E′) *Tg(ela3l:cre); (hsp70l:loxP:mCherrySTOP:loxP:H2B-GFP)* embryos were heat-shocked at 60 hpf (B,B′) or 4.5 dpf (C–E′) and analyzed 24 h post heat-shock. (F–I′) *elastase:cre-Ptf1aEN* (F,F′,H,H′,J,J′) and *Tg(ela3l:cre)* negative control embryos (G,G′,I,I′,K,K′) were heat-shocked at 4.5 dpf and analyzed at 5.5 dpf (H–I′) or 6.5 dpf (F–G′, J–K′). (B,B′) *Tg(ela3l:cre)* did not mark pancreatic cells by 60 hpf (dotted outline). (C,C′) By 4.5 dpf, *Tg(ela3l:cre)* marked a large fraction of pancreatic cells (dotted outline). Cells that were marked by *Tg(ela3l:cre)* at 4.5 dpf co-expressed the acinar marker Elastase (D,D′, arrow) but never co-expressed the endocrine marker Insulin (E,E′) at 5.5 d pf. (F,F′) *elastase:cre-Ptf1aEN* induction caused down-regulation of *Tg(elastase:*eGFP*)* expression in a subset of acinar cells (F′, inset). Some acinar cells that expressed low levels of *Tg(elastase:*eGFP*)* co-expressed *Tg(insulin:*dsRED) (F, inset). (G,G′) *Tg(insulin:*dsRED) expression was neverdetected in *Tg(ela3l:cre)* negative controls. (H–I′) *elastase:cre-Ptf1aEN* expression induced expression of the endocrine progenitor marker *Tg(pax6b:*eGFP*)* in cells outside of the principal islet (arrow s, H,H′). In *Tg(ela3l:cre)* negative controls at this stage, *Tg(pax6b:*eGFP*)* was expressed in the principal islet and in endocrine cells that differentiated in the extra-pancreatic duct (arrow head s, I,I′). (J–K′) *elastase:cre-Ptf1aEN* also induced Tg*(gcga:*GFP*)* expression in the exocrine compartment (J,J′), whereas Tg*(gcga:*GFP*)* expression was restricted to the principal islet in *Tg(ela3l:cre)* negative controls (K,K′).

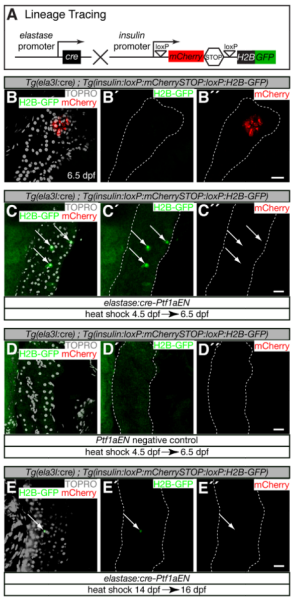

Figure 4. Genetic lineage tracing of acinar to insulin+ cell fate conversion

(A) *Tg(ela3l:cre); Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP)* lineage tracing. Expression of Cre in acinar cells excises the mCherry -STOP cassette and perm its expression of H2B-GFP in cells that activate the *insulin* promoter. (B–E″) Confocal sections through pancreata marked with fluorescent transgenes and TOPRO to mark DNA. Scale bars = 20μm. (B–B″) *Tg(ela3l:cre); Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP)* did not mark any pancreatic cells in wild-type animals. (C–C″) *elastase:cre-Ptf1aEN* expression induced scattered H2B-GFP positive cells throughout the pancreatic domain (arrows), which were not induced in *Ptf1aEN* negative controls (D–D″). (E–E″) *elastase:cre-Ptf1aEN* expression induced isolated H2B-GFP positive cells at 14 dpf (arrow).