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Fate mapping of *ptf1a*-expressing cells during pancreatic organogenesis and regeneration in zebrafish

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Abstract

Background—Pancreas development in zebrafish shares many features with mammals, including the participation of epithelial progenitor cells expressing pancreas transcription factor 1a (*ptf1a*). However, to date it has remained unclear whether, as in mammals, *ptf1a*-expressing zebrafish pancreatic progenitors are able to contribute to multiple exocrine and endocrine lineages. To delineate the lineage potential of *ptf1a*-expressing cells, we generated *ptf1a:creER*^{T2} transgenic fish and performed genetic-inducible lineage tracing in developmental, regenerating, and *ptf1a*-deficient zebrafish pancreas.

Results—In addition to their contribution to the acinar cell lineage, *ptf1a*-expressing cells give rise to both pancreatic Notch-responsive-cells (PNCs) as well as small numbers of endocrine cells during pancreatic development. In fish with *ptf1a* haploinsufficiency, a higher proportion of *ptf1a* lineage-labeled cells are traced into the PNC and endocrine compartments. Further reduction of *ptf1a* gene dosage converts pancreatic progenitor cells to gall bladder and other non-pancreatic cell fates.

Conclusions—Our results confirm the presence of multipotent *ptf1a*-expressing progenitor cells in developing zebrafish pancreas, with reduced *ptf1a* dosage promoting greater contributions towards non-acinar lineages. As in mammals, loss of *ptf1a* results in conversion of nascent pancreatic progenitor cells to non-pancreatic cell fates, underscoring the central role of *ptf1a* in foregut tissue specification.

Keywords

ptf1a; pancreas; pancreatic Notch-responsive cells; endocrine; lineage tracing

Introduction

The zebrafish has emerged as a highly informative system for the study of pancreatic organogenesis and has provided new insights regarding cellular signaling, lineage

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hierarchies and other developmental mechanisms including β-cell specification during pancreas morphogenesis (Yee et al., 2005; Kinkel and Prince, 2009; Tiso et al., 2009).

Pancreas development in zebrafish shares many features with mammalian pancreas development. The mammalian pancreas develops in two stages. During the first wave of development, referred to as the “primary transition”, there is early formation of an endocrine population (Rall et al., 1973; Herrera et al., 1991). Starting around embryonic day 13.5 (E13.5) in mouse, dramatic morphogenetic changes occur. This process is referred to as the “secondary transition” and is characterized by rapid cellular proliferation and differentiation. Mature endocrine cell populations also start to appear at this stage (Kemp et al., 1972; Pictet et al., 1972).

Pancreas transcription factor 1a (*Ptf1a*) is one of the earliest genes expressed in the pancreatic field (Hald et al., 2008). Lineage analysis in mice has shown that *Ptf1a* marks a multi-lineage progenitor pool that generates both endocrine and exocrine progeny during early pancreatic organogenesis. Between E13.5 to E18.5, the lineage contributions of the *Ptf1a*-expressing progenitor pool become progressively restricted, and by E18.5, *Ptf1a* exclusively labels cells that are destined to become acinar cells (Kawaguchi et al., 2002; Pan et al., 2013). In *Ptf1a* null mice, growth of the pancreas is severely retarded; there is a complete lack of acinar cells, and endocrine cells become redistributed to spleen (Krapp et al., 1998; Burlison et al., 2008). In addition, in the *Ptf1a*-deficient state, progenitor cells that would normally be directed to the pancreatic fate instead become incorporated into the duodenum or common bile duct (Kawaguchi et al., 2002; Burlison et al., 2008).

Similar to mammals, the zebrafish pancreas develops from two distinct anlagen arising from foregut endoderm. Although these have been termed the dorsal and ventral pancreatic buds (Field et al., 2003), their evolutionary relationship to the mammalian dorsal and ventral buds remain unknown. While in mammals these buds contain admixed endocrine and exocrine elements, there seems to early spatial segregation of zebrafish pancreatic lineages (Field et al., 2003; Ward et al., 2007). The fish dorsal bud develops first and gives rise to a primary endocrine cluster known as the principal islet, while the ventral bud appears later and has traditionally felt to be responsible for generating the exocrine ductal and acinar lineages as well as a later population of endocrine cells (Field et al., 2003; Lin et al., 2004; Zecchin et al., 2004; Hesselson et al., 2009; Wang et al., 2011). In zebrafish, the early dorsal bud-derived endocrine population may represent the equivalent of those endocrine cells that originate during the primary transition in the mammalian pancreas, perhaps corresponding to the “Brockman body” observed in jawless fishes (Epple and Brinn, 1975; Slack, 1995). In any case, these early cells have limited proliferation potential, and, the vast majority of mature hormone-producing cells are derived from the secondary transition (Hesselson et al., 2009; Parsons et al., 2009; Wang et al., 2011). With respect to progenitor cells responsible for generating differentiated pancreatic cell types, two populations have been defined in zebrafish: a Notch-responsive population and a *ptf1a*-expressing population (Lin et al., 2004; Wang et al., 2011). Lineage tracing studies have demonstrated that zebrafish Notch-responsive cells (PNCs) differentiate to ducts, centroacinar cells and endocrine cells during the secondary transition (Parsons et al., 2009; Wang et al., 2011).

In contrast, the precise contribution of *ptf1a*-expressing progenitor cells to different pancreatic lineages has yet to be determined. Previous work has clearly established that zebrafish *ptf1a* plays an important role in the establishment of acinar cell fate, equivalently to its mammalian counterpart (Lin et al., 2004; Zecchin et al., 2004). Furthermore, the early endocrine population arising in the "dorsal bud" is independent of *ptf1a* (Lin et al., 2004; Zecchin et al., 2004). However the role of *ptf1a* in the specification of endocrine cells arising during secondary transition remains unclear. Data has shown that a reduced level of *ptf1a* is more favorable for endocrine differentiation (Dong et al., 2008). Nevertheless, it is uncertain whether these endocrine cells are directly derived from a *ptf1a*-expressing progenitor population. To address these issues and to determine the fate of zebrafish *ptf1a*-expressing cells, we generated an inducible *ptf1a:creER*^{T2} transgenic fish line using BAC recombineering, and employed this line to complete lineage tracing studies. Early lineage labeling confirmed that *ptf1a*-expressing progenitors contribute primarily to the acinar cell lineage. Furthermore, we identified the contributions of *ptf1a* lineage to pancreatic Notch-responsive cells (PNCs) and endocrine cells during development. We also demonstrated that *ptf1a* lineage-labeled cells gave rise to newly formed β cells during regeneration. Interestingly, heterozygous *ptf1a*$^{sa126/wt}$ mutant fish displayed enhanced contributions of *ptf1a* lineage-labeled cells to the PNC and endocrine cell fates. In addition, we observed that, in the absence of functional Ptf1a, *ptf1a* lineage-labeled cells were converted into gall bladder and other non-pancreatic cell types. In summary, we showed that early *ptf1a*-expressing cells in zebrafish pancreas displayed limited but demonstrable multipotency during development and regeneration. Under conditions where *ptf1a* dosage was reduced, the contribution of *ptf1a*-expressing progenitor cells to non-acinar lineages was increased. These findings confirm conservation of general mechanisms for pancreas development among vertebrates, while highlighting qualitative differences.

Results

Validation of the ptf1a:creER^{T2} driver line

To lineage trace *ptf1a*-expressing cells in a temporarily controlled manner, we engineered a large genomic bacterial artificial chromosome (BAC) spanning the *ptf1a* locus. As similar BAC in which GFP replaced *ptf1a* coding sequence has been shown to faithfully recapitulate endogenous *ptf1a* expression in pancreas, hindbrain, retina and spinal cord (Park et al., 2008). We replaced the GFP sequence in the *ptf1a:eGFP* BAC with a DNA sequence encoding creER^{T2}. To facilitate integration of the BAC construct into the genome, an additional cassette containing the inverted left and right arm of the *Tol2* transposable element was used (Suster et al., 2011). Several independent F1 *ptf1a:creER*^{T2} transgenic founder lines were established and were crossed onto the *ubi:loxP-CFP-loxP-nuc-mCherry* reporter line.

In order to screen lines for inducible cre activity, *ptf1a:creER*^{T2}; *ubi:loxP-CFP-loxP-nuc-mCherry* double transgenic larvae were treated for 24 hours with 5 μM 4-hydroxytamoxifen (4-OHT), beginning at 1 day post fertilization (dpf). Larvae were then fixed for imaging at 5 dpf (Fig. 1A). Untreated control larvae were used as comparisons. At 5 dpf, strong nuc-mCherry signal was observed in 4-OHT-treated larvae, indicating cre-dependent

recombination (Fig.1B, d2 and g2). Rare to non-existent pancreatic or hindbrain nuc-mCherry signals were detected in larvae without 4-OHT treatment (Fig.1B, d1 and g1). The continued detection of CFP signal observed in Fig. 1B, c1 and f1) is due to the fact that our transgenic fish line carries multiple insertions of *ubi:loxP-CFP-loxP-nuc-mCherry* reporter, with not all copies undergoing cre-mediated recombination and CFP excision.

A single transgenic line with the highest fluorescent intensity upon 4-OHT treatment and lowest incidence of recombination events without 4-OHT treatment ("leakage") was selected and expanded for further use.

Detailed characterization of recombination efficiency in ptf1a:creER^{T2}; ubi:loxP-CFP-loxP-nuc-mCherry transgenic embryos

The expression of *ptf1a* is first detectable around 32 hours post fertilization (hpf). It has been reported that ligand-mediated recombination could be detected as early as 2 hours post 4-OHT treatment in transgenic zebrafish expressing *creER^{T2}* (Hans et al., 2009). We therefore treated *ptf1a:creER^{T2}; ubi:loxP-CFP-loxP-nuc-mCherry* embryos with 4-OHT at 30–54 hpf in an effort to label the earliest pool *ptf1a*-expressing pancreatic progenitor cells (Fig. 2A). We subsequently imaged the fish at 6 dpf, when pancreatic architecture is fully formed.

In our selected transgenic line, the frequency of embryos displaying tamoxifen-independent creER^{T2} activity displayed significant clutch-to-clutch variability, ranging from 5% –30%. However, tamoxifen-independent recombination events were confined to very small numbers of cells. Among 32 fish with detectable 4-OHT-independent recombination, we observed an average of only 19 (±10) cells per fish with detectable nuc-mCherry signals, as assessed at 6 dpf. All cells displaying 4-OHT-independent reporter recombination also expressed markers of pancreatic acinar cell differentiation. In comparison, among 32 fish that had been treated with 4-OHT, there were on average 377 (± 82) lineage labeled cells per fish, representing 85% of total acinar cells. Stated differently, cells displaying 4-OHT-independent cre activity represented only a small proportion of total transgene expressing cells (<5%), and were limited to the acinar lineage. We therefore concluded that our study would not be significantly confounded by 4-OHT-independent recombination, especially as it related to the contribution of *ptf1a*-expressing cells to non-acinar lineages.

The early ptf1a lineage has a limited contribution to non-acinar cell fate in the zebrafish pancreas

We next sought to evaluate the contribution of *ptf1a*–expressing progenitor cells to different pancreatic lineages. By 6dpf, we observed broad contributions to the acinar cell lineage, confirming that *ptf1a*–expressing progenitor cells represent the major and perhaps exclusive source of these cells. We also observed lower magnitude contributions to the PNC and endocrine (principal islet) cell lineages. For the 36 fish we examined, 26 of them (72%), did not have any detectable *ptf1a* lineage contribution to PNCs (Fig. 2B; Fig. 5F, G). For the remaining 10 fish, there were on average 8 PNCs marked by the *ptf1a* lineage label (Fig. 2C; Fig. 5F, G). At this stage, there are an average 137 PNCs per fish, hence, cells of *ptf1a* lineage contribute to approximately 6% of the total PNC population.

We subsequently surveyed the *ptf1a* lineage contribution to endocrine cells in the principal islet. At 6 dpf, the principal islet contains endocrine cells directly descended from the dorsal bud as well as cells differentiated during the secondary transition (Biemar et al., 2001; Hesselson et al., 2009). For the 21 larvae we assessed, 11 of them (52%) did not have any *ptf1a* lineage-labeled cells traced into the principal islet (Fig. 2D; Fig. 5H, I). For the 10 remaining larvae where we did observe contributions from *ptf1a-expressing* progenitors to the endocrine lineage, there were an average of 3.4 lineage labeled cells observable in the principal islet (Fig. 2E; Fig. 5H, I). This represents approximately 10% of the principal islet endocrine population at 6 dpf.

To confirm that the current 30–54 hpf 4-OHT treatment window does not preferentially label acinar cells, we compared lineage analysis results from different 4-OHT pulse intervals, varied from as early as 6 hpf, a time point when ptf1a is not yet expressed, to 6 dpf (Fig. 2F, G). In all cases, the lineage tracing results were comparable.

The ptf1a lineage contributes to endocrine cells in juvenile and adult zebrafish

To determine the contribution of the ptf1a lineage to later arising endocrine cells, we pulsed 4-OHT again from 30–54 hpf, and further traced the *ptf1a* lineage into juvenile and adult fish (Fig. 3A). In 4-OHT treated fish, at 30 dpf, we saw widespread lineage labeling of acinar cells. In addition, we noted a contribution of *ptf1a*-expressing progenitors to endocrine cells within secondary islets (Fig. 3B, C). Interestingly, for some of the secondary islets in which we observed *ptf1a* lineage labeling, almost all endocrine cells within the islets were labeled (Fig. 3C). We theorized that the *ptf1a* lineage gave rise to the progenitors of those secondary islets, which subsequently had gone through clonal expansion process to form entire secondary islets. We further quantified the frequency of *ptf1a* lineage labeled cells based on their principal or secondary islet location (Fig. 3D). We observed no differences in the percentage of *ptf1a*-lineage labeled cells between these two populations (Fig. 3D). Similarly, in the adult pancreas, we observed isolated as well as clustered *ptf1a* lineage labeled cells located with islet tissue (Fig. 3E, F).

ptf1a lineage contributes to endocrine β-cell regeneration

Having assessed the fate of *ptf1a*-expressing progenitor cells during normal pancreas development, we proceeded to determine whether these cells could contribute to β-cell regeneration. To this end, we employed a well-established nitroreductase (NTR) cell-ablation system to eliminate β cells (Pisharath et al., 2007). The NTR enzyme, encoded by the bacterial gene *nfsb*, can convert prodrugs such as metronidazole (Met) to cytotoxins (Lindmark and Muller, 1976; Anlezark et al., 1992). In our system, we expressed the *nfsb* gene under the control of *insulin* promoter. Subsequently, upon administration of Met, the insulin secreting β cells are ablated, with cell death occurring within 6 hours post Met treatment. Following β-cell ablation, regenerating insulin-expressing cells can be observed within 36 hours after Met removal (Pisharath et al., 2007). To determine the contribution of the *ptf1a* lineage to regenerating β-cells, we crossed *ptf1a:creER^{T2}*; *ubi:loxP-CFP-loxP-nuc-mCherry* fish with *sst:CFP*; *ins:ds YFP-2TA-nsfB* fish (Fig. 4A). Besides the *ptf1a* lineage tracing constructs, the triple transgenic fish carries the *nfsB* transgene construct intended for β-cell ablation. In these fish, the *insulin* promoter drives expression of destabilized YFP

(dsYFP) and *nfsB*, while the *somatostatin* (*sst*) promoter drives the expression of CFP (Fig. 4A).

We treated the resulting triple transgenic fish with 4-OHT from 30–54 hpf and applied Met from 82–106 hpf (Fig. 4B). In the control pancreata, YFP-expressing β cells form a compact inner core, intermingled with somatostatin-secreting δ cells and surrounded by other endocrine cell types (Fig. 4C). In the Met-treated pancreata, much weaker YFP signals were detected within the principal islet (Fig. 4D). Some cells contained aggregates with high YFP fluorescence intensity. However, these cells were detached from the rest of endocrine cells and the fluorescence most likely represent cellular debris. (Fig. 4D). We assessed β-cell regeneration at 6 dpf, 48h after Met removal. At this time point, β cells started to regenerate but there was still a distinct reduction of β-cell numbers in the Met-treated fish (Fig. 4E, F). Interestingly, we observed that cells derived from the early *ptf1a* lineage contributed to the newly generated β cells (Fig. 4F). The level of contribution, however, was similar to the control pancreas (Fig. 4G), suggesting that *ptf1a* lineage was not the main source for new β cells. To be noted, in the current study, β-cell ablation was carried out from 82–106 hpf, a time when the endocrine population is rapidly proliferating. Our unpublished observation indicates that during this developmental time window, newly generated β cells mainly come from pre-existing endocrine cells and from the differentiation of PNC progenitors, likely explaining the modest contribution from *ptf1a*-expressing progenitor cells.

Reduction in ptf1a gene dosage enhances the contribution of ptf1a-expressing progenitor cells to non-acinar cell fates

In the mouse, pancreatic cells with different *Ptf1a* gene dosages seem to have altered cell fates (Fukuda et al., 2008; Pan and Wright, 2011). During early mouse pancreas development, it has been shown that intermediate expression of *Ptf1a* marks multipotent progenitor cells, whereas higher *Ptf1a* expression levels are observed in pancreatic progenitors undergoing restriction towards an acinar cell fate (Pan et al., 2013). Similarly, in zebrafish, studies have shown that reduced levels of *ptf1a* seem to promote endocrine differentiation (Dong et al., 2008). Furthermore, in the adult fish, reduction of *ptf1a* activity induces the expression of endocrine-specific genes in mature acinar cells (Hesselson et al., 2011). To formally characterize the *ptf1a* lineage allocation under conditions of reduced *ptf1a* dosage, we took advantage of the $ptf1a^{sa126/wt}$ mutant fish generated by the Sanger Zebrafish Mutation Project (Kettleborough et al., 2013). The $ptf1a^{sa126}$ allele contains a nonsense mutation within exon1 of the *ptf1a* coding region (Fig. 5A). This mutation is predicted to result in either the expression of a truncated protein or the induction of nonsense-mediated decay (NMD) (Randlett et al., 2013).

We first characterized pancreatic morphology in $ptf1a^{sa126/wt}$ heterozygous and $ptf1a^{sa126/sa126}$ homozygous fish. Homozygous *ptf1a* mutations phenocopied the previously reported *ptf1a* morpholino phenotype (Fig. 5B, C) (Lin et al., 2004). At 5 dpf, $ptf1a^{sa126/sa126}$ fish did not have an exocrine pancreas, as assessed by *ptf1a:GFP* transgene expression; whereas the principal islet appeared normal or slightly reduced in size (Fig. 5B, C). In contrast, the pancreatic morphology of $ptf1a^{sa126/wt}$ heterozygous fish was undistinguishable from wildtype controls at 5dpf (Fig. 5D, E). These data are consistent with

the prediction that the *ptf1a*^{sa126} mutation represents either a hypomorphic or functionally null allele.

We next proceeded to perform lineage tracing experiments in *ptf1a*$^{sa126/wt}$ heterozygous fish. Intriguingly, with reduced *ptf1a* dosage, more *ptf1a* lineage labeled cells were traced into PNCs and the principal islet (Fig. 5D, E). In the 40 fish examined, 19, (48%) did not have any *ptf1a*-lineage labeling in PNCs. The fraction of unlabeled fish is lower than in the wildtype condition, where the corresponding percentage is 72% (Fig. 5F, G). Among the 21 remaining *ptf1a*$^{sa126/wt}$ fish where we observed *ptf1a*-lineage labeling in PNCs, an average of 17 cells per pancreas, or 13.5% of the total number of PNCs, were labeled as descendants of the *ptf1a* lineage. In comparison, in wildtype fish, we observed 5.8% of the PNCs were labeled by *ptf1a* lineage (Fig. 5F, G). These differences in the frequency of *ptf1a*-lineage contributions to PNCs were statistically significant.

Similarly, we observed that in *ptf1a*$^{sa126/wt}$ heterozygotes, 22% had no *ptf1a* lineage-labeled cells within the principal islet (compared to 52% of wildtype fish) (Fig. 5H, I). In fish in which we did observe *ptf1a*-lineage labeling within the principal islet, there was a higher percentage of endocrine cells labeled (19% compared with 10% in the principal islet of *ptf1a* wildtype fish) (Fig. 5H, I). Each of these differences was statistically significant.

The total number of PNCs and total area of the endocrine population showed no difference between wildtype and *ptf1a*$^{sa126/wt}$ heterozygous pancreas (data not shown). To exclude the trivial explanation that *ptf1a* lineage labeling was more efficient in *ptf1a*$^{sa126/wt}$ heterozygotes compared to wildtype fish, we compared labeling efficiencies in the acinar population. We observed that, as is the case with wildtype fish, 85% of acinar cells were labeled in *ptf1a*$^{sa126/wt}$ heterozygotes. All the above data suggest that haploinsufficiency for *ptf1a* promotes an enhanced contribution of *ptf1a*-expressing pancreatic progenitors to non-acinar cell fates, with higher level of regulatory mechanisms acting to determine the actual number of PNCs and endocrine cells.

Further cell fate conversion in the ptf1a$^{sa126/sa126}$ homozygous fish

In homozygous *ptf1a*$^{sa126/sa126}$ fish, there are no detectable acinar cells and no secondary islets (Fig. 5C). Nonetheless, even in the absence of a morphologically discernible exocrine pancreas, we still observed a *ptf1a* lineage-labeled population in the region of the intestinal bulb. In order to characterize the identity of these cells, we performed immunolabeling using the 2F11 antibody that recognizes enteroendocrine cells, intrahepatic bile ducts, gall bladder, hepatopancreatic ducts and PNCs (Dong et al., 2007). 2F11 antibody staining indicated that some of the *ptf1a* lineage labeled cells were located within the gall bladder (Fig. 6A, B). Moreover, in the homozygous mutant fish, *ptf1a* lineage labeled cells could also be detected among PNCs as well as within the principal islet (Fig. 6C, D). Of note, in the *ptf1a*$^{sa126/sa126}$ fish, PNCs were properly specified (Fig. 6D). This observation was consistent with the notion that the *ptf1a* lineage and PNCs are independently specified (Wang et al., 2011). However, in the homozygous mutant fish, PNCs remained as a cluster of cells adjacent to the principal islets, and the total number of PNCs was significantly reduced (Fig. 6D). These data suggest that *ptf1a* lineage-derived acinar cells may provide a supporting cellular framework required for the proliferation and migration of PNCs and normal establishment of

ductal network. The reduced number of PNCs is not likely to reflect a direct requirement for *ptf1a* in generating the PNC lineage, since under wildtype *ptf1a* conditions, only ~5% of PNCs were labeled by the *ptf1a* lineage mark. Alternatively, other cell types required for normal PNC expansion and migration might also be compromised in the *ptf1a*$^{sa126/sa126}$ fish.

Discussion

In the present study, we generated a new *ptf1a* fate-mapping fish and carried out *ptf1a* lineage analysis in developing, regenerating and *ptf1a*-deficient pancreas. We observed a major contribution of the *ptf1a* progenitor lineage to later appearing acinar cells, and minor contributions to PNCs and endocrine cells during development. The *ptf1a* lineage also contributed to β cells during regeneration. Furthermore, *ptf1a* gene dosage was found to influence the magnitude of non-acinar lineage contributions. In heterozygous *ptf1a*$^{sa126/wt}$ fish, we observed a larger contribution from the *ptf1a* progenitor lineage to the PNC and endocrine compartments. As in the mouse, a complete absence of *ptf1a* lead to trans-fating of *ptf1a*-expressing cells into gall bladder epithelium and other foregut tissues.

Limited contribution of the ptf1a lineage to non-acinar cell fates

In the current work, we observed limited contribution of the *ptf1a* lineage to non-acinar cell fates. One explanation for this observation might be that the earliest multi-lineage progenitor population may be characterized by only low-level expression of *ptf1a*, resulting in inefficient labeling of this population. However, when we changed our 4-OHT treatment time to 6–32 hpf, a time period that only covers the earliest low level expression of *ptf1a* (Lin et al., 2004; Zecchin et al., 2004) we still observed robust labeling (Fig. 2F). Similarly, when 4-OHT treatment was extended from 6h-6dpf, we did not observed any further enhancement of labeling (Fig. 2G). In addition, the *ubi:loxP-CFP-loxP-nuc-mCherry* reporter line has shown robust labelling in the PNC and endocrine population upon crossing with a TP1 lineage tracing fish (unpublished observation). Together, these results suggest that the low frequency contribution of the *ptf1a* lineage to non-acinar fates is unlikely to be due to low labeling efficiency for the progenitor population. Thus it is probable that pancreatic-Notch-responsive cells (PNCs) represent the dominant progenitor population required for generating non-acinar pancreatic cell types, functioning largely independent of *ptf1a* (Wang et al., 2011).

Clonal expansion of secondary islets

In some of the secondary islets where we detected *ptf1a* lineage labeling, clusters of lineage-traced cells were observed. The low probability of *ptf1a* lineage labeling in the endocrine population made it almost certain that these cell clusters were generated by clonal expansion, implying that a fraction of secondary islets originated from single *ptf1a* positive progenitors. This is consistent with previous studies showing that newly generated secondary islets can also arise from single PNCs, and that these cells are highly proliferative (Matsuda et al., 2013)

ptf1a lineage and Notch-responsive lineage

We used Nkx6.1 as a marker for PNCs in the current study. We have previously observed that in the zebrafish pancreas, immunofluorescent labeling for Nkx6.1 completely overlaps with PNCs marked directly by the transgene *TP1:eGFP* (Huang et al., 2014). In mouse, it has been shown that Ptf1a and Nkx6.1 reciprocally repress each other's expression (Schaffer et al., 2010). This appears to represent a significant mechanism for determining the allocation of cells between acinar and endocrine cell fates. Ongoing expression of Ptf1a directs cellular programming towards the acinar fate, while Nkx6.1 directs cells towards a ductal/endocrine fate (Schaffer et al., 2010). Our data suggest that the same mechanism may be applicable in the zebrafish pancreas. Specifically, we observed that in the setting of *ptf1a* haploinsufficiency, there was a significantly greater *ptf1a* lineage contribution to the Nkx6.1-expressing PNC compartment. PNCs have been shown to be the precursors of the endocrine population in zebrafish, and inhibition of Notch signaling enhances endocrine differentiation (Parsons et al., 2009; Wang et al., 2011). These results may imply that the increased contribution of *ptf1a*-expressing progenitor cells to the endocrine compartment in *ptf1a* heterozygous fish may proceed through a PNC intermediate.

Different levels of ptf1a regulate the multi-lineage potential of ptf1a-expressing progenitors

With little-to-no *ptf1a* activity in the $ptf1a^{sa126/sa126}$ fish, we observed a complete absence of exocrine pancreas, while early endocrine cell differentiation and principal islet structure was maintained. This result is similar to the *ptf1a* morpholino phenotype (Lin et al., 2004), but different from observations in human and mouse in which low level of *ptf1a* also reduces endocrine cell numbers and alters their distribution (Stoffers et al., 1997; Burlison et al., 2008; Fukuda et al., 2008). This may reflect the different developmental programming of zebrafish pancreas (Tiso et al., 2009), as well as fundamental differences between the development of principal and secondary islets. Another possible scenario is that in both *ptf1a* morphant and $ptf1a^{sa126/sa126}$ homozygous fish, there is still enough expression and function of *ptf1a* to support proper endocrine lineage differentiation. Evidence that supports this hypothesis includes a recent study on the function of *ptf1a* in synaptic neuropil, where it was demonstrated that the combination of $ptf1a^{sa126/sa126}$ allleles and a *ptf1a* translation-blocking morpholino were required to reveal a true *ptf1a* null phenotype (Randlett et al., 2013). In the $ptf1a^{sa126/sa126}$ fish, we also observed that the *ptf1a* lineage became trans-fated to gall bladder and other non-pancreatic fates. This cell-fate conversion is in keeping with observations in mice (Burlison et al., 2008; Fukuda et al., 2008).

Overall, it is apparent from the current work that, as in mouse, different zebrafish pancreatic cell fates require different threshold levels of *ptf1a*, at least for a defined subset of cells. Further studies investigating intermediate dosages/activities of *ptf1a* will be needed to address the function of Ptf1a in a more quantitative way. This might be done by the combination of *ptf1a* mutant alleles varying amounts of morpholino against *ptf1a*; or, by combining the $ptf1a^{sa126}$ Sanger allele with the $ptf1a^{akreas}$ hypomorphic allele (Dong et al., 2008). Certainly, more work is required to characterize downstream mechanisms that are responsive to different levels of *ptf1a*.

Our findings have provided an important understanding on the mechanisms of pancreatic development in zebrafish, and further clarify both similarities and differences between pancreas development in fish and mammals. Notably, they also underscore fundamental differences between “dorsal bud” and “ventral bud” development in zebrafish, with the ventral bud displaying a more mammalian-like program involving ptf1a-dependent development and the participation of multi-lineage ptf1a-expressing progenitor cells.

Experimental Procedures

Generation of transgenic zebrafish lines

We followed established BAC recombineering methods (Suster et al., 2011) to generate the BAC transgene *tg*(*ptf1a:creER*^{T2}), abbreviated to *ptf1a:creER*^{T2}, by replacing GFP with *creER*^{T2} elements in the *ptf1a:GFP* construct. The *ptf1a:GFP* construct is derived from genomic BAC CH211-142H2 (Park et al., 2008; Sharan et al., 2009), which encompasses the zebrafish *ptf1a* coding sequence. The *ptf1a:GFP* transgene has been shown to accurately recapitulate the endogenous *ptf1a* expression (Park et al., 2008; Pashos et al., 2013).

The lineage responder line *tg(ubi:loxP-CFP-loxP-nuc-mCherry)*, abbreviated to *ubi:loxP-CFP-loxP-nuc-mCherry* was modified from the previously published line *Tg(T2Kβactin:loxP-stop-loxP-hmgb1-mCherry)*^{ih15} (Wang et al., 2011), where the *βactin* promoter was replaced by the *ubiquitin* promoter (*ubi*) (Mosimann et al., 2011). CFP was also included in the construct to facilitate the identification of the transgenic fish.

Ptf1a$^{sa126/wt}$ fish were obtained from the Sanger Institute Zebrafish Mutation Project (Kettleborough et al., 2013).

Stable *sst:CFP*; *ins:dsYFP-2TA-nsfB* transgenic fish was established to express CFP under *somatostatin (sst)* promoter, and *nsfB* encoding Nitroreductase was expressed under *insulin (ins)* promoter (Pisharath et al., 2007; Walker et al., 2012).

All fish were maintained under standard conditions. All procedures were performed under the approval of the Johns Hopkins University School of Medicine Animal Care and Use Committee guidelines.

Drug treatment

For the induction of creER activity, 4-Hydroxytamoxifen (4-OHT, T176, Sigma) treatment was performed as previously described (Wang et al., 2011). Briefly, 4-OHT was dissolved in 100% ethanol to create a stock solution of 10 mM. Embryos were placed in a 5 μM solution of 4-OHT in E3 medium from 30 to 54 hours post fertilization (hpf), unless otherwise stated in the text.

For the β-cell ablation experiment, Metronidazole (Met, Sigma, M3761) treatment was performed as previously described (Pisharath et al., 2007). Briefly, Met was dissolved in E3 medium at a concentration of 10mM. Embryos were incubated in this solution for 24 hours from 82 to 106 hpf. Embryo-containing petri dishes were kept in the dark.

Immunofluorescence

For whole mount immunofluorescent staining, larvae were fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, the whole gut region was dissected out and blocked for 1 hour with PBS with 0.2% Triton (PBST) and 10% fetal bovine serum. For cryosections, fish were similarly fixed and the gut-intestine system was dissected. Tissues were then immersed in 30% sucrose/PBS, embedded in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen, and sectioned in 10 μm thickness using a cryostat. Primary antibodies used in this study included: mouse anti-Nkx6.1 (Developmental Studies Hybridoma Bank, F55A12), 1: 100. Guinea pig anti-Insulin (Dako, A0564), 1: 500. Rabbit anti-Glucagon (Dako, A0565), 1:400. Rabbit anti-Somatostatin (Dako, A0566), 1:500. Rabbit anti-DsRed (Clontech, 642496), 1:100. Mouse anti-2F11 (Abcam, ab71286), 1:200. Primary antibodies were incubated at 4°C overnight. After washing with PBST, samples were incubated with secondary antibodies (Jackson Immunoresearch, 1:300) in blocking buffer. Fluorescent images were acquired with Nikon A1 scanning confocal microscope. Cell counting was carried out manually. Briefly, whole mount tissues were scanned by confocal microscope and maximum projections were assembled from Z-stacks. Cell numbers were counted from reconstructed images. Students t-test was implemented for statistical analysis.

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Key findings

- In the zebrafish pancreas, early *ptf1a*-expressing progenitor cells are multipotent.
- The *ptf1a* lineage contributes to new β cells during regeneration.
- *The ability of the ptf1a* lineage to contribute to non-acinar cell types is enhanced when *ptf1a* dosage is reduced.

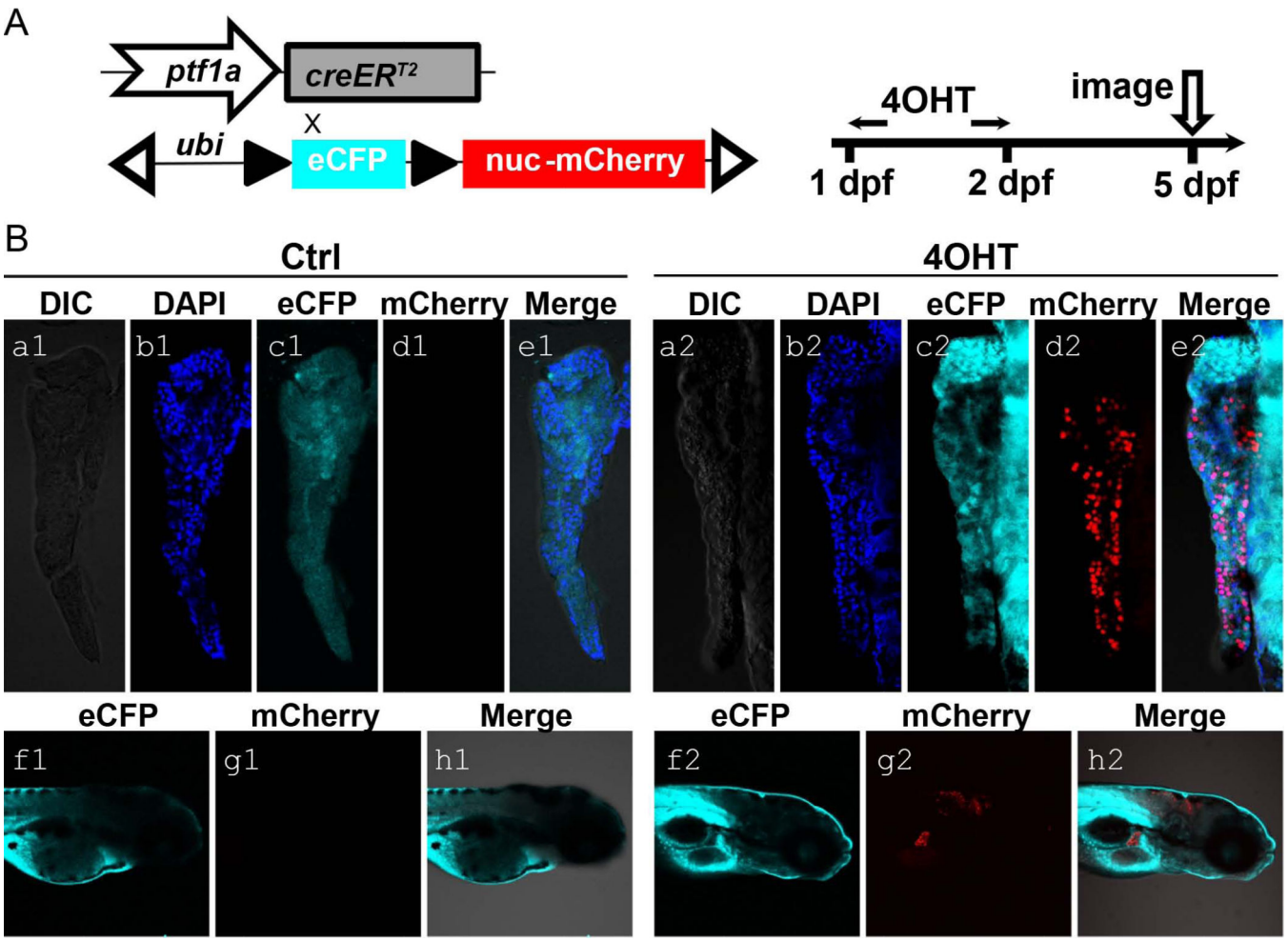

Figure 1. Generation of the *ptf1a* lineage tracing system and its initial characterization

(A) Schematic diagram of *ptf1a:creER*^{T2} driver and responder *ubi:loxP-CFP-loxP-nuc-mCherry*. Double transgenic fish larvae were treated with 5 μM 4-OHT in E3 medium at 1 dpf for 24 hours and then fixed for imaging at 5 dpf. These larvae were raised along with untreated controls. (B) At 5 dpf, No pancreatic and hindbrain nuclear-mCherry signals were detected in control larvae (a1-e1, f1-h1). 4-OHT-treated larvae showed nuclear-mCherry signal (red) in exocrine pancreas (a2-e2) and hindbrain (f2-h2), indicating *ptf1a*-dependent Cre activity. CFP signal (cyan) could be detected ubiquitously (c1, f1, c2, f2). DAPI (blue) stains for nuclei (b1, b2).

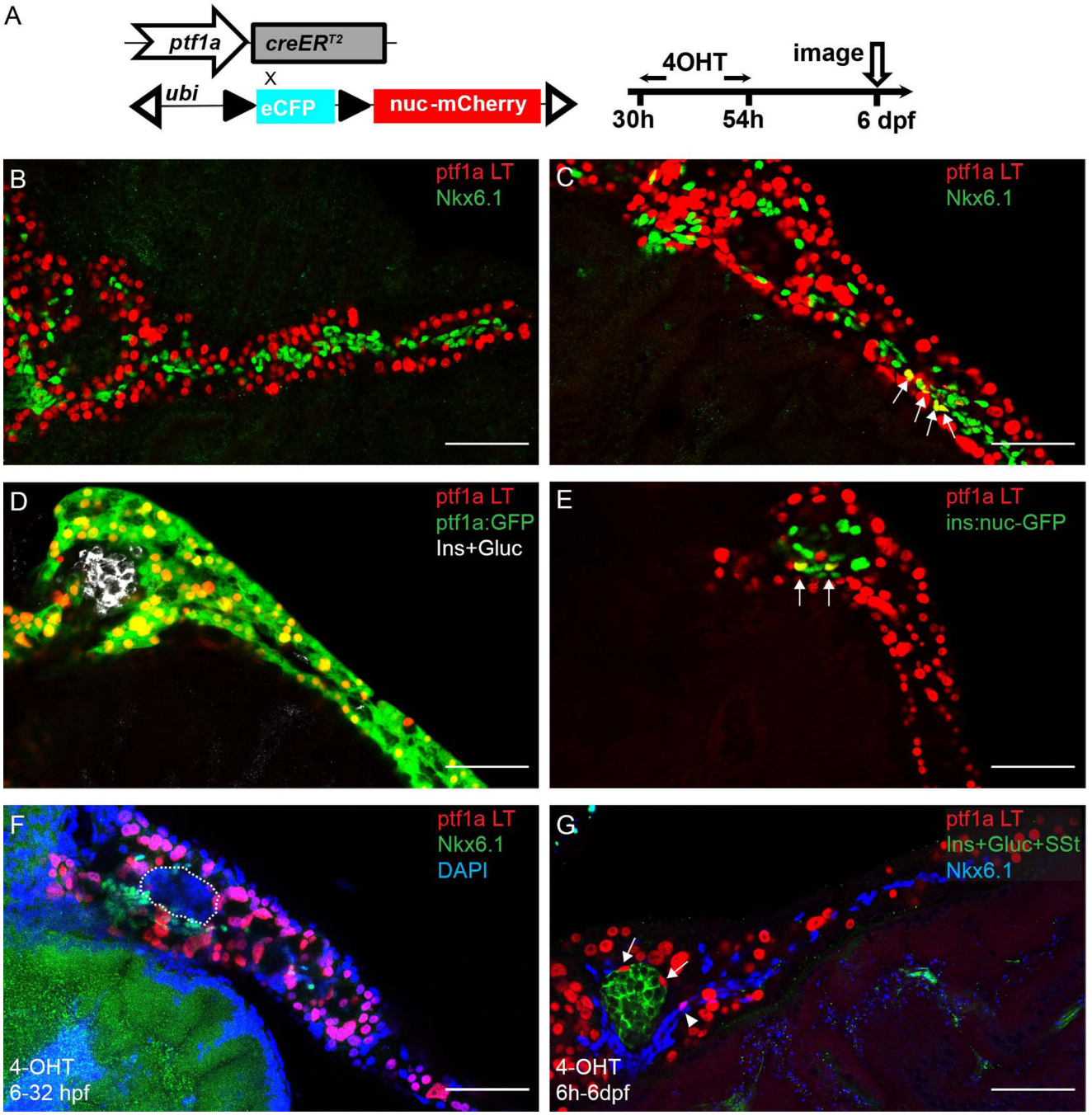

Figure 2. The early *ptf1a* lineage generates a small fraction of PNC and endocrine cells

(A) Experimental setup. (B–G) In all the panels, *ptf1a* lineage is indicated by nuclear-mCherry expression (red). (B, C) Immunofluorescence for Nkx6.1 (green) labels PNCs. (B) In some pancreata, the *ptf1a* lineage and PNCs show no overlap. (C) In some pancreata, *ptf1a*-lineage labeled cells are traced into PNCs. Arrows point to colabeling events. (D) In some pancreata, the *ptf1a* lineage has no demonstrable contribution to endocrine cell types. *ptf1a:GFP* transgene (green) shows current expression of *ptf1a*, which, at 6 dpf, is limited to acinar cells. Endocrine cells are labeled by a mixture of antibodies against Insulin (Ins) and

Glucagon (Gluc), white. (E) In a few pancreata, some of the cells derived from the *ptf1a* lineage are co-labelled by the *ins:nuc-GFP* transgene (green), which is visualized in the nuclei of Insulin-secreting cells. Arrows point to co-labeling events. (F) 4-OHT treatment at 6h-32hpf. In the pancreas shown, there are no *ptf1a* lineage-labeled cells observed in the endocrine compartment (outlined) nor PNCs (Nkx6.1, green). (G) 4-OHT treatment at 6h-6dpf. Nkx6.1, blue. Arrows point to *ptf1a* lineage-labeled endocrine cells. Arrowhead points to *ptf1a* lineage-labeled PNCs. Scale bar, 50 μm.

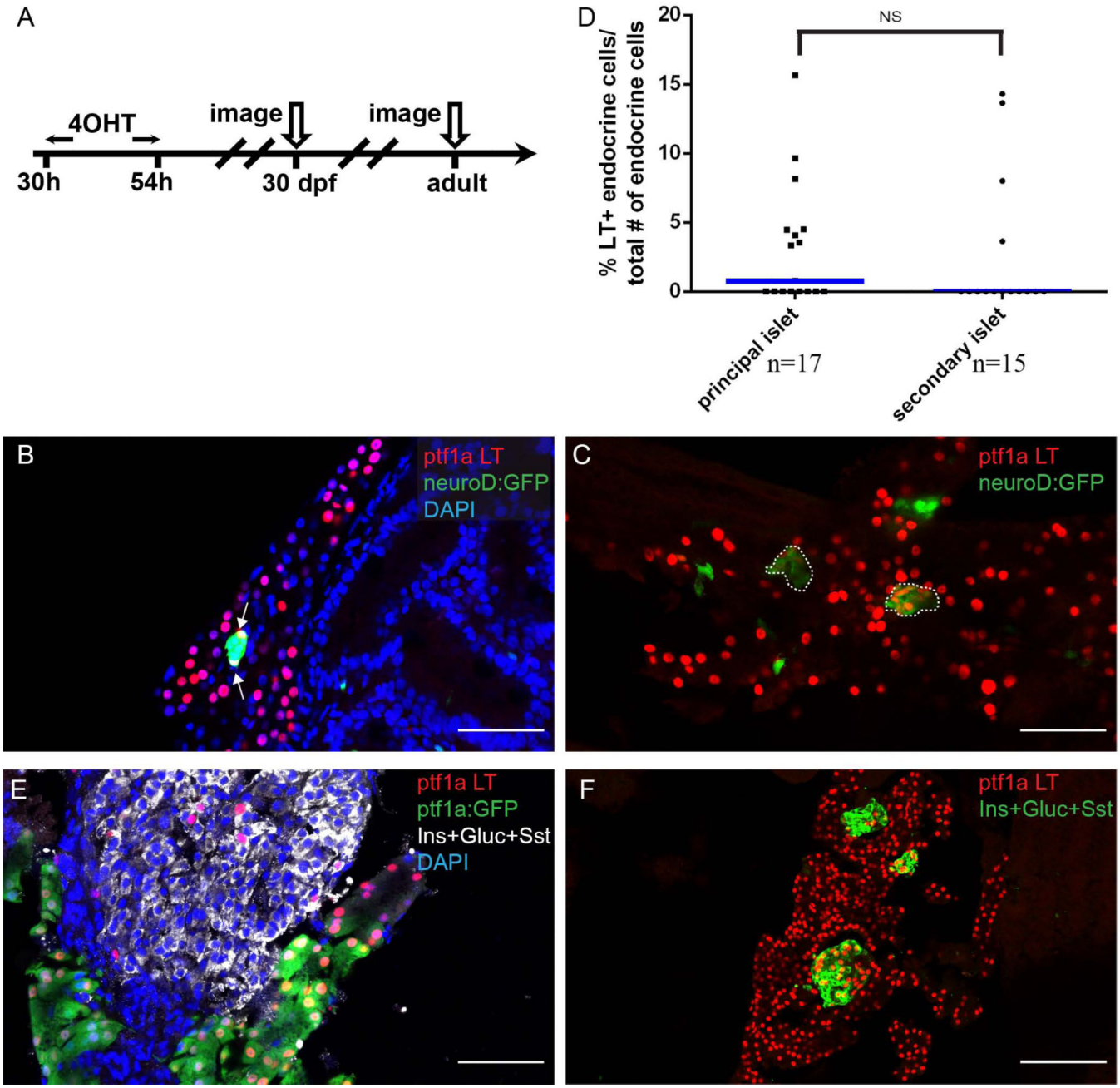

Figure 3. The early *ptf1a* lineage contributes to secondary islet and adult endocrine cells

(A) Experimental setup. (B, C, E, F) The *ptf1a* lineage is labeled by nuclear-mCherry (red). (B, C) Sections from 30 dpf juvenile lineage-tracing fish. The secondary islet is labeled by the transgenic marker *neuroD:GFP* (green). (B) Arrows point to endocrine cells expressing the *ptf1a* lineage mark. Notice that not all nuclei of within the secondary islet are colabeled by the *ptf1a* lineage mark. DAPI, blue. (C) Secondary islets are labeled by *neuroD:GFP* (green) and outlined. The secondary islets consist of a clusters of *ptf1a*-lineage labeled cells. (D) Scatter plot shows frequency of *ptf1a* lineage labeled cells traced into principal or secondary islets. T-test shows no significance. Blue bars show population median. “n”

indicates the number of slides quantified for each population. (E, F) Sections from adult lineage-tracing fish. The endocrine population is labeled by a mixture of antibodies against Insulin (Ins), Glucagon (Gluc) and Somatostatin (Sst). (E) Some of the endocrine cells express the *ptf1a* lineage label. The majority of *ptf1a* lineage-labeled cells co-express *ptf1a:GFP* transgene (acinar marker). (F) Selected islets consist of a cluster of *ptf1a*-lineage labeled cells. (F) DAPI, blue. Scale bar, 50 μm.

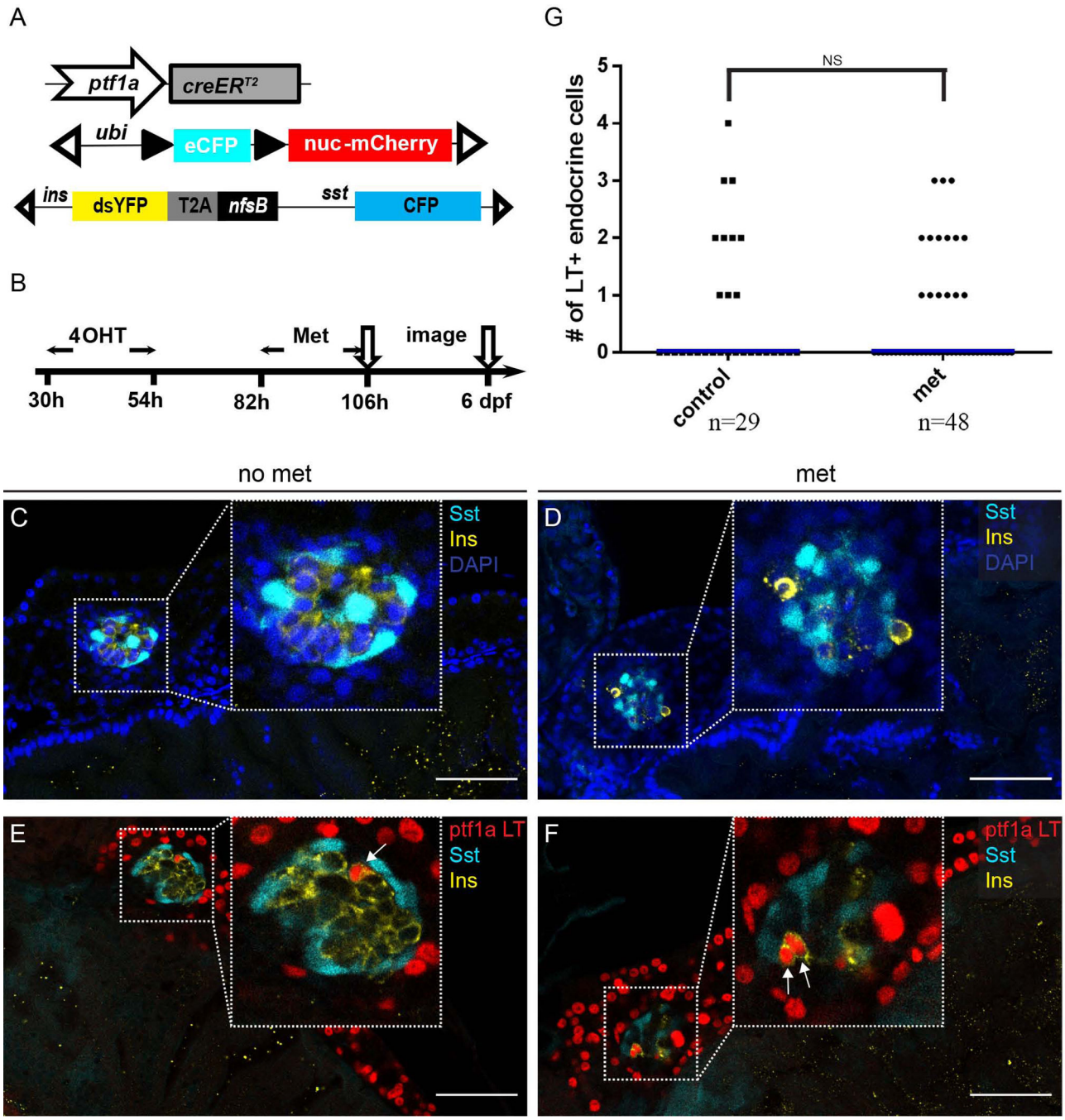

Figure 4. The *ptf1a* lineage contributes to β-cell regeneration

(A) Schematic depicting the triple transgenic line utilized in the experiment. (B) Experimental setup. (C) In control fish with no Metronidazole (Met) treatment, the principal islet has compact architecture. Insulin (Ins), yellow. Somatostatin (Sst), cyan. Nuclei are labeled by DAPI (blue). (D) Pancreatic region following 24h of Met treatment. The majority of Insulin-secreting cells are gone. Insulin (Ins), yellow. Somatostatin (Sst), cyan. DAPI, blue. (E, F) The *ptf1a* lineage is labeled by nuclear-mCherry expression (red) 48 h after removal of Met. (E) In untreated fish, there are rare *ptf1a* lineage cells traced into the

principal islet. Insulin (Ins), yellow. Somatostatin (Sst), cyan. (F) In the Met treated fish, two regenerated β cells express the *ptf1a* lineage label. Insulin (Ins), yellow. Somatostatin (Sst), cyan. In (E) and (F), arrows point to Insulin+ cells that are labeled with the *ptf1a* lineage mark. (G) Scatter plot shows quantification of *ptf1a* lineage labeled cells within the endocrine compartment. No significant differences in the frequency of *ptf1a* lineage contributions are observed between normal and regenerating β cells. “n” indicates the number of fish quantified for each condition. Blue bars show population medium.

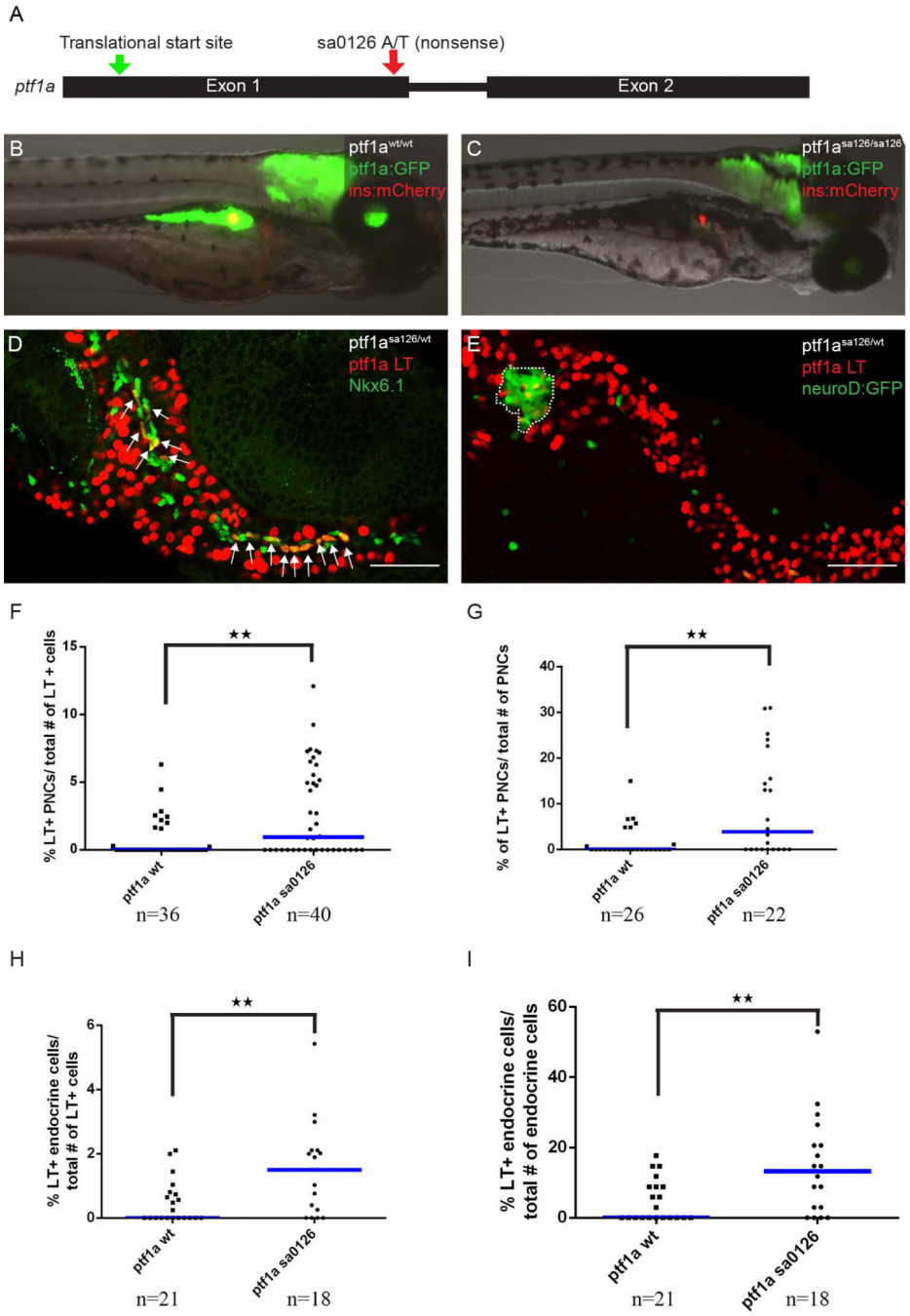

Figure 5. Ptf1a haploinsufficiency increases the the allocation of pancreatic progenitor cells to non-acinar lineages

(A) The *ptf1a*^{sa126} allele. This allele has an A to T transition in the first exon of *ptf1a* coding sequence, introducing a premature stop codon. (B) Wildtype larvae, with *ptf1a:GFP* (green) marking acinar cells and *ins:mCherry* (red) transgene marking β cells. (C) *ptf1a*$^{sa126/sa126}$ homozygous fish do not have exocrine pancreas. (D, E) The *ptf1a* lineage is labeled by nuclear-mCherry expression (red). (D) In *ptf1a*$^{sa125/wt}$ fish, more lineage-labeled cells can be traced into Nkx6.1+ (green) PNCs. Arrows point to co-labeling events. (E) In *pf1a*$^{sa126/wt}$ fish, more lineage-labeled cells can be traced into the *neuroD:GFP+* endocrine compartment.

The principal islet is outlined. (F, G) Quantification of *ptf1a* lineage-labeled cells contributing to PNCs, normalized by total number of *ptf1a*-lineage labeled cells (F), or by the total number of PNCs (G). (H, I) Quantification of *ptf1a*-lineage labeled cells contributing to the endocrine compartment, normalized by total number of *ptf1a*-lineage labeled cells (H), or by the total number of endocrine cells (I). (F-I) “n” indicates the number of fish quantified for each genotype. Blue bars show population median.

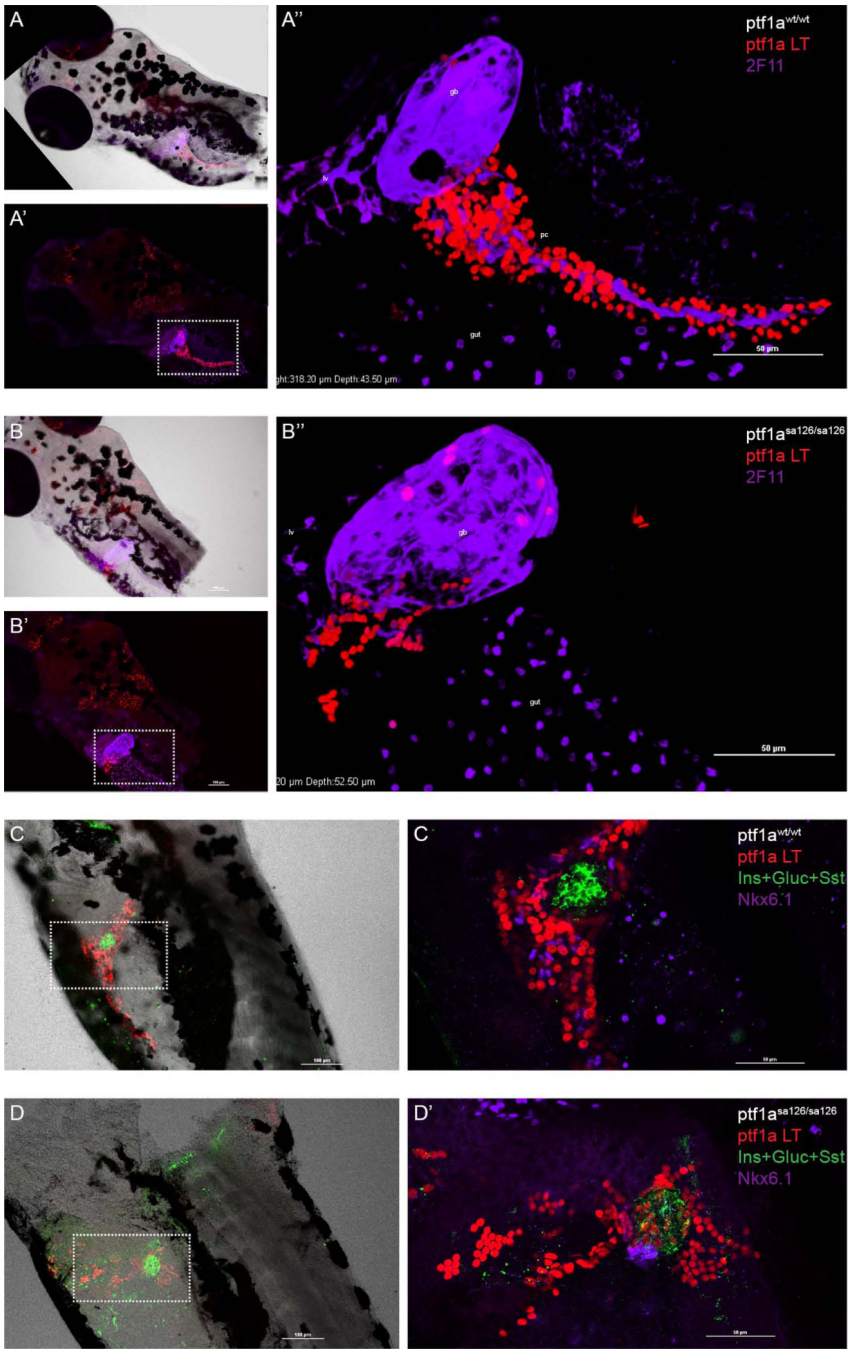

Figure 6. *ptf1a* lineage analysis in homozygous *ptf1a*$^{sa126/sa126}$ fish

In all panels, *ptf1a* lineage-labeled cells are shown in red. (A) In wildtype zebrafish, *ptf1a* lineage labeling can be observed in the hindbrain and pancreas (A'). A'' is a high magnification, 3D-reconstructed view of the boxed region in A'. 2F11 (purple) labels gall bladder, liver, pancreatic PNCs, and some structures in the gut bulb. (B) In *ptf1a*$^{sa126/sa126}$ fish, there is no exocrine pancreatic structure. Some lineage labeled cells can be observed in the wall of gall bladder, as well as in another unidentified population adjacent to the gall bladder. B'' is a zoomed-in view of the boxed region in B'. 2F11, purple. (C, D) A mixture

of antibodies against Insulin (Ins), Glucagon (Gluc) and Somatostatin (Sst) (green) labels the endocrine cell population. Nkx6.1 (purple) labels PNCs. (C) In the wildtype zebrafish pancreas, the majority of lineage labeled cells are acinar cells. (D) In homozygous *ptf1a* mutant fish, the number of PNCs is reduced and they appear at an ectopic location. *ptf1a* lineage labeled cells can be detected in the principal islet and some of the PNCs. gb, gall bladder. lv, liver.