ABSTRACT
The exocrine pancreas displays a significant capacity for regeneration and renewal. In humans and mammalian model systems, the partial loss of exocrine tissue, such as after acute pancreatitis or partial pancreatectomy induces rapid recovery via expansion of surviving acinar cells. In mouse it was further found that an almost complete removal of acinar cells initiates regeneration from a currently not well-defined progenitor pool. Here, we used the zebrafish as an alternative model to study cellular mechanisms of exocrine regeneration following an almost complete removal of acinar cells. We introduced and validated two novel transgenic approaches for genetically encoded conditional cell ablation in the zebrafish, either by caspase-8-induced apoptosis or by rendering cells sensitive to diphertheria toxin. By using the ela3l promoter for exocrine-specific expression, we show that both approaches allowed cell-type-specific removal of >95% of acinar tissue in larval and adult zebrafish without causing any signs of unspecific side effects. We find that zebrafish larvae are able to recover from a virtually complete acinar tissue ablation within 2 weeks. Using short-term lineage-tracing experiments and EdU incorporation assays, we exclude duct-associated Notch-responsive cells as the source of regeneration. Rather, a rare population of slowly dividing ela3l-negative cells expressing ptf1a and CPA was identified as the origin of the newly forming exocrine cells. Cells are actively maintained, as revealed by a constant number of these cells at different larval stages and after repeated cell ablation. These cells establish ela3l expression about 4-6 days after ablation without signs of increased proliferation in between. With onset of ela3l expression, cells initiate rapid proliferation, leading to fast expansion of the ela3l-positive population. Finally, we show that this proliferation is blocked by overexpression of the Wnt-signaling antagonist dkk1b. In conclusion, we show a conserved requirement for Wnt signaling in exocrine tissue expansion and reveal a potential novel progenitor or stem cell population as a source for exocrine neogenesis after complete loss of acinar cells.

KEY WORDS: Exocrine pancreas, Regeneration, Ablation systems, Wnt pathway, Ptf1a, Zebrafish

INTRODUCTION
The pancreas is a vertebrate-specific endodermal organ executing major functions in food digestion and glucose homeostasis. The mature organ is composed of an exocrine compartment with acinar and duct cells that produce and transport digestive enzymes into the gut, and an endocrine compartment from which metabolism-regulating peptide hormones including insulin are secreted into the blood stream. Studies on pancreas regeneration have focused mainly on the endocrine compartment, with the aim of gaining knowledge on β-cell regeneration. More recently, exocrine pancreas regeneration has been receiving more attention in the context of cancer and diabetes research because experimental models revealed a high capacity for cell fate plasticity (Murtough and Keefe, 2015).

Regeneration of exocrine tissue has been mainly studied in mouse models, where the loss of cells can be induced by caerulein treatment or partial duct ligation (Aghdassi et al., 2011; Lerch and Gorelick, 2013). Using these procedures, repair mechanisms such as acinar cell proliferation along with de-differentiation and re-differentiation processes were described (Desai et al., 2007; Strobel et al., 2007). Because of the lack of lineage-tracing experiments, the origin of regenerating acinar cells could not be determined. Possible sources include acinar cells, centroacinar cells, duct cells or adult progenitor cells (Siveke et al., 2008). The latest findings suggest that more extreme acinar loss in ElaCre-ERT2;R26DTR mice is not only repaired by acinar cell proliferation but also by differentiation of non-acinar cells (Criscimanna et al., 2011). The nature of these cells has not been clarified, although the data suggest involvement of duct or duct-associated cells.

Here, we used the zebrafish as an alternative model for studying exocrine pancreas regeneration. Importantly, the pancreata in mammalian systems and fish have not only conserved physiological functions and similar cellular compositions and structures, but also conserved expression and function of most genes involved in organ development (Argenton et al., 1999; Biemar et al., 2001; Eames et al., 2010; Jurczuk et al., 2011; Lin et al., 2004; Yee et al., 2005; Zecchin et al., 2004). Similar to mammals, the zebrafish pancreas arises from two progenitor domains called the dorsal bud and the ventral bud (Field et al., 2003; Hesselson et al., 2009). The dorsal bud develops after 24 hours post fertilization (hpf) and consists exclusively of clustered early endocrine cells known as the principal islet. The ventral bud appears after 36 hpf, grows to engulf the principal islet (Field et al., 2003; Wang et al., 2011) and gives rise to later-forming endocrine cells and all exocrine compartments (Field et al., 2003; Hesselson et al., 2009; Lin et al., 2004; Wang et al., 2011; Zecchin et al., 2004). Development of the exocrine pancreas, as described by Yee et al. (2005) using histological, immunohistochemical and ultrastructural approaches, can be followed by the successive induction of trypsin (48 hpf), carboxypeptidase A (CPA) (60 hpf) and elastase 3-like (ela3l).
However, the molecular cascades of events involved in exocrine pancreatic differentiation and regeneration in zebrafish are not well defined.

As part of this study, we also introduced two novel approaches for genetically induced cell ablation into the zebrafish. One method uses the application of diphtheria toxin (DT) in combination with transgene-driven, exocrine-cell-specific expression of the human DT receptor (HB-EGF), rendering cells sensitive to DT (Furukawa et al., 2006; Saito et al., 2001). In the second approach, cell-type-specific apoptosis can be induced by conditional dimerization of a membrane-bound caspase-8-FKBP fusion protein, which then initiates the caspase cascade. Both techniques have been successfully established in mouse models to ablate β-cells of the endocrine pancreas, exocrine cells and adipocytes (Criscimanna et al., 2011; Pajvani et al., 2005; Thorel et al., 2010), but have not previously been applied in zebrafish.

By introducing these systems into the pancreas of zebrafish, we were able to efficiently ablate acinar cells in larval through to adult stage animals, and we proceeded with a detailed examination of the exocrine regeneration process. Studies on larval pancreas regeneration revealed different mechanisms and dynamics of regeneration depending on the time point of treatment. While our studies found no evidence for an involvement of duct and centroacinar cells in exocrine regeneration, they revealed the presence of a previously uncharacterized ptf1α−, ela3l− cell population and propose that these cells are a novel type of pancreatic progenitor cell. Following a virtually complete removal of acinar gland cells, these ptf1α, ela3l cells differentiated into exocrine cells and restored exocrine cell mass by subsequent Wnt-signaling-dependent proliferation.

RESULTS

Complete exocrine cell ablation using two novel cell-ablation approaches in zebrafish larvae and adults

Currently, the most efficient system for genetically encoded conditional cell ablation in zebrafish is based on the transgenic expression of bacterial nitroreductase (NTR) to sensitize specific cells to the antibiotic metronidazole (Met; Curado et al., 2007; Pisharath et al., 2007). The possibility of unwanted side effects from metronidazole interfering with microbiota led us to explore two alternative ablation methods, which had been shown to enable almost complete removal of pancreatic and other cell types in adult mice. These approaches either utilize induction of apoptosis through caspase 8 or they render cells sensitive to diphtheria toxin (Carlotti et al., 2005; Criscimanna et al., 2011; Pajvani et al., 2005; Thorel et al., 2010). To test these techniques in the context of exocrine pancreas regeneration, we generated transgenic lines expressing the mediator proteins together with the in vivo reporter E2Crimson under control of the acinar-specific ela3l promoter (Wan et al., 2006). In Tg(ela3l:casps8;ela3l:E2Crimson) embryos (termed ela:casp8) the mediator protein consists of the p20 and p10 catalytic domains of human caspase 8 fused to serial FKBPv dimerization domains and a myristoylation site to provide membrane attachment (Pajvani et al., 2005) (Fig. 1A). Incubation of 5 dpf ela:casp8 embryos in 5 µM dimer-inducing agent AP20187 (termed Dim) resulted in loss of E2Crimson signal within 2 days of treatment (0 days post ablation or 0 dpa) (Fig. 1B). To determine optimal conditions for ablation and to test for potential side effects, embryos were treated with 3 different concentrations of Dim (1.6 µM, 5 µM and 8 µM) for two different time periods (48 and 96 h). Quantification of ela3l and trypsin mRNA expression levels via RT-qPCR confirmed a dose- and time-dependent removal of exocrine tissue (Fig. 1C). The lowest concentration of 1.6 µM Dim resulted in 85% and 77% reduced ela3l and trypsin levels after 48 h of treatment and a 95% reduction of these RNAs after 96 h. Treatment with 8 µM Dim caused an ela3l and trypsin reduction of 95% and 94% after 48 h and 98% to 99% (96 h), respectively. Importantly, the prolonged treatment for 96 h did not cause any obvious side effects as revealed by careful macroscopic analyses of more than 100 embryos and larvae (data not shown). Caspase-8-induced apoptosis was detected in exocrine cells as early as 8 h after administration of Dim, as revealed by TUNEL assays (Fig. 1D). Time-lapse analysis of an ela:casp8 embryo treated with 5 µM Dim recorded between 6 and 16 h after the start of treatment visualization the accumulation of E2Crimson and the concurrent loss of E2Crimson-expressing cells (Movie 1). A apoptotic ablation of exocrine tissue was confirmed by the loss of cell membrane integrity in cldn:lyn-eGFP embryos (Haas and Gilmour, 2006), in which all epithelial cells are marked by membrane-tagged eGFP (Fig. S1A,B). Based on these results, treatment with 5 µM Dim for 48 h was used for further experiments.

The second ablation system uses the human diphtheria toxin receptor HB-EGF (Furukawa et al., 2006) (DTR) to make cells sensitive to administrated diphtheria toxin (DT) (Fig. 1E). Neither the expression of human DTR in Tg(ela3l:DTR;ela3l:E2Crimson) (termed ela:DTR) animals nor the incubation of wild-type animals in DT concentrations up to 50 µg/ml caused any distinguishable phenotypes in more than 100 analyzed control embryos and larvae (data not shown). However, incubation of ela:DTR transgenic fish in 10 µg/ml and 15 µg/ml DT caused reduced E2Crimson signals at 72 h of treatment. Moreover, an almost complete loss was achieved after 96 h (Fig. 1F). RT-qPCR analyses showed that expression of exocrine markers was unchanged after 48 h of treatment with 15 µg/ml DT, while after 96 h of treatment, ela3l mRNA was reduced by 93% (10 µg/ml DT) and 96% (15 µg/ml DT), and trypsin levels were reduced by up to 92% (Fig. 1G). Further analyses of DT-treated ela:DTR animals by TUNEL and in a cldn:lyn-eGFP background (Fig. 1H; Fig. S1C) revealed the first apoptotic cells after 72 h of treatment. The data show that both ablation approaches allow an almost complete removal of larval exocrine tissue within 4 days of treatment and that kinetics of apoptosis induction were slightly slower in DT-treated ela:DTR fish compared with Dim-treated ela:casp8 animals.

To determine if neighboring cells are affected by the ablation of exocrine cells, treatment using either ela:casp8 (with 5 µM Dim) or ela:DTR transgenes (with 15 µg/ml DT) were performed in GFP-reporter backgrounds for Notch-responsive cells (NRCs) (Tpal: GFP, Fig. 2A-C) and β-cells (ins:CD4-GFP, Fig. 2D). Notably, pancreatic NRCs have recently been revealed to be centroacinar cells serving as endocrine progenitors (Beer et al., 2016; Delaspre et al., 2015; Ghaye et al., 2015). Removal of exocrine cells in the Tpal:eGFP background resulted in the disruption of the net-like organization of the NRCs. However, pancreas-specific GFP volumes in these fish were not affected at any time point after treatment, suggesting that the total number of NRCs was unaffected by the treatment (Fig. 2C). Using a transgenic line labeling β-cells via eGFP fused to the membrane-localized human CD4 protein, we also found that neither the islet morphology nor the number of β-cells was affected by acinar cell ablation (Fig. 2D).

As the examination of repair and regeneration, especially in adult stages, is becoming more important, we tested the ability of our alternative ablation methods to remove exocrine cells in >6-month-old adult fish carrying either the ela:casp8 or the ela:DTR transgene. In ela:casp8 animals, a single injection of 20-30 ng/g Dim achieved...
a nearly complete ablation after 14 days (n=5, data not shown), whereas the 75 ng/g Dim injection caused the loss of fluorescent signals within 4 days (Fig. S2A). Adult ela:DTR fish were injected once with 20 ng/g DT intraperitoneally and subsequently displayed strongly reduced fluorescence 3-4 days post injection (Fig. S2B). The removal of exocrine cells was confirmed in histological sections of inner organs (Fig. S2A′,B′). In addition, we established transgenic lines for the ablation of the insulin-producing β-cells (Fig. S2C,D). Both treatments led to the loss of E2Crimson-expressing β-cells (97% for the caspase-8-induced ablation and 96% for the DT-mediated ablation) and correspondingly reduced relative expression of insulin mRNA (96% for the caspase-8-induced and 91% for the DT-mediated ablation) (Fig. S2E). In conclusion, these studies demonstrate that both the inducible activation of caspase 8,
as well as the expression of human DTR, enable efficient cell-type-specific ablation in embryonic and adult stages in zebrafish.

**Dynamics of exocrine cell regeneration**

The analyses of ela:casp8;Tp1:eGFP and ela:DTR;Tp1:eGFP larvae at 10 dpa revealed that both lines had re-established E2Crimson-positive cells directly adjacent to GFP-positive NRCs (Fig. 2A,B). For a more detailed understanding of the regeneration dynamics, ela:casp8 embryos that had been treated with Dim from 5 to 7 dpf were analyzed at 2 day intervals from 0 to 8 dpa (Fig. 3A-G). In addition, EdU treatments starting 2 days before fixation were used to correlate proliferation behavior with regeneration dynamics (Hesselson et al., 2009; Kramer-Zucker et al., 2005). Fixed and stained larvae were documented by 3D confocal microscopy (Fig. 3B,E). As clumping of E2Crimson protein prevented accurate cell assignments, a volumetric approach was used for quantification of E2Crimson staining (Fig. 3C,F; Fig. S3). In addition, experiments were performed in ela3l:casp8/ela3l:H2B-GFP double transgenic animals to enable cell-counting based on nuclear-specific H2B-GFP expression (Fig. 3B,D,E,G; Table S1). Consistent with the RT-qPCR analyses, volumetric analyses (Fig. 3C,F) and counting of cells (Fig. 3D,G) showed a 94% reduction of the ela3l+ cells at 2 dpa. Between 2 and 4 dpa, ela3l+ cell mass increased to 8% of the control larvae, while between 6 and 8 dpa a rapid increase of exocrine tissue occurred reaching 18% and 26% of control levels, respectively. Unlike control embryos, which mainly lacked EdU+, ela3l+ cells between 9 and 17 dpf (Fig. 3B-D), Dim-treated embryos showed elevated proliferation rates at 6 dpa and lower rates at 8 dpa (Fig. 3E-G). The data imply proliferation of the remaining E2Crimson+ cells as a mechanism of exocrine tissue repair. We speculated that these cells were either resistant to Dim or they may have escaped ablation because of a later onset of caspase-8/E2Crimson expression only after Dim exposure. We considered resistance unlikely as the

**Fig. 2. Ablation of exocrine cells does not affect neighboring NRCs and β-cells.** (A,B) Confocal image projections of ela:casp8;Tp1:eGFP (A) and ela:DTR;Tp1:eGFP (B) in untreated control animals (7 dpf for ela:casp8 and 9 dpf for ela:DTR), animals directly after treatment and 10 dpf animals. Note that newly established exocrine cells are found next to NRCs. (C) Volume of pancreatic NRCs, determined by GFP expression, is not significantly changed after ablation and during regeneration of exocrine cells in ela:casp8 embryos (n=5 larva for each time point). (D) β-cells labeled using Tg(ins:CD4-eGFP). The number of β-cells was unaffected by treatment with dimerizer (n=6). Values are mean±s.e.m. Scale bars: 100 µm (A,B) and 20 µm (D).
Fig. 3. See next page for legend.
reduction of E2Crimson+ tissue volume between 0 dpa (16% of control levels) and 2 dpa (6%) suggests that eventually all ela:cas8-expressing cells undergo apoptosis, but with slightly variable dynamics. Based on the high proliferation rate of ela3l cells seen between 5 and 7 dpf in control embryos (Fig. 3B-D), we hypothesized that some cells were not fully matured and therefore did not express ela3l during the treatment. In this case, a treatment starting at 7 dpf, which is after the drop in proliferation, should result in a more complete removal of exocrine tissue. To better distinguish between an ablation-induced regeneration response and the normal development based exocrine differentiation, regeneration experiments and 2 day EdU treatments were repeated in ela:cas8 larvae treated with Dim at 7-9 dpf (Fig. 3H-J). Consistent with our expectation, this ‘late’ treatment resulted in a virtually complete loss of ela3l+ cells at 2 and 4 dpa (Fig. 3LJ). This was confirmed by RT-qPCR analyses, which revealed 2.6-times lower ela3l mRNA levels after 7-9 dpf Dim treatment compared with ‘early’ treatments (Fig. S4). Unlike the situation in early-treated larvae, very few proliferating E2Crimson+ cells were found before 6 dpa (Fig. 3LJ). However, at 8 dpa, rapidly increasing numbers of ela3l+ cells were observed in late-treated larvae and at 8 dpa, these larvae had recovered about 23% tissue compared with control animals (Fig. 3H″″,LJ).

While exocrine tissue repair in larvae ablated at 5-7 dpf may happen through self-replication of late-maturing ela3l+ cells, the data for the ‘late’ treatment suggest the existence of an alternative regeneration path using ela+ cell neogenesis.

Regeneration of exocrine cells does not involve ductal cells

Studies on exocrine differentiation in mouse showed different modes of regeneration. A partial loss of exocrine tissue appears to be compensated mainly by the proliferation of remaining exocrine cells (Desai et al., 2007; Strobel et al., 2007), whereas complete removal induces exocrine neogenesis from a currently undefined progenitor population (Criscimanna et al., 2011). Pancreatic duct cells had been suggested as a potential source of this progenitor population, even though lineage-tracing studies supporting this hypothesis are lacking (Criscimanna et al., 2011).

To clarify whether duct cells contribute to exocrine tissue regeneration in zebrafish, we performed short-term tracing experiments with the duct-specific reporter lines Tg(Tp1:eGFP), Tg(Tp1:H2B-mCherry) and Tg(nks2.2a:eGFP) (Ninov et al., 2012; Parsons et al., 2009; Pauls et al., 2007). In these experiments, ela:cas8 larvae, treated with Dim from 7 to 9 dpf were analyzed at different time points during regeneration. We found no evidence for expression of NRCs or duct cell reporters in newly forming exocrine cells at 8 dpa (Fig. S5). A loss of lineage tracer by protein decay is unlikely as H2B-mCherry had been reported to be stable for weeks (Bremand et al., 2007; Hesselson et al., 2009). To exclude the dilution of lineage trace by massive proliferation, we also performed 2 day EdU treatment starting directly after Dim treatment (Fig. 4, Fig. S6). We found that the majority of eGFP/mCherry-positive cells in 2 dpA larvae had no EdU signal. Hence, we concluded that neither Notch-responsive cells nor other duct cells are a source for newly established exocrine cells.

Identification of ptf1a+, CPA+, ela3l+ cells during exocrine regeneration

In order to identify regenerating cells at an earlier stage of maturation, we performed antibody staining for the early maturation marker CPA (Zhou et al., 2007). Analyses at 2 dpA revealed rare CPA+, E2Crimson+ cells (5-8 cells per embryo) next to the eGFP- or H2B-mCherry-labeled duct cells (Fig. 4). Few of these CPA+ cells contained EdU label, indicating a low proliferation rate (in total 6 cells in 36 embryos; Fig. 4B-D). Therefore, we hypothesized that CPA staining marks slowly proliferating progenitors of the regenerating exocrine cells. As pancreas-specific transcription factor 1a (Ptf1a) is essential for differentiation of exocrine pancreas (Dong et al., 2008; Lin et al., 2004; Zacchini et al., 2004), we expected a regulation of ptf1a in such progenitors cells at some stage of exocrine regeneration. To correlate CPA expression with that of ptf1a during pancreatic regeneration, we performed ablation studies combined with EdU incorporation experiments in the ptf1a:eGFP background. We found that Dim treatment did not remove all ptf1a:eGFP+ cells and that CPA expression and the rare EdU labels were restricted to the pool of these remaining GFP+ cells (Fig. 4E). Genetic lineage-tracing studies previously showed that Notch-responsiveness and ptf1a expression correlates with mainly different pancreatic lineages (Wang et al., 2011, 2015). Consistently, no overlap for GFP and mCherry was found in ptf1a:eGFP/Tp1:H2B-mCherry and ela3l:H2B-GFP/tp1:H2B-mCherry control animals between 3 and 7 dpf (Fig. S7). By investigating 2 dpA larvae in triple transgenic ela:cas8;ptf1a:eGFP;tp1:H2B-mCherry animals, we further found that ptf1a+ E2Crimson+ cells reside next to NRCs at 2 dpA and 8 dpA but that they lack the NRC-lineage marker H2B-mCherry and therefore are also not derived from NRCs (Fig. 4F).

Presence of ptf1a+, ela3l+ cells during normal development and exocrine regeneration

Detailed analyses of confocal images taken from ptf1a:eGFP:ela:cas8 animals at different stages of development showed GFP+, E2Crimson+ cells, not only in early larval stages, but also in juvenile fish (Fig. 5A-C). Corresponding cells preferentially localized to the pancreas periphery where they were found intermingled with E2Crimson+ cells. Manual examination of confocal image stacks taken from pancreatic head of regions of 5 dpf to 30 dpf animals was used to estimate their frequency of occurrence. The studies also revealed that eGFP+, E2Crimson+ cells lacked enzyme granules seen in all E2Crimson+ cells, providing an additional morphological criterion for their identification (inset in Fig. 5A). In 5 dpf embryos, we counted 180 eGFP+ cells per embryo.
including 7-9 cells (5%) that lacked E2Crimson. The proportions of E2Crimson− cells decreased to ∼3% at 7 dpf, 2% at 9 dpf, down to 1-2% in 20 dpf and 30 dpf larva (Fig. 5D). Taking into account the rapid growth of the pancreas during these stages (Wan et al., 2006), this suggests that the total number of ptf1a+, ela3l− cells per pancreas remains relatively constant. The data highlight ptf1a+, ela3l− cells as a developmentally maintained rare subpopulation within the ptf1a:eGFP+ pool.

To determine whether and how these cells contribute to exocrine cell recovery we followed regeneration in ela:casp8;ptf1a:eGFP larvae over a period of 8 days after a 7-9 dpf ablation procedure. Incubation of larvae in EdU over 2 days before fixation was used to
At 6 dpa, a reduced number of eGFP + E2Crimson− cells could be
distinguished, while the number eGFP+ E2Crimson+ cells rapidly
increased and reached around 30 cells/embryo at 8 dpa (Fig. 6D).
Consistent with our earlier observation, very few EdU signals were
detected in eGFP+ E2Crimson− cells (7%, n=97 cells), whereas
after 6 dpa, more than 52% of eGFP+ E2Crimson+ cells showed
EdU signals (Fig. 6B-E, see also Fig. 4). The data confirm the
slowly proliferating ptf1a+, ela3l− cells residing in the pancreas as
the source for exocrine cell regeneration. They further suggest that
concurrent with or after onset of ela3l expression at around 6 dpa,
these cells switch to rapid proliferation.

As the number of eGFP+ E2Crimson− cells appeared to decrease
with onset of E2Crimson expression, we were interested to find out
if all ptf1a− cells eventually differentiated into acinar tissue during
regeneration. In this case, a second ablation treatment shortly
after onset of ela3l expression (10-12 dpa) was expected to cause the
complete removal of ptf1a−, ela3l− cells. In contrast to this
expectation, the corresponding experiment using ela:casp8 larvae
revealed no difference between the number of ptf1a−, ela3l− cells
after single or after repeated ablation (Fig. S9). In both cases,
analyses at 0 dpa revealed about 6-7 ptf1a−, ela3l− cells (n=7
embryos). This shows that a constant number of ptf1a−, ela3l− cells
is maintained during regeneration and that only a subpopulation of
ptf1a−, ela3l− cells contributes to exocrine cell neogenesis.

Wnt signaling is required for proliferation of exocrine cells
during the later regenerative phase
Canonical Wnt signaling has been reported to play a key role in
exocrine cell proliferation and regeneration in mouse (Keefe et al.,
2012; Murtaugh et al., 2005; Murtaugh and Keefe, 2015; Nakhai
et al., 2008; Wells et al., 2007). To test whether Wnt signaling has
similar functions in zebrafish, we used the Tg(hsp70l:dkk1-eGFP)
line for conditional activation of the Wnt-signaling inhibitor
dickkopf 1 (Dkk1) (Stoick-Cooper et al., 2007). Consistent with a
conserved requirement for Wnt signaling in normal late pancreatic
development, in hsp70l:dkk1-eGFP embryos that received two heat
shocks at 5 and 6 dpa, ela3l expression was reduced by 26% at 7 dpa
(Fig. S10). To determine the functions of Wnt during regeneration,
ela:casp8:hsp70l:dkk1-eGFP animals were treated with Dim at 7 to
9 dpa and then heat shocked twice either at 4 and 5 dpa or at 6 and
7 dpa (Fig. 7A). EdU treatment started 1 hour after the first heat
shock and continued until fixation 1 day after the second heat shock;
these were used as a quantitative measure of mitosis (Fig. 7B-D).
While induction of dkk1 had only mild effects on the volume of
exocrine tissue, we observed significantly reduced numbers of EdU+
cells in dkk1-induced embryos compared with sibling larvae lacking
the hsp70l:dkk1-eGFP transgene. The number of EdU+ E2Crimson−
cells per pancreatic head region was reduced by more than 83% in
animals heat treated at 4 and 5 dpa, and by 95% in animals heat
treated at 6 and 7 dpa (Fig. 7B,C). In conclusion, this suggests that
proliferation of exocrine cells during development and regeneration
is dependent on Wnt signaling.

DISCUSSION
In this study we used the zebrafish as a model system to study
exocrine pancreas regeneration. As part of these experiments we
introduced two genetically inducible cell ablation systems into the
zebrafish and we demonstrate that both systems allow highly
efficient and specific removal of exocrine tissue in larvae and adult
animals. Furthermore, we show that zebrafish larvae are able to
recover from a complete loss of exocrine tissue in less than 2 weeks,
and we identified a developmentally maintained rare population of

define sites of cell proliferation (Fig. 6). Between 0 and 4 dpa, the
pancreatic head region contained around 5-7 cGFP-expressing cells
lacking E2Crimson and a similar number of spots with overlapping
cGFP and E2Crimson signals (Fig. 6A,D). While these spots were
counted as cells, their abnormal morphology (Fig. 6A,B), the
absence of trypsin expression as revealed by qPCR and in situ
hybridization analyses (Fig. 1C; Fig. S8) and the complete lack of
EdU in a total of 73 cells (Fig. 6E), suggest that most of these signals
correspond to apoptotic cells and post-apoptotic cell debris. Starting

Fig. 5. Ptf1a+, ela3l− cells are present at different developmental stages.
(A-C) Confocal images of the anterior pancreatic region of Tg(ela:casp8; ptf1a:
cGFP) in 5 dpf (A), 9 dpf (B) and 30 dpf (C) animals (arrows indicate selected
GFP+, e2Crimson− cells). (D) Relative amount of ptf1a+, ela3l− cells at different
developmental stages (n>5). Mean+s.e.m. Scale bar: 20 µm.
$ptf1a^+$, $ela3l^-$ cells as the source of this regeneration. Our data suggest that exocrine regeneration requires an initial proliferation-independent maturation process in these cells, which is then followed by a phase of Wnt-regulated rapid proliferation and cell expansion.

Introduction of two alternative methods for genetically encoded inducible cell ablation to zebrafish

The zebrafish embodies a remarkable regenerative capacity as it is able to regenerate liver (Choi et al., 2014), endocrine $\beta$-cells in embryonic and adult stages (Curado et al., 2007; Delaspre et al., 2015; Ghaye et al., 2015; Moss et al., 2009; Pisharath et al., 2007), heart, neurons and even whole fins, including bone and cartilage tissue (reviewed in Gemberling et al., 2013). Conditional targeted ablation is a powerful tool to study the role of specific cell lineages, tissues or physiological responses involved in regenerative processes. Several genetically encoded systems have been tested transiently in zebrafish embryos (reviewed in Curado et al., 2008), but until now, the only efficient and reliable method was the NTR-mediated conversion of antibiotic metronidazole into a cytotoxic agent (Curado et al., 2007; Pisharath et al., 2007). Here, we introduce and validate two alternative approaches for inducible, genetically encoded cell ablation in zebrafish. We show that both approaches are highly efficient in removing cells of the exocrine compartment of the pancreas without affecting adjacent tissues. Treatment of embryos expressing FKBP-caspase-8 with Dim induced apoptosis of cells within 8 h and resulted in >95% reduced expression of exocrine markers within 48 h. The DT-mediated approach was similarly efficient but slower, probably since it is not directly interacting with the cellular apoptosis machinery (Morimoto and Bonavida, 1992). In this approach, the first apoptotic cells appeared after 60 h of treatment, and a virtually complete removal of cells was achieved between 72 and 96 h. Furthermore, both methods were also highly efficient and specific in ablating exocrine tissue in adult animals and in removing the insulin-producing $\beta$-cells of the endocrine pancreas. Dim-mediated ablation as well as DT-induced removal of cells in adult animals was achieved after a single injection within a few days, as revealed by the
loss of fluorescent cells and by the lack of these cells in histological sections. Regeneration models using juvenile or adult animals rather than embryos become more important as they better resemble human pathologies. Furthermore, regeneration events in embryonic stages potentially represent a continuation of developmental events and might not be the same as regeneration in adult animals (Hesselson et al., 2009). While additional experiments will be required for a detailed evaluation and comparison of potentially missed side effects, our results highlight both ablation approaches as an attractive alternative to NTR/Met for future ablation studies using zebrafish. Unlike NTR/Met, both methods utilize eukaryote-specific pathways for cell ablation that should not interfere with microorganisms. Therefore, these methods might be of particular interest for studying the role of internal microbiota, which have recently gained major attention as important modulators of physiology, disease and regeneration (Bates et al., 2006; Chang and Lin, 2016; Cheesman et al., 2011; Hill et al., 2016; Kostic et al., 2013; Reikvam et al., 2011; Shanahan, 2013).

Distinct modes of exocrine cell regeneration
Regeneration studies have revealed an amazing flexibility and plasticity in tissue recovery. For example, observations of β-cells and liver regeneration showed that different sources for newly forming cells can be mobilized depending on the extent, timing and cell-specificity of tissue disruption (Aloia et al., 2016; Chera and Herrera, 2016; Kopp et al., 2016). Different modes of regeneration have also been described for exocrine tissue recovery in mouse. Following partial ablation by caerulein treatment or duct ligation, the exocrine tissue was re-established primarily by proliferation of residual exocrine cells (Desai et al., 2007; Strobel et al., 2007). Another study using genetically encoded expression of the human DTR in exocrine cells and administration of DT induced almost complete ablation of exocrine cells and showed regeneration by differentiation from the mature duct, terminal duct cells, centroacinar cells or another unidentified progenitor cell population (Criscimanna et al., 2011). While our data suggest a different source for the regenerating acinar cells in exocrine tissue-
depleted zebrafish larvae, they also revealed two distinct modes or phases of regeneration. In particular, we find that the complete removal of ela3l+ cells resulted in a phase of about 4 days with minor pancreatic proliferation and no exocrine maturation, which was then followed by onset of ela3l expression and massive proliferation of ela3l+ cells. These results are consistent with a two-phase regeneration process, with phase 1 being the slow neogenesis of ela3l+ cells, and phase two, the rapid proliferation of mature or partly matured ela3l+ cells as the major mechanism of exocrine tissue recovery. In this context, it is interesting to note that the faster partly matured exocrine cells in zebrafish larvae was then followed by onset of minor pancreatic proliferation and no exocrine maturation, which ablation treatments not after later ablation (Yee et al., 2005). Our ela3l these cells during larval stages and after repeated ablation argue option, the time frame of differentiation and the maintenance of expressing ordinary differentiating exocrine cells not yet (or weakly) expressing ela3l:E2Crimson signal. As CPA and Ptf1a expression have been reported to precede that of zymogens such as elastase in normal exocrine maturation (Gittes and Rutter, 1992; Guerrero et al., 2015; Han et al., 1986; Schick et al., 1984), these cells might represent ordinary differentiating exocrine cells not yet (or weakly) expressing ela3l. While our data do not entirely exclude this option, the time frame of differentiation and the maintenance of these cells during larval stages and after repeated ablation argue against it. Based on the reported delay of 1-2 days between CPA and ela3l expression, a maturation-dependent recovery of ela3l should start immediately after ablation, as it was only the case after 5-7 dpf ablation treatments not after later ablation (Yee et al., 2005). Our data suggest ptf1a+, ela3l+ cells as a previously unrecognized progenitor population for acinar cell neogenesis. These cells could be monopotent progenitor cells similar to the STMN1+ cells recently identified in the adult mouse pancreas (Wollny et al., 2016). While STMN1+ and ptf1a+, ela3l+ cells both share a low prevalence in the mature pancreas, STMN1+ cells, unlike the ptf1a+, ela3l+ cells, express mature acinar markers such as trypsin and elastase. Alternatively, the ptf1a+, CPA+ cells might represent residual multipotent potential progenitor cells with the potency to form endocrine, exocrine and duct cells, which have been reported during the early phase of mouse pancreas development (Zhou et al., 2007). Recently, lineage-tracing studies in zebrafish revealed the presence of a similar multipotent progenitor population in early pancreatic development (Wang et al., 2015). Using a ptf1a:CreERT2 line, the contribution of early stage ptf1a+ cells to ~6% of NRCs in 6 dpf embryos and to adult acinar cells was shown. Corresponding mouse lineage-tracing studies using Cpa1CreERT2 and Ptf1aCreERTM also revealed a shift from multipotency to acinar-specific unipotency after secondary transition (Zhou et al., 2007), while multipotency was found to be (re)activated in Ptf1a+ cells by injury, e.g. partial duct ligation (Pan et al., 2013). Interestingly, earlier regeneration studies using ElastaseCreERT2 mice for cell tracking indicated an acinar-restricted differentiation potential in pre-existing acinar cells (Desai et al., 2007). It had been suggested that the lower Cre/loxP labeling efficiency of ElastaseCreERT2 compared with Ptf1aCreERTM might have hindered detection of the rare endocrine contributions in the ElastaseCreERT2 experiments (Pan et al., 2013).

Alternatively, Ptf1aCreERT2 might label additional cell populations and the potential for multipotency could be restricted to an Elastase−, Ptf1a− subpopulation. While future studies using genetic tracing approaches in combination with tissue-specific or ptf1a-regulated cell ablation will be required to clarify this option, it is tempting to speculate that ptf1a+, ela3l+ cells resemble a conserved multipotent pancreatic progenitor population.

**Maintenance of ptf1a+, ela3l+ cells during development and regeneration**

We find that the proportion of ptf1a+, ela3l− cells decreases with age, whereas the total number appears to be unaffected by age and even by repeated acinar ablation. This highlights ptf1a+, ela3l− cells as a maintained cell population of the pancreas. Currently, we can only speculate about the molecular and cellular mechanism regulating their maintenance, proliferation or induction of acinar differentiation. The constant number of ptf1a+, ela3l− cells during regeneration suggests asymmetric cell division with only one daughter cell initiating ela3l expression as the likely mechanism for progenitor maintenance. While this type of proliferation normally depends on lateral inhibition through Notch signaling, our short-term NRC-tracking analyses, in agreement with genetic lineage-tracing studies (Wang et al., 2011, 2015), excluded an involvement of canonical Notch signaling in exocrine regeneration. Interestingly, the lack of Notch responsiveness in acinar cells contrasts with the various requirements for Notch signaling and individual Notch signaling mediators in exocrine differentiation and regeneration (Ensi et al., 2004; Hidalgo Sastre et al., 2016; Kopinke et al., 2012; Siveke et al., 2008; Yee et al., 2005; Zecchin et al., 2007). Activation of the TP1-Notch reporter requires interaction of the intracellular part of Notch receptors (NICD) with its cofactor RBP-Jx (Parsons et al., 2009). As NICD and Ptf1a directly compete for RBP-J binding (Beret et al., 2006; Masui et al., 2007), Notch signaling might regulate ptf1a+, ela3l− fates without inducing TP1 reporter by reducing interactions between Ptf1a and RBP-J (Ensi et al., 2004; Gradwohl et al., 2000; Heremans et al., 2002). Consistent with this option, Ptf1a levels had been found to be crucial for the differentiation potential of ptf1a-expressing cells (Wang et al., 2015).

**Proliferation of ela3l+ cells and requirement of Wnt signaling in exocrine recovery**

The 4- to 5-fold increase of ela3l+ cell numbers between 4 and 6 dpf (Figs 3 and 6) demonstrate an amazing proliferation capacity of these cells, particularly as not all cells appear to contribute to acinar cell recovery. The lack of EdU signals in more than 50% of these cells, particularly as not all cells appear to contribute to acinar cell recovery. The lack of EdU signals in more than 50% of cells suggests that only a subset of these cells is proliferating with a rate above 1 division per day. In agreement with the rare division events observed in ptf1a+, ela3l− cells, this further suggests that very low numbers of newly formed ela3l− cells are sufficient to explain the observed recovery dynamics. In this context, the accumulation of newly forming ela3l− cell in a few cell clusters of 8 dpf animals (for example, 3 clusters with 6-32 cells in Fig. 3H′′) is consistent with the formation of these clusters from individual ela3l− cells. The heterogeneity in the proliferation behavior among the ela3l− cells reveals a rapid functional diversification. In analogy to adult stem cell systems, the newly formed ela3l+ cells might first expand as transit-amplifying cells before maturation into non-proliferating acinar cells. However, the striking similarities to the recently described heterogeneity among adult mouse acinar tissue (Wollny et al., 2016) support an alternative explanation for the different proliferation behavior of ela3l−. Accordingly, newly
formed ela3l− cells could be mono-potent acinar-specific progenitors with long-term self-renewal capacity. Finally, we found that proliferation of newly generated exocrine cells was strongly impaired upon induction of the Wnt antagonist Dkk1. As mouse studies previously established canonical Wnt signaling as a key regulator of differentiation, proliferation, maintenance and regeneration of acinar tissue (Keefe et al., 2012; Morris et al., 2010; Murtaugh et al., 2005; Murtaugh and Keefe, 2015; Nakhai et al., 2008; Wells et al., 2007), our results are consistent with a conserved requirement for Wnt signaling in acinar cell proliferation.

In early zebrafish development, Wnt signaling has been associated with hepatic specification and proliferation, and the inhibition of pancreatic specification (Goessling et al., 2008; Poulian and Ober, 2011). More recently, it was shown that prostaglandin E2 has the same effects in early development as activated Wnt signaling, but when applied at later stages, it also promotes expansion of the exocrine pancreas (Nissim et al., 2014). Considering the genetic interaction of prostaglandin E2 and Wnt signaling (Goessling et al., 2009), this suggested a conserved connection with Wnt signaling and late embryonic expansion of exocrine tissue. Consistent with this notion and in agreement with Wnt-specific interference, we found that induction of dkk1 reduced expression of the liver and acinar marker. Opposing effects of Wnt signaling on preventing early pancreas specification and supporting exocrine expansion have also been shown in mouse, where an early upregulation of β-catenin prevents normal formation of exocrine and endocrine compartments whereas upregulation at a later time point causes enhanced proliferation and increase in pancreas size (Heiser et al., 2006). Interestingly, the volume of E2Crimson− regenerated tissue was not reduced in the dkk1-induced animal as would be expected. Possibly, the short time window after heat treatment was sufficient for preventing S-phase-dependent EdU incorporation in the dkk1-GFP-induced embryo, but not long enough to interfere with a full division cycle. As increased Wnt activity resulted in smaller acinar cells (Heiser et al., 2006), the unchanged acinar volume might also result from interference with exocrine cell size.

Conclusions
We introduced two alternative approaches enabling complete ablation of pancreatic cells in the zebrafish and by using these techniques, we found ptf1a+ cells not expressing ela3l as a novel source of pancreatic regeneration. Furthermore, we identify Wnt signaling as important for the expansion of exocrine cell mass. Our data suggest a two-phase regeneration. Furthermore, we identify Wnt signaling as important for unchanged acinar volume might also result from interference with exocrine cell proliferation.

MATERIALS AND METHODS
Cloning of transgenic constructs
Transgenic lines were generated using the ‘Tol2-Kit’ (Kwan et al., 2007). 1.9 kb of the elastase3l promoter (Wan et al., 2006) and 1 kb of the insulin promoter (provided by Francesco Argenton, University of Padova, Italy) were cloned into the pSE-MCS. The DTR constructs were prepared by cloning the human HB-EGF(L117V/L148V) cDNA (Furukawa et al., 2006) into pSE-ela3l and the p5E-ins plasmids. The FKBP-caspase8 fusion cDNA (Pajvani et al., 2005) a gift from Philipp Scherer, University of Texas Southwestern Medical Center, USA) was also cloned downstream into the p5E-ela3l and p5E-ins plasmids. E2Crimson (pE2Crimson-N1 Vector, 632554, Clontech) under the control of ela3l or insulin promoters were cloned into the pME-MCS plasmid. For the ela3l:H2B-eGFP construct H2B-eGFP cDNA (obtained from Joachim Wittbrodt, University of Heidelberg, Germany) was cloned in the pME-MCS plasmid. Constructs were fused together by the LR recombination reaction as described (Kwan et al., 2007) in the pDestTo2pa to generate Tg(ela3l:DTR; ela3l:E2Crimson), abbreviated to ela:DTR, Tg(ins:DTR;ins:E2Crimson), abbreviated ins:DTR, Tg(ela3l:caspa8;ela3l:E2Crimson), abbreviated to elacasp8 and Tg(ins:caspa8;ins:E2Crimson), abbreviated to ins:caspa8. Corresponding DNAs were injected with transposase mRNA into fertilized eggs to generate transgenic fish.

Zebrafish maintenance and fish lines
Zebrafish (Danio rerio) were maintained according to standard protocols. Most lines are kept in the Mifebio breeding facility (background) (a gift from Wolfgang Drierer, University of Freiburg, Germany).

Drug dependent cell ablation
Diphtheria toxin (DT, D0564, Sigma) was reconstituted in sterile distilled water to 1 mg/ml and diluted to the indicated concentrations in eggwater. AP20187 (dimerizer, Dim) is supplied at 500 µM in 100% ethanol (635059, Clontech) and this stock was directly diluted in eggwater. Control embryos were exposed to corresponding volumes of ethanol. Treated embryos were incubated at 28°C in the dark. F2 transgenic embryos were used to study the dose response of DT and Dim in ablating the exocrine pancreas. At 5 dpf, embryos were incubated in 10 and 15 µg/ml DT and 1.6-8 µM Dim. Adult ela:DTR and ela:casp8 zebrafish were injected intraperitoneally with 20 ng/g DT and 75 ng/g Dim (both diluted in 5 mmol/l citrate, pH 5) and loss of fluorescence was monitored via epifluorescence microscopy.

Whole-mount TUNEL staining
Embryos were fixed for 1-2 h in 4% paraformaldehyde (PFA), 1% DMSO and permeabilized by incubation in 1×PBS, 0.2% Triton X-100, heads and tails were cut off and the gut was cut open. After proteinase K digestion (10 µg/ml) (Invitrogen, C10085) was used. In all experiments, animals were incubated at 28°C in the dark. F2 transgenic embryos were used to study the dose response of DT and Dim in ablating the exocrine pancreas. For EdU-based detection of proliferating cells, the Click-IT 647 Kit (Invitrogen, C100085) was used. In all experiments, animals were incubated in EdU for 48 h before fixation. Treatments were started with an injection of 5 nL EdU solution (100 µM EdU, 2% DMSO) into the common cardinal vein (5-11 dpf) (Kramer-Zucker et al., 2005) and at later stages (~11 dpf) in the gut of anaesthetized animals (0.6 mM tricaine). Subsequently, the injected fish were incubated in 50 µM EdU, kept in the dark for 48 h and then harvested. Samples were fixed in 4% PFA for 1-2 h at RT. After three washes in 1×PBS, 0.2% Triton X-100, heads and tails were removed and the gut was cut open. EdU staining was performed according to manufacturer’s instructions.

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Immunofluorescence
Embryos were fixed for 1-2 h at room temperature in 4% PFA, washed for 3×5 min with 1×PBS, 0.2% Triton X-100, head and tail were cut off and the gut was cut open. Embryos were incubated in blocking buffer containing 1% DMSO, 1% sheep serum, 1% BSA and 1% Triton X-100 in 1×PBS for at least 60 min at room temperature. The embryos were then incubated overnight at 4°C with primary and secondary antibodies, using 1:200 and 1:100 dilutions, respectively. Primary antibodies: rabbit anti-carboxypeptidase A/CPA (Chemicon, AB1213), mouse anti-eGFP (Roche, 11814460001), rabbit anti-dsRed (Clontech, 632496), Alexa Fluor 488 and Alexa Fluor 546 conjugated anti-rabbit and anti-mouse were used as secondary antibodies (Invitrogen, A11001, A11100).

Whole-mount in situ hybridization
*In situ* hybridization was performed with digoxigenin-labeled antisense RNA probes (DIG RNA Labelling Mix, Roche) and anti-digoxigenin-AP antibody (1:4000, Roche) using previously published protocols (Hauptmann and Gerster, 2000). The *trypsin* antisense RNA probe [obtained from Francesco Argenton (Biemar et al., 2001)] was generated after linearizing the corresponding plasmid and using Sp6 RNA polymerase.

RT-qPCR
Total RNA samples were prepared from pools of 5-10 embryos or larvae at indicated time points using Trizol Reagent (Ambion). cDNA was prepared using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1641). HOT FirePol EvaGreen qPCR Mix Plus (Solis BioDyne, 08-24-00020) was used for qPCR reactions in a CFX Connect Real-Time System (Bio-Rad). Reactions were performed on at least two biological samples with two technical replicates each. Primers were designed using 'QuantPrime' (Arvidsson et al., 2008) as follows (5’-3’ orientation): *β-actin*: F, CGTGC-TGTATGGCGCATGAC and R, GTTAGACAACTACTTTGCG; *elaa3l*: F, GGTGTCGCTGATGAATGAGG and R, TGGGCGTGAGTGCTTGGATCG; *ttxpsin*: F, ACCAGCTGTCTGTCTGGATG and R, CAGACGCTGGTTGTGAGCGTC; *casp8*: F, TGGACCATGCCCATGCTCTTCTAG and R, GAGACGCAAGGCTCAATGGT; *casp9*: F, TGGACCTGGCAAACTTACAGACAT and R, AGACTTGGATGTGGTACG; *ins*: F, GC-CCAAAGCAGCTTCTACAC and R, GCAGATTAGAGGAGAAGCC.

Histological analysis
Inner organs of adult fish were fixed in 4% PFA overnight at 4°C, then specimens were serially dehydrated with ethanol solutions and embedded in paraplast wax. Serial sections for histological studies were 8 µm thick and stained with Hematoxylin and Eosin (H&E).

Image acquisition
Confocal images were generated as previously described (Arkhipova et al., 2012) with a Zeiss LSM Exciter5 microscope equipped with 25 mW Argon laser (468, 488 and 514 nm), 1 mW 543 nm He-Ne laser, and a 5 mW 633 nm He-Ne laser. Fluorescence detection was performed with the following filters: BP 480-510 (eGFP and Alexa 488), LP 560 (E2Crimson, mCherry) and LP 650 (Alexa 633, Click-IT 647). Additional confocal images were generated with a Leica TCS SP5 II Laser using 488 nm and 561 nm excitation wavelengths. Fluorescence detection was performed on a bandwidth of PMT 500 to 550 nm and a Hyd 3 bandwidth from 600 to 650 nm. Adobe Photoshop CS5 was used for image arrangement and adjustment. Epifluorescence images were taken at the Leica MZ16A stereomicroscope using either a Leica DFC320 camera or a Zeiss AxioCam ICC1 camera.

Statistics
Volumetric analyses of E2cristom- and eGFP-expressing tissue and automatic cell counting of ptf1a:eGFP+, EdU+ cells and elaa3l:H2B-GFP+ cells were performed using Imaris software (v.7.3, Bitplane). Endocrine cells marked by GFP (ins:CD4-GFP) and E2Crimson expression (ins:casp8, ins:DTR) were counted using the Cell Counter plugin of ImageJ (http://rsweb.nih.gov/ij/plugins/cell-counter.html). Statistical analysis and graphs were performed with SigmaPlot (v.12.5, Systat Software) using Student’s t-test or one-way analysis of variance (ANOVA) and pairwise multiple comparison using the Holm–Šidák method. P-values for statistical significance are stated. All graphs show mean±s.e.m.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceiving and designing the experiments: N.S., D.M. Performing the experiments and analyzing the data: N.S. Providing the pTreck construct: K.K. Writing the manuscript: N.S., D.M. Editing the manuscript and general advice: D.M.

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