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## Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E.coli* nitroreductase

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### Abstract

In order to generate a zebrafish model of  $\beta$  cell regeneration, we have expressed an *E.coli* gene called *nfsB* in the  $\beta$  cells of embryonic zebrafish. This bacterial gene encodes a nitroreductase (NTR) enzyme, which can convert prodrugs such as metronidazole (Met) to cytotoxins. By fusing *nfsB* to *mCherry*, we can simultaneously render  $\beta$  cells susceptible to prodrug and visualize Met dependent cell ablation. We show that the neighboring  $\alpha$  and  $\delta$  cells are unaffected by prodrug treatment and that ablation is  $\beta$  cell specific. Following drug removal and 36hrs of recovery,  $\beta$  cells regenerate. Using *ptf1a* morphants, it is clear that this  $\beta$  cell recovery occurs independently of the presence of the exocrine pancreas. Also, by using photoconvertible Kaede to cell lineage trace and BrdU incorporation to label proliferation, we investigate mechanisms for  $\beta$  regeneration. Therefore, we have developed a unique resource for the study of  $\beta$  cell regeneration in a living vertebrate organism, which will provide the opportunity to conduct large-scale screens for pharmacological and genetic modifiers of  $\beta$  cell regeneration.

### Introduction

Zebrafish embryos develop morphologically simple pancreata and when compared to mammalian systems, there is a high degree of conservation of both expression and function of genes involved in pancreas development (Argenton et al., 1999; Biemar et al., 2001; Lin et al., 2004; Yee et al., 2001; Zecchin et al., 2004). Utilizing transgenics allows the detailed observation of interactions between pancreatic tissues in living zebrafish embryos (Godinho et al., 2005; Huang et al., 2001; Wan et al., 2006). For instance, using lines transgenic for either *gut:eGFP* (Field et al., 2003) or *ptf1a:eGFP* (Godinho et al., 2005) permits the visualization of ventrally derived exocrine cells migrating toward the dorsal pancreas, enveloping the endocrine cells (or principal islet) and forming the first rudimentary pancreas by 44hrs post fertilization (hpf). The cellular composition and structure of the zebrafish principal islet is reminiscent of mammalian adult islets consisting of a central core of  $\beta$  cells and a peripheral mantle of  $\alpha$  cells (Argenton et al., 1999). Thus, the molecular pathways, as well as the cellular

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components and physiology, are conserved between the zebrafish and mammalian model systems. For these reasons the zebrafish has increasingly been used to study the development and diseases of the pancreas. For instance, it has been shown recently that pancreatic ductal adenocarcinoma can be induced in the zebrafish by recapitulating the same signal transduction errors that occur commonly in pancreas cancer in humans (Park and Leach personal communication).

Juvenile diabetes (diabetes type I) is a debilitating disease caused by the chronic loss of the insulin producing  $\beta$  cells of the pancreas. Research into a cure for type I diabetes is centered on islet cell replacement. Considerable success in alleviating the symptoms of juvenile diabetes has been achieved using human islet transplantation; however, the availability of donors is very limiting and the need for continued immunosuppression with its risks and side effects has limited the clinical utility of this approach (Rood et al., 2006). Expanding  $\beta$  cells in tissue culture or inducing proliferation following transplantation could be a potent method to alleviate the paucity of donor tissue available. Another approach to recovering glucose homeostasis would be to induce endogenous regeneration of  $\beta$  cells. Type 1 diabetes in humans and Non Obese Diabetic (NOD) mice is caused by an autoimmune T cell dependent destruction of  $\beta$  cells. In long standing type 1 diabetes in humans, there are occasional  $\beta$  cells scattered in the pancreas along with continued apoptosis of  $\beta$  cells (Meier et al., 2005). Similarly,  $\beta$  cell mass can be restored to cure type 1 diabetes in NOD mice when subjected to a complex treatment protocol to restrain autoimmunity (Chong et al., 2006). Together these results suggest some capacity for regeneration of endogenous  $\beta$  cells exists in patients with type 1 diabetes. It is hoped that elucidating the factors controlling  $\beta$  cell regeneration, therefore, will be useful in providing therapeutic solutions to diseases such as diabetes.

We have turned to the embryonic zebrafish to generate a model of  $\beta$  cell regeneration, as the small size and biology make this organism ideal for screening for both genetic and chemical modifiers of the regeneration process. To create such a model, first an inducible system to cause  $\beta$  cell ablation is required. Injection of the alkylating agent, streptozotocin into mammals causes apoptosis of the  $\beta$  cells (O'Brien et al., 1996); however, addition of the drug to the water of embryonic zebrafish has no detectable effect on  $\beta$  cell mass as judged by in situ hybridization or *ins:eGFP* transgene expression (data not shown). Another way to have temporal control of  $\beta$  cell ablation is to drive expression of a prodrug converting enzyme to the insulin producing cells using transgenesis. Under the action of the exogenous enzyme in the  $\beta$  cell, the prodrug is converted to a cytotoxin and causes cell death. By incorporating the *E.coli* gene *nfsB* into transgenes, mice have been created that express the enzyme nitroreductase (NTR). Exogenous NTR converts added prodrug CB1954 to a cytotoxin, leading to cell death (Bridgewater et al., 1995; Drabek et al., 1997). This system has had limited use in cell ablation in the mouse, but luminal cells of the mammary gland (Clark et al., 1997; Cui et al., 1999), and stem cells within the developing prostate (Wang et al., 2004) have been successfully ablated. The restricted use of this system might be due to the diffusible cytotoxic metabolites of CB1954, which causes bystander effects (Helsby et al., 2004).

In this report we have used metronidazole (Met) as a substrate for NTR-mediated cell ablation for the first time within a live vertebrate. By expressing NTR in  $\beta$  cells in zebrafish embryos we demonstrate prodrug dependent cell ablation, without affecting neighboring cells. In so doing, we have generated a model in which to study  $\beta$  cell regeneration. We also demonstrated the utility of using photo-convertible Kaede in transgenic zebrafish to trace cell lineage and investigate mechanisms of  $\beta$  cell recovery.

## Results

### Metronidazole induces apoptosis in a NTR dependent manner in embryos

To test the capability of NTR to elicit drug dependent cell ablation, the bacterial *nfsB* gene was cloned from *E. coli* genomic DNA into the plasmid T2KXIGΔIN (Kawakami lab), replacing the eGFP marker. With this new construct *EF1a:nfsB* (Fig. 1A), *nfsB* is transcribed under the control of the ubiquitous *EF1a* promoter. This plasmid was co-injected with Tol2 transposase mRNA into single cell embryos, which created embryos with widespread expression of NTR, albeit in a mosaic fashion. In initial experiments these transient transgenic embryos were incubated from 56hpf to 96hpf in 7.5mM or 10mM of Met. By 80hpf there was a prodrug and gene dependent effect on the embryos as assessed by gross pathological changes including edema, lack of heart beat and massive necrosis in the somatic musculature. When used alone, neither the gene *nfsB* nor the prodrug was toxic to zebrafish embryos.

To test how the apparent tissue damage occurred, similar *EF1a:nfsB* transient transgenic embryos were incubated in Met from 6 to 48hpf and then tested for the presence of apoptotic cell death. Two negative controls were used; 1) embryos similarly injected with *EF1a:nfsB* not incubated in drug, and 2) embryos injected with *EF1a:eGFP* (T2KXIGΔIN) and incubated in drug. At 48hpf all embryos were fixed and assayed for apoptosis using TUNEL. Negative control embryos displayed a small amount of apoptosis; however, embryos injected with the *EF1a:nfsB* plasmid and incubated in Met displayed widespread and significantly elevated levels of apoptosis (Figs. 1B–D). It was concluded that *nfsB* encoded NTR can cause drug dependent apoptosis in cells of developing zebrafish embryos.

### Insulin promoter reliably drives expression to the $\beta$ cells of the developing zebrafish

In order to cause drug dependent ablation of the  $\beta$  cells, NTR needs to be specifically expressed in these cells. The earliest known marker of  $\beta$  cells in the developing zebrafish is the *preproinsulin* (*ins*) gene and the *ins* promoter is known to confer expression in the nascent endocrine pancreas (Huang et al., 2001). A 995bp fragment upstream of the ATG of the *ins* gene was amplified by PCR and cloned into the plasmid T2KXIGΔIN, replacing the existing promoter sequence (*EF1a*) (Fig. 2A). Utilizing transposon mediated DNA integration, lines of transgenic fish were established and characterized in detail for expression of eGFP. In *ins:eGFP* transgenic lines, made using this construct, the earliest time point in embryogenesis we could detect fluorescence was around 20hpf just prior to the  $\beta$  cells coalescing as the principal islet forms. Fluorescence was maintained through development and the *ins:eGFP* was localized to the pancreas in both *ins:eGFP* larvae (Figs. 2B–F) and adult fish (Figs. 2G–L). To verify that the fluorescence was localized specifically to  $\beta$  cells, we performed immunofluorescent staining for insulin on *ins:eGFP* larvae, 10 days post fertilization (dpf). By confocal microscopy, we saw that only the insulin expressing cells were also fluorescent for GFP (Fig. 2F). In adult *ins:eGFP* fish, the fluorescence could still be observed transcutaneously (Fig. 2G). Dissection, sectioning, and immunofluorescent imaging demonstrated that  $\beta$  cells in adult pancreas reside in three kinds of aggregations: a large principal islet, secondary islets and very small accumulations of  $\beta$  cells (Fig. 2H). Co-staining for both insulin and eGFP, confirmed that the *ins:eGFP* transgene was reliably labeling  $\beta$  cells (Fig. 2L). Altogether, these data indicate that the insulin promoter in the context of the Tol2 transposable element provides an accurate label of  $\beta$  cells in live zebrafish throughout their life span.

### Expression of NTR via the insulin promoter leads to $\beta$ cell loss

In order to test the ability of NTR to render  $\beta$  cells susceptible to drug dependent ablation, two new constructs were made (Figs. 3A and 3B); *ins:mCherry* drives expression of mCherry to the  $\beta$  cells (Fig. 3C) and *ins:nfsB-mCherry* drives expression of a fusion protein, mCherry-NTR, to the  $\beta$  cells (Fig. 3D). *Ins:nfsB-mCherry* F1 transgenic embryos were used to study the

dose response of Met in ablating  $\beta$  cells. In initial studies, 56hpf embryos were incubated in a range of Met concentrations (1mM to 10mM). After 24hrs of incubation in prodrug, only a concentration of 10mM resulted in complete loss of fluorescence. The relatively high concentration of Met needed to ablate  $\beta$  cells most likely reflects the low affinity of prodrugs toward NTR (Johansson et al., 2003). Another explanation is low prodrug penetration, as suggested by the low octanol to water partition coefficient for Met (Mahfouz and Hassan, 2001). This concentration (10mM) was used in all later studies. To further assess the effects of Met, individual 24hpf embryos were kept in wells of a 24 well plate and incubated in 10mM of the prodrug and examined over time using an epi-fluorescence microscope. Addition of the prodrug for 24hrs (*i.e.* until 48hpf) had no effect on the fluorescence in *ins:mCherry* embryos (n=8), demonstrating that there were no apparent off target effects of this drug on  $\beta$  cell survival (Fig. 3C). Similarly, *ins:nfsB-mCherry* embryos incubated for an identical time without prodrug, displayed the expected fluorescence in the  $\beta$  cells (Fig. 3D, n=30). However, sibling embryos incubated in the prodrug for 24 hrs, showed no detectable fluorescence (Fig. 3E, n=31). Identical results were obtained by starting drug treatments at later developmental time points, namely 36 hrs or 56 hrs and incubating again for 24hrs, until 60hpf or 80hpf respectively (n=23 and n=46 respectively, data not shown and Fig. 3K). These results confirmed that expressing NTR via the *insulin* promoter causes a prodrug dependent loss of fluorescence in the pancreatic  $\beta$  cells.

Confocal microscopy was used to visualize the extent of cell ablation in greater detail. By crossing the *insulin* transgenic lines with a *pf1a:eGFP* line (Godinho et al., 2005), we could visualize the cells of the islet as marked by the boundary with the surrounding green fluorescent exocrine cells. Two separate developmental stages were examined for the ability to undergo cell ablation; embryos were exposed to Met for 24hrs beginning at either 24 or 56hpf. At the time of harvest (48 or 80hpf), embryos were fixed and then dissected to remove the gut and pancreatic endoderm. Two control groups of embryos were used in these experiments, *ins:mCherry* embryos incubated in prodrug for 24hrs (Figs. 3F, I) and *ins:nfsB-mCherry* kept without prodrug addition (Figs.3G, J). In all controls the  $\beta$  cells were detected by red fluorescence of mCherry. The fluorescence was diffuse throughout the cells and also punctate in appearance, which could be due to the exogenous protein being maintained as inclusion bodies within the cytoplasm. As seen in whole embryos using the epi-fluorescence microscope, following 24hr of prodrug treatment, few cells appear red within the region of the islet. By increasing the strength of the laser, weakly red fluorescent cells could be seen within the pancreas of treated embryos at 48hpf (Fig. 3H). These cells do not possess the punctate distribution of fluorescence seen in control embryos and many cells no longer appear within the region of islet, or indeed the pancreas. When embryos were examined at the later time point of 80hpf under maintained laser strength, fluorescent cells were not detected in the islet (Fig. 3K).

Detailed inspection of the cells in the islet revealed the presence of weakly red cells containing fragmented nuclei (Fig. 3L). This morphology is indicative of cells undergoing apoptosis. Cells presenting this appearance were rarely detected. Although streptozotocin also kills  $\beta$  cells in the mouse by inducing apoptosis, cells undergoing apoptosis are hard to detect as the process is rapid and there are a relatively small number of  $\beta$  cells (O'Brien et al., 1996). In addition to occasional apoptotic cells, other evidence of cellular degradation was detected following prodrug exposure. Red fluorescence can also be detected in debris smaller than cells. These particles are often located outside of the pancreas (Fig. 3M) and following 24hrs of prodrug treatment, were frequently distributed across the surface of the yolk (data not shown). Together these data strongly suggest that there is a drug dependent loss of  $\beta$  cells expressing the NTR protein.

## Ablation of $\beta$ cells does not affect neighboring cell populations

In order to validate the specificity of NTR-induced cell death, we looked at other cells within the developing pancreas for signs of abnormality. Using the *ptfla:eGFP* transgene as a marker of nascent exocrine pancreas, we saw no difference in the localization, polarization or length of the pancreas as a whole, whether the  $\beta$  cells were ablated from 24–48 hrs (Fig. 3H) or 56–80 hrs (Fig. 3K). Expression of the *trypsin* gene is an indicator of differentiated exocrine tissue and the development of acinar tissue. Using *in situ* hybridization to detect *trypsin* transcripts, we similarly observed no difference between treated and untreated sibling embryos (data not shown). Taken together these results suggest that  $\beta$  cell ablation has no detectable effect on exocrine formation, migration or differentiation.

Next we looked at markers of other endocrine pancreatic cell types. *Ins:nfsB-mCherry* embryos were raised until 56hpf, then incubated in Met for 24hrs until 80hpf. Untreated sibling embryos were kept as a control. At 80hpf all these embryos were fixed and examined by either *in situ* hybridization or immunofluorescence to detect endocrine markers. At 80hpf insulin expression is robust and limited to the islet as shown in controls (n= 20 example in Fig. 4A). Following Met treatment, no *ins* transcripts were detected in 23 out of 24 embryos by *in situ* hybridization (Fig. 4D); lending further evidence that the  $\beta$  cells have been ablated. As in mammalian systems, the zebrafish pancreas contains Glucagon expressing  $\alpha$  cells at the islet periphery (Argenton et al., 1999). There is no difference in the localization or number of these cells between controls (n=18, Fig. 4B) and prodrug treated embryos (n=20, Fig. 4E). Unlike  $\alpha$  cells, the Somatostatin producing  $\delta$  cells are intercalated with  $\beta$  cells within the core of the islet (Argenton et al., 1999). Immunofluorescent staining for Somatostatin demonstrated that  $\delta$  cells were present in comparable numbers in both prodrug treated (n=10, Fig. 4F) and untreated embryos (n=10, Fig. 4C). These results indicate that prodrug induced ablation of  $\beta$  cells has no detectable effect on closely associated cell neighbors within or immediately outside the islet.

## $\beta$ cell numbers recover following ablation

In order to determine whether the *ins:nfsB-mCherry* transgenic fish might serve as a model of  $\beta$  regeneration, we assayed if  $\beta$  cell number recovers over time following prodrug treatment. An *ins:nfsB-mCherry* embryo at 24hpf is shown in Fig. 5B; and as seen previously, expression of the mCherry fusion protein could clearly be seen by epi-fluorescence microscopy. Treatment with Met for 24hrs until 48hpf, lead to a loss of insulin positive cells (n=31, Fig. 5F), whereas untreated controls showed no effect (n=15, Fig. 5C). After prodrug removal and a further incubation of 36hrs, fluorescence could again be detected in the pancreatic field in all embryos tested (31/31, Fig. 5G). By repeating these experiments in *ptfla:eGFP; ins:nfsB-mCherry* double transgenic embryos and observing the islet following recovery, we could clearly see red fluorescent cells within the islet (Figs. 5K and 5L). These cells exhibited the same kind of punctate distribution of fluorescence observed in control embryos. There was still a reduction in the absolute number of cells that were positive for red fluorescence, indicating that regeneration was not completely compensating for the loss of ablated  $\beta$  cells at this timepoint (*c.f.* Figs. 5I, L). We subsequently maintained both prodrug treated and untreated embryos for longer periods of recovery. At 8 dpf we re-examined the embryos for fluorescence in the pancreas. As shown (Fig. 5J, M) there was only a small residual reduction in the number of fluorescent cells in embryos that had been previously treated in drug (n=12, controls n=3). We maintained these larvae which in due time grew into fertile adults demonstrating that neither  $\beta$  cell ablation nor Met treatment for 24hrs in the larvae have any long term deleterious effects on viability.

## $\beta$ cell recovery is independent of the exocrine pancreas

In mammals, several studies have raised the possibility that differentiated exocrine cells might serve as a source for  $\beta$  cell regeneration (Hao et al., 2006; Lardon et al., 2004). To test the role of the exocrine pancreas in zebrafish  $\beta$  cell recovery, we performed prodrug dependent cell ablation in embryos lacking exocrine pancreas. To do this, single cell *ins:nfsB-mCherry* embryos were injected with a *ptfla* antisense morpholino to prevent exocrine pancreas development (Lin et al., 2004; Zecchin et al., 2004). These embryos were cultured until 56hpf and then incubated in Met as before. The  $\beta$  cell fluorescence is extinguished within the pancreas following 24hrs of treatment, both in morphant embryos without exocrine pancreas (n= 24, Fig. 6F) and in non-morpholino controls (n= 18, Fig. 6B). Following 36hrs of prodrug removal, an equivalent amount of  $\beta$  cell recovery can be observed compared to non-morphant controls which possess exocrine pancreas. To verify that exocrine pancreas formation was completely inhibited by the morpholino injections, we assayed for Carboxypeptidase (CPA - a marker of differentiated acinar cells of the exocrine pancreas) by immunofluorescence. CPA immunoreactivity was absent in all *ins:nfsB-mCherry*, *ptfla* morphants examined (n=16, example shown in Fig. 6H), while CPA was present in all non-morphant controls (n=16, Fig. 6D). This result demonstrates that  $\beta$  cell recovery is independent of the exocrine pancreas. Hence, no cell type or extracellular factor from the exocrine pancreas is required for  $\beta$  cell recovery in this system.

### Mechanism of $\beta$ cell recovery

One possible explanation for our observed results is that non-insulin expressing progenitors escape cell ablation as they are not producing NTR; and that later in development, concurrent with prodrug removal, these cells differentiate to generate insulin positive  $\beta$  cells. To define a temporal window during which  $\beta$  cell progenitors normally differentiate, we generated a stable transgenic line of zebrafish where a fluorescent protein called Kaede is expressed in  $\beta$  cells. The green fluorescent Kaede photoconverts to red when exposed to UV light (Ando et al., 2002; Sato et al., 2006). The time at which the embryo is exposed to UV light determines when the red fluorescent marker is formed. Following removal of UV light, the photoconverted red Kaede persists as red fluorescent protein, while newly translated Kaede will be green. The red fluorescence is durable and inherited by daughter cells, allowing this utility of temporal labeling via photoconversion to be applied to cell lineage tracing experiments.

Following exposure of *ins:kaede* embryos to UV light, the  $\beta$  cells fluoresce red (represented in Fig. 7B and seen in 7D). New  $\beta$  cells created from the division of other  $\beta$  cells inherit pre-converted red fluorescent protein and also produce their own green fluorescent Kaede protein (red and green cells are represented as yellow in Fig. 7B and seen in the islet in 7H). Alternatively, a newly formed  $\beta$  cell originating from the differentiation of a non- $\beta$  cell will inherit no red fluorescent protein at all, but will produce green fluorescent protein (green in Fig. 7B). We photoconverted 6 F1 embryos at 48hpf and looked for the appearance of green cells at 120hpf by confocal microscopy. In two embryos we observed distinct green fluorescent cells at the islet periphery (Fig. 7H). This result indicated that new  $\beta$  cells can mature during the time we are seeing  $\beta$  cell recovery. This maturation of  $\beta$  cells is obviously variable but represents a possible mechanism by which new  $\beta$  cells can be formed between 48 and 120hpf. This result also demonstrates that non- $\beta$  cells continue to contribute to the  $\beta$  cell pool, even after exocrine/endocrine cell clustering in the zebrafish.

Recovery of  $\beta$  cell mass following prodrug dependent ablation could also occur from proliferation. To test this hypothesis, *ins:nfsB-mCherry* 56hpf embryos were first treated in prodrug for 24hrs, then washed into BrdU solution and left to recover for 36hrs until 118hpf. Following detection of BrdU incorporation, pancreata were dissected and visualized via confocal microscopy. In dissected pancreata from *ins:nfsB-mCherry* embryos, BrdU positive

$\beta$  cells can clearly be detected. This observation was made for both embryos recovering from prodrug treatment (n=6, Fig. 7K) and in untreated controls (n=4, Fig. 7J). These results indicate that proliferation of either  $\beta$  cells or their precursors is occurring during the time in which we observe  $\beta$  cell recovery. Whether there is an increase in proliferation that compensates for  $\beta$  cell loss will require detailed further investigation.

In summary we have presented a new method to ablate  $\beta$  cells and by combining microdissection with confocal microscopy can monitor in detail cell behavior following perturbation. By expressing and photoconverting the protein Kaede in the  $\beta$  cells, we have demonstrated the ability to map in time when cell differentiation occurs. Together these techniques provide valuable new tools to investigate  $\beta$  cell biology in the zebrafish.

## Discussion

Experiments designed to elucidate the process of regulative development or regeneration often focus on the removal of a tissue and the observation of tissue recovery. The most remarkable examples include the amputation and complete recovery of amphibian limbs (Kintner and Brockes, 1984; Wallace et al., 1981). Such classical ablation studies have contributed immensely toward our understanding of cell interactions and their involvement in tissue patterning. For instance, studies in *C.elegans* have revealed the range of developmental effects resulting from cell ablation, such as, cell autonomy, induction, lineage regulation (Sulston and Horvitz, 1977), and provision of supporting (Kimble and White, 1981) and repressive factors (Seydoux et al., 1990). Each of these classical ablation methods has some inherent caveats. For example, microsurgical techniques can perturb tensional integrity (Ingber, 1993) and dynamic reciprocal interactions (Bissell et al., 1982) that contribute heavily toward proper gene regulation and cell behavior; while laser ablation can leave debris that can affect downstream interactions, and is often limited by the numbers of cells that can be ablated at one time (Yang et al., 2004). Finally, it is often impossible to be certain which cells are being removed without specific molecular markers. Chemical ablation using pharmaceutical agents that target specific cell types can avoid these problems, allowing investigation into the mechanisms and components of regeneration. Examples include the use of (2-morpholinobutyl)-4-thiophenol which causes melanocytotoxicity in zebrafish (Yang and Johnson, 2006) and streptozotocin which causes apoptosis in mammalian  $\beta$  cells (O'Brien et al., 1996).

In this report we describe a prodrug dependent system in the zebrafish that allows for spatial and temporal control of cell ablation, with minimal disturbance to the tissue environment. We used a 1kB fragment from the insulin promoter to drive the expression of NTR to the  $\beta$  cells, and demonstrated  $\beta$  cell ablation following prodrug exposure. Examination of molecular markers showed that damage was limited to the insulin positive cells with no detectable injury to the surrounding tissues. After 24hrs of prodrug treatment, we saw a loss of *ins* transcript; however, there was variability in the localization of mCherry fluorescence. Residual fluorescent elements were often detected throughout the embryo and occasionally within the pancreas. These elements might represent weakly expressing cells, dead cells or cell debris. Continuing work includes trying to establish whether this movement of fluorescent particles out of the islet is coincident with the action of an early immune response by cells such as macrophages. We were only able to make a limited association of cell death markers with ablation, most likely due to the speed of death and the removal of cells from the islet. To further investigate the method of cell death, we are currently developing transgenic reporters, which in conjunction with confocal microscopy are designed to analyze mitochondrial and nuclear morphology. It is hoped this will allow a more thorough investigation of  $\beta$  cell ablation in our zebrafish model and if the mechanism of cell death is reminiscent of that seen in type I diabetes (Steer et al., 2006).

By following  $\beta$  cell ablation and 36hrs of recovery in the absence of prodrug, we observed a steady increase in fluorescence within the islet. During this same stage of development we observed cell proliferation, as marked by BrdU incorporation, and the differentiation of non- $\beta$  cells to  $\beta$  cells, as marked by cell lineage tracing using photo-convertible Kaede. We have, therefore, shown that two potential mechanisms exist for the observed replacement of the ablated  $\beta$  cells, namely; differentiation of non- $\beta$  cells and proliferation (reviewed by (Gu et al., 2003). Photoconversion of Kaede has been used to lineage trace neurons (Sato et al., 2006), but this is the first example of using changes in Kaede fluorescence to determine the timing of cellular differentiation during embryogenesis. The *ins:kaede* transgenic fish will, therefore, provide a useful tool in assaying the kinetics of  $\beta$  cell differentiation throughout the zebrafish lifespan.

The observation that  $\beta$  cell numbers recover implies the existence of a mechanism for sensing and maintaining  $\beta$  cell mass. In other words the  $\beta$  cells en masse are behaving as a robust system (Nijhout, 2002) and are resistant to perturbation. For the observed regeneration to occur, the decrease in  $\beta$  cell number or a recruitment signal must first be detected by the responding cells. This detection may take the form of assessing changes in cellular contact, changes in autocrine signaling or indeed a systemic change caused by loss of  $\beta$  cells. Muscle mass in mammals is controlled in part by the amounts of a negative regulator called myostatin that circulates through the blood (Lee, 2004; Magee et al., 2006); and there is evidence that this negative regulator inhibits the action of the growth factor IGF-1 (Shyu et al., 2005). It is possible that such a negative regulator is also released by  $\beta$  cells either locally or throughout the embryo to regulate  $\beta$  cell proliferation.

Taking advantage of the small size of zebrafish larvae, it is hoped the *ins:nfsBmCherry* transgenic fish can be used to identify genetic or pharmacological modifiers of  $\beta$  cell recovery. Such studies could be in the form of mutagenesis or chemical screens and assaying the degree or speed of  $\beta$  cell recovery following ablation. Any candidate gene product or pharmacological agent shown to influence  $\beta$  cell regeneration could then be validated in a recently developed mouse model where  $\beta$  cell ablation can be easily induced in the adult (Pedro Herrera, personal communication).

While the pancreas has been the focus of intense study for many years, we are still learning of new cell types that occupy the islet (Wierup et al., 2004) and how the endocrine and exocrine compartments may interact during zebrafish pancreas development. Our studies suggest that a resident insulin negative population can contribute to  $\beta$  cell regeneration. We have established that the exocrine pancreas is not essential for the regeneration of new  $\beta$  cells, but the identity of the progenitor population remains to be clarified. A stem cell niche consists of enclosed progenitors capable of sensing requirements to proliferate (Fuchs et al., 2004). We have shown that pancreatic cells do proliferate and differentiate quickly in response to perturbation, suggesting the embryonic zebrafish islet might contain stem cell niches. Variation seen in the Kaede experiments indicating that  $\beta$  cells can differentiate between 80 and 116hpf could be explained by small fluctuations in the careful balance that maintains these niches.

Recently, an unbiased screen using ENU mutagenesis has identified several classes of mutations that affect islet morphogenesis, although no mutations that only affect the insulin positive cells were found (Kim et al., 2006). Morpholino analyses have also implicated non-canonical Wnt/Frz interactions in organizing the clustering behavior of the islet, but a careful study of the individual cell populations showed that this mechanism is likely to be indirect (Kim et al., 2005). The *ins:nfsB-mCherry* zebrafish lines provide not only a model system to study  $\beta$  cell regeneration, but also an alternative strategy to manipulate cell interactions during pancreas development. Using this method, we have shown that the insulin positive population



is dispensable for both exocrine cell migration and the maintenance of the islet as a discrete cluster.

The method of prodrug dependent ablation demonstrated in this report has been previously shown to be effective in a number of tissues in the mouse (Clark et al., 1997; Drabek et al., 1997; Wang et al., 2004) and this NTR/Met method is not restricted to  $\beta$  cells in the zebrafish. Recent work has also demonstrated effective ablation of liver and heart cells (D.Stainier personal communication) and the creation of a *UAS:nfsB-mCherry* line has allowed several recently established Gal4 driver lines to be utilized for ablation of floor plate, notochord and muscle cells (J.Davison personal communication). It is therefore likely that prodrug dependent cell ablation in zebrafish will play an important role in our understanding of system requirements for the control of cell behavior, and ultimately for the treatment of diseases.

## Experimental Procedures

### Cloning of transgenic constructs

The *E. coli* gene *nfsB* was amplified by PCR from bacterial genomic DNA using the primers shown below. Coding regions are capitalized with restriction sites underlined and endonucleases shown in parentheses.

Forward primer (Nco I) 5' ccATGGATATCATTTCTGTCGCCTTA 3'

Reverse primer (Cla I) 5' atcgaTTACACTTCGGTTAAGGTGATGT 3'

Reverse primer removing stop codon (Cla I) 5'

atcgatCACTTCGGTTAAGGTGATGTTT 3'

*NfsB* was cloned into T2KXIGΔIN(Kawakami, 2004) as a Nco/Cla fragment, placing *nfsB* downstream of the *Ef1 $\alpha$*  promoter. 995bp immediately upstream from the ATG of *preproinsulin (ins)* (ENSDARG00000035350) was amplified by PCR from zebrafish genomic DNA using the primers below.

Forward primer (BamH I) 5' ggatccatttaacttcagcccacagtct 3'

Reverse primer (Nco I) 5' CACTGCCATggtcacact

*EGFP* and *mCherry* were amplified by PCR with primers containing Cla restriction sites in both primers. PCR products were designed to make inframe fusions on C terminus of NTR (minus stop codon). Constructs were assembled from these components into BamHI/ClaI cut T2KXIGΔIN vector backbone(Kawakami, 2004) (Figs. 3A, B). Backbone includes a SV40 poly adenylation site and flanks the insert (promoter, genes and polyA) with the Tol2 transposon arms (500bp left and 500bp right). The Kaede open reading frame was amplified from pKaede-S1 (CoralHue™, MBL®) using Forward primer (Nco I) 5' ccATGGGTGAGTCTGATTAAACC and reverse primer (Cla I) atcgATTACTTGACGTTGTCCGGCAATCC. Kaede was then cloned downstream of the insulin promoter as shown in Fig. 7a. These constructs were used to make transgenic fish (Kawakami, 2004) and F2 progeny from incrossed F1 fish were used in drug ablation studies.

### Drug dependent cell ablation

Metronidazole (Met, M3761, Sigma®), was used due to its efficacy over CB1954 in ablating  $\beta$  cells. Met was dissolved in E3 media (Westerfield, 1995) by vigorous agitation. Embryos were incubated at 28°C in the dark. *Ins:nfsB-mCherry* F1 transgenic embryos were used to

study the dose response of Met in ablating  $\beta$  cells. In initial studies, 56hpf embryos were incubated in a range of Met concentrations (1mM to 10mM). After 24hrs of incubation in prodrug, only a concentration of 10mM resulted in complete loss of fluorescence. A concentration of 10mM was used in all later studies and had no deleterious effects on the larvae (*n.b.* this concentration is highly toxic to adult fish). *Ptf1a* morphants were created by as previously reported (Lin et al., 2004).

### In situ hybridization and Immunofluorescence

Whole-mount in situ hybridization of zebrafish embryos was performed using digoxigenin labeled antisense riboprobes (Thisse, 1998). Probes against zebrafish *insulin*, *glucagon* and *px1* have been described previously (Biemar et al., 2001; Wallace and Pack, 2003). For whole-mount immunofluorescence, embryos were treated as per (Lin et al., 2004) and incubated overnight in 10% goat serum with 1:100 guinea pig anti-insulin, rabbit anti-carboxypeptidase A (Linco Research) and rabbit anti-somatostatin (Dako), washed briefly, and then incubated overnight in 10% goat serum with 1:100 Cy2- and Cy3-conjugated secondary anti-guinea pig or anti-rabbit antibodies. For immunofluorescence on adult fish sections, tissues were fixed in 10% formalin, embedded in paraffin and 5 $\mu$ M sections cut and processed as per standard procedures (Cell signaling technology) followed by double labeling using rabbit polyclonal anti-GFP (Chemicon international; 1: 1000): goat anti rabbit Cy3 (Jackson ImmunoResearch; 1:300) and guinea pig polyclonal anti-insulin (Linco Research Inc; 1:1000): goat anti guinea pig Cy2 (Jackson ImmunoResearch; 1:300).

### Whole Mount TUNEL assay

Fixed and methanol permeabilized embryos were rehydrated and treated for 15 minutes with 1 $\mu$ g/ml proteinase K, followed several washes in PBS 0.1% Tween and then a 1 hr incubation at 37°C in a cell death detection reagent (in situ cell death Detection Kit-TMR Red, Roche Diagnostics). Washed embryos were visualized for fluorescence.

### BrdU staining

Embryos were placed in 10mM solution of BrdU (Sigma) in E3 and kept in dark at 28° C for 36 hrs. Then embryos were fixed in 4% PFA for 2 hrs at room temperature, washed and incubated in 1 N HCl for 1hr. The washed embryos were blocked using 5% goat serum for 1 hr and incubated over night at 4°C in 1:100 mouse anti-BrdU monoclonal antibody (Chemicon). After washing, embryos were incubated over night at 4°C in 1:100 Cy2 conjugated donkey anti-mouse antibody (Jackson Immunoresearch) and dissected pancreata visualized via confocal microscopy.

### Image acquisition

Kaede protein was photoconverted by 30 second exposure of widefield UV using a DAPI filter ( $\lambda=375$ nm). Widefield and confocal sections were acquired using an Axiovert 200M microscope coupled to the Zeiss LSM 5 Pascal system. A Plan-Apochromat 10X/0.45 lens for widefield or Plan-Neofluar 40X/1.3 Oil DIC objective for confocal sections were used. Blue, green and red channels were excited using a UV, Argon and He/Ne laser, and emissions were detected using BP 420–480, BP 505–530 and LP 560 filters, respectively, and controlled through the Multi-track mode on Zeiss AIM software. All rendering and quantitation used the AIM software. Fixed pancreata were dissected using pulled glass needles on a bed of agarose and mounted on a cover slip for imaging.

## Acknowledgements

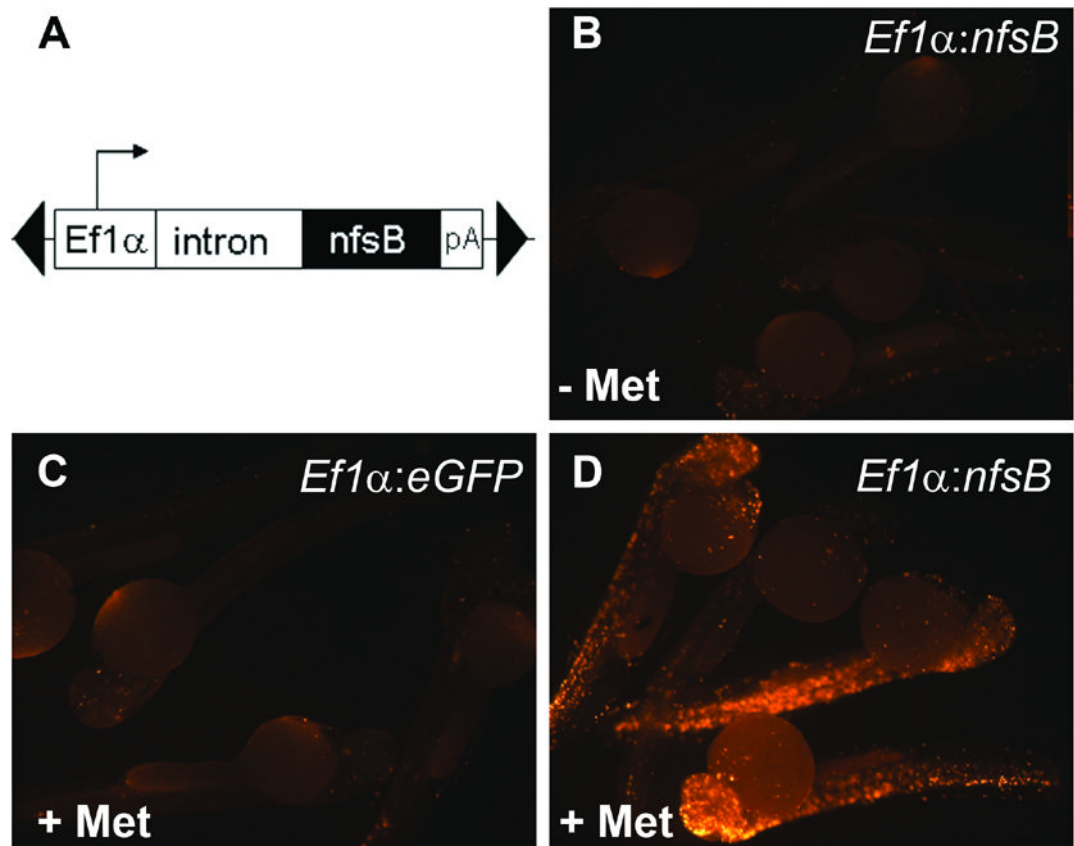
This work was supported by a pilot project from Johns Hopkins Dept of Surgery (MJP), and by NIH grants DK61215 and DK56211 (SDL). HP is supported by NIH NCRR T32 grant 07002. The authors thank Koichi Kawakami for the T2KXIG IN plasmid and Yangseon Park for expert technical support.

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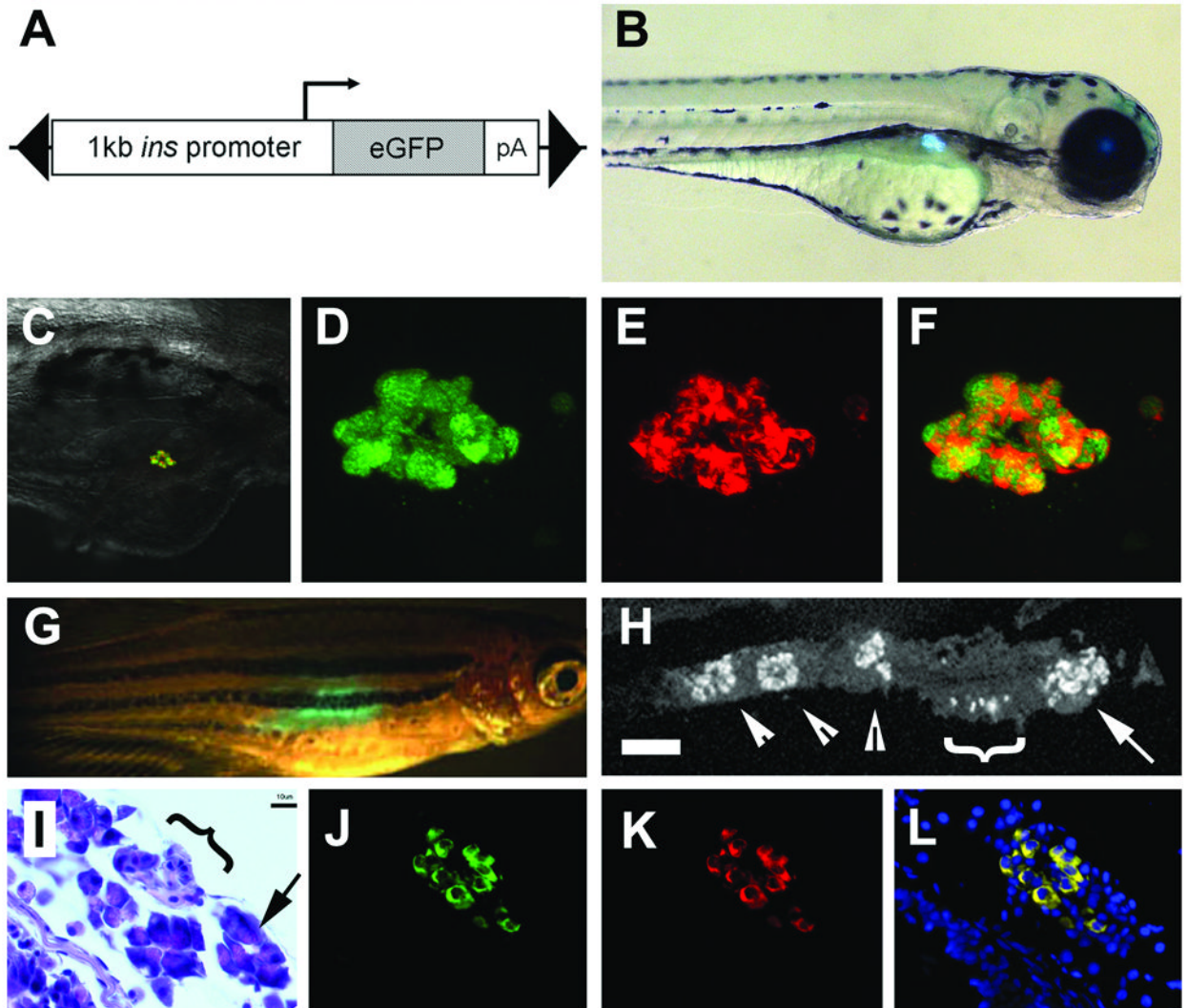
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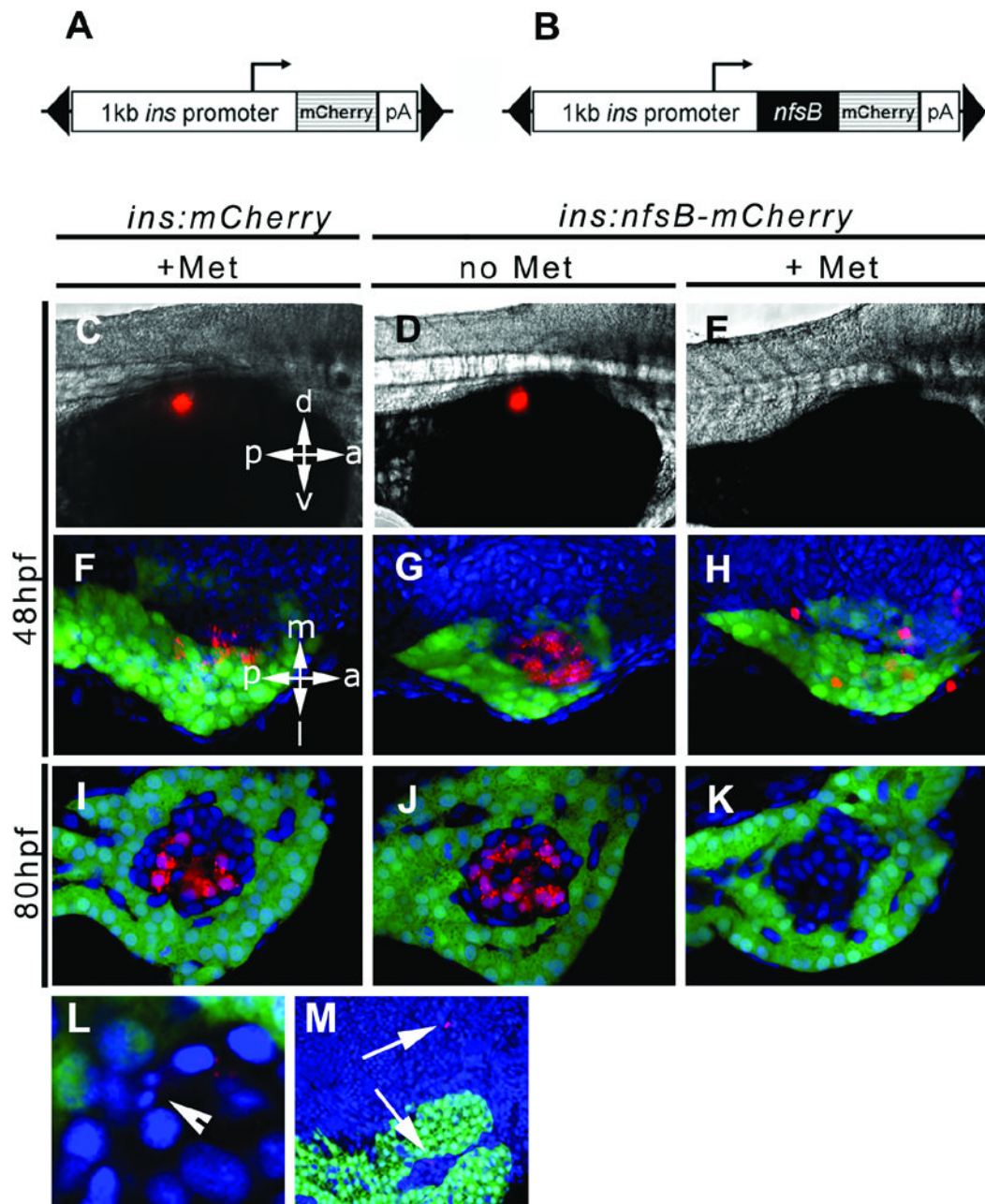
**Fig. 1.**

Met induces apoptosis in a NTR dependent manner. (A) Schematic of construct used to drive ubiquitous expression of NTR. Construct includes two arms from the Tol2 transposable element (black triangles), the *EF1α* promoter and intronic sequence from the rabbit  $\beta$ -globin gene (Kawakami, 2004). *EF1α* drives ubiquitous expression of NTR. Embryos injected at 1 cell stage and incubated with or without Met were fixed at 48hpf and assayed for apoptosis using fluorescent TUNEL. (B) Negative control, mosaic for *Ef1α:nfsB* with no prodrug treatment. (C) Negative control, mosaic for *Ef1α:eGFP* incubated in Met (6 to 48hpf). (D) Mosaic embryos for *Ef1α:nfsB*, incubated in Met. (6 to 48hpf). Results demonstrate increased apoptosis when NTR is expressed in presence of Met.



**Fig. 2.**

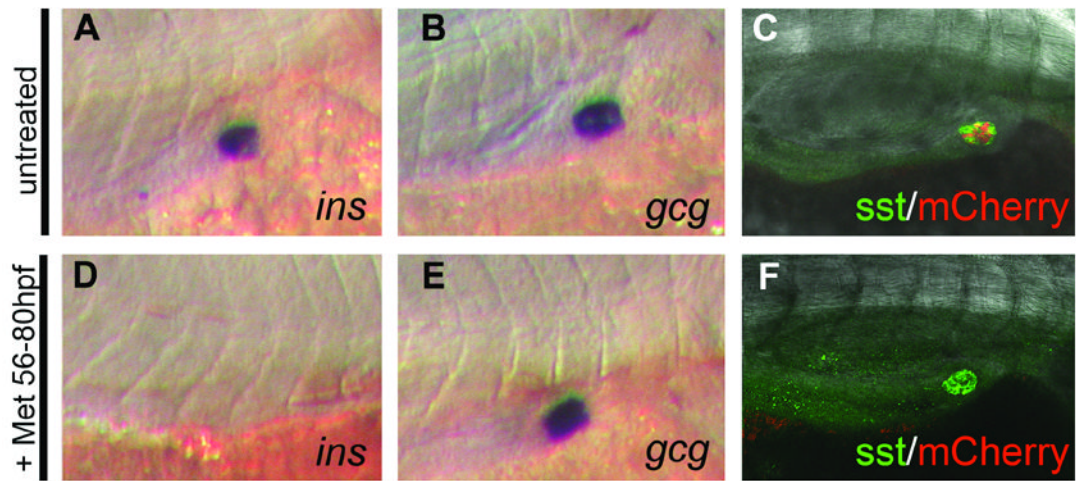
Insulin promoter reliably drives expression to the  $\beta$  cells. (A) Schematic of construct used including the 1kb promoter region of *preproinsulin*, and gene encoding eGFP, all within a Tol2 transposable element (black triangles). (B) Fluorescent image of a 3 day old *ins:eGFP* embryo showing expression within the islet of the pancreas. (C) Rendered composite of confocal images of a 10 day old *ins:eGFP* embryo following immunofluorescence to detect insulin, with overlaid 20x bright field. (D) Close up images of eGFP fluorescence and (E) immunofluorescence to detect Insulin and (F) merged image demonstrating co-localization of eGFP with Insulin. (G) 3 month *ins-eGFP* adult fish showing transcutaneous fluorescence. (H) Section through dissected pancreas showing immunofluorescence to detect Insulin (scale bar = 100 $\mu$ M). The principal islet can be seen in the anterior head of the pancreas (arrow). More posterior are small aggregations of  $\beta$  cells (white bracket) and three secondary islets (arrowheads). (I) High magnification, the morphology of the smaller islets can be seen (black Targeted ablation of  $\beta$  cells bracket) surrounded by acinar tissue (arrow), by H&E staining (scale bar = 10 $\mu$ M). Immunofluorescence detection for eGFP in (J), insulin in (K). (L) Merged image demonstrate co-localization in adult tissues (Hoescht staining for nuclei in blue). In (B to H) anterior is to the right and posterior to the left.

**Fig. 3.**

The *ins:nfsB-mCherry* transgene leads to prodrug dependent loss of  $\beta$  cells. Schematic of constructs used to drive  $\beta$  cell expression of mCherry in (A) and the fusion protein NTR-mCherry in (B). The expression cassettes were cloned inside of a Tol2 transposable element (black triangles). In the experiment shown here, two groups of controls were used, *ins:mCherry* embryos incubated in Met (+Met, C, F, I) and *ins:nfsB-mCherry* without prodrug (no Met, D, G, J). Experimental groups consisted of *ins:nfsB-mCherry* embryos incubated for 24 hrs in Met (+Met, E, H, K). Two separate time points were chosen for addition of prodrug as shown, incubation starting at 24hpf and finished by 48hpf (48hpf, C to H) and starting at 56hpf and finishing at 80hpf (80hpf, I to K). Embryos were photographed by either epifluorescence in (C) to (E), or by confocal microscopy on isolated pancreata plus adjacent

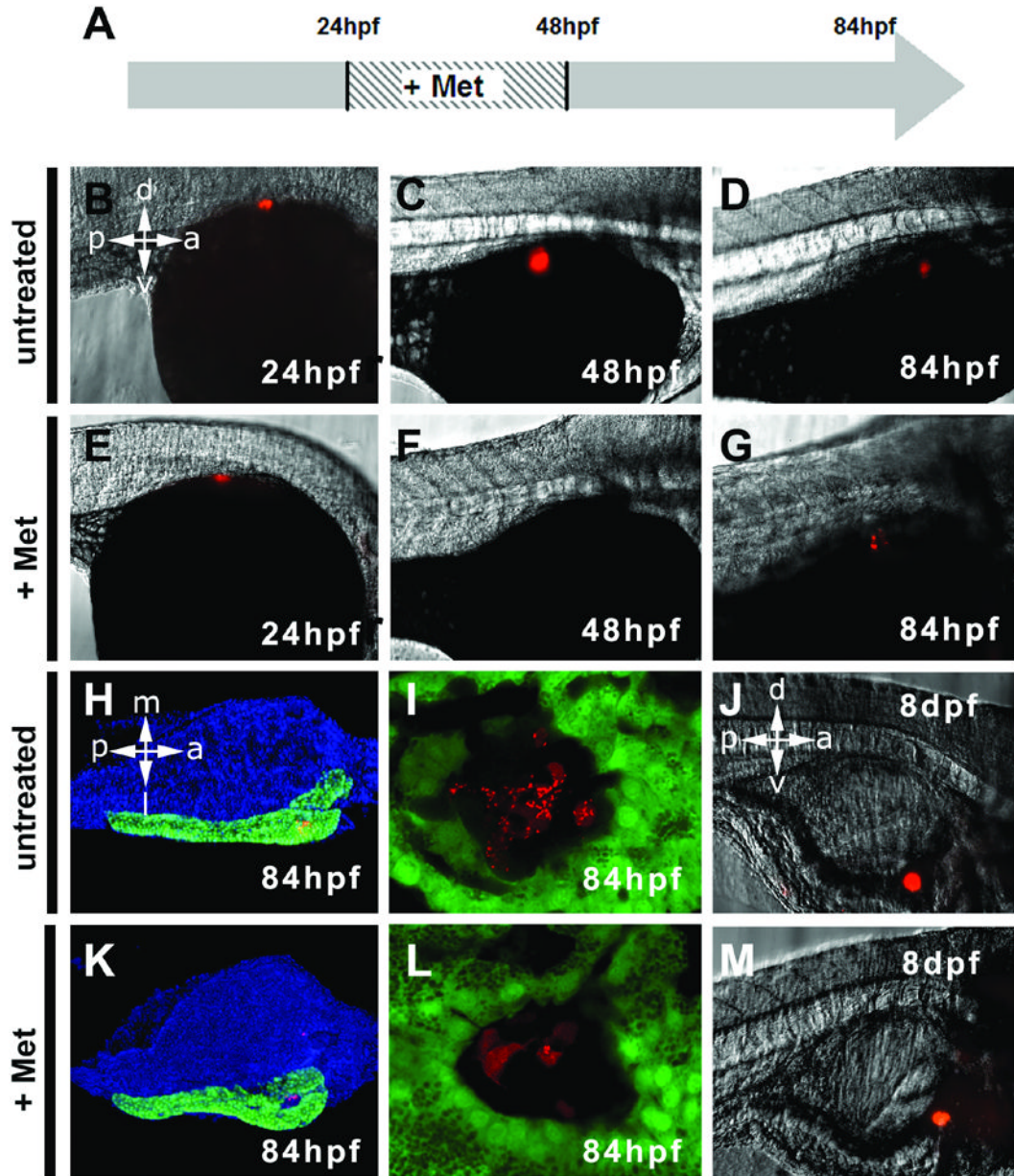


endoderm in (F) to (M). Red fluorescence indicated presence of mCherry (C, F, I) or NTR-mCherry fusion protein (D, E, G, H, J, K, L and M). eGFP marks the exocrine pancreas with green fluorescence and nuclei are stained blue (Hoescht). (L) Example of fragmented nucleus indicative of apoptosis (arrowhead) and cell debris inside and outside of islet in (M) (arrows). Orientation as indicated, d = dorsal, v = ventral, a = anterior, p = posterior, m = medial and l = lateral.

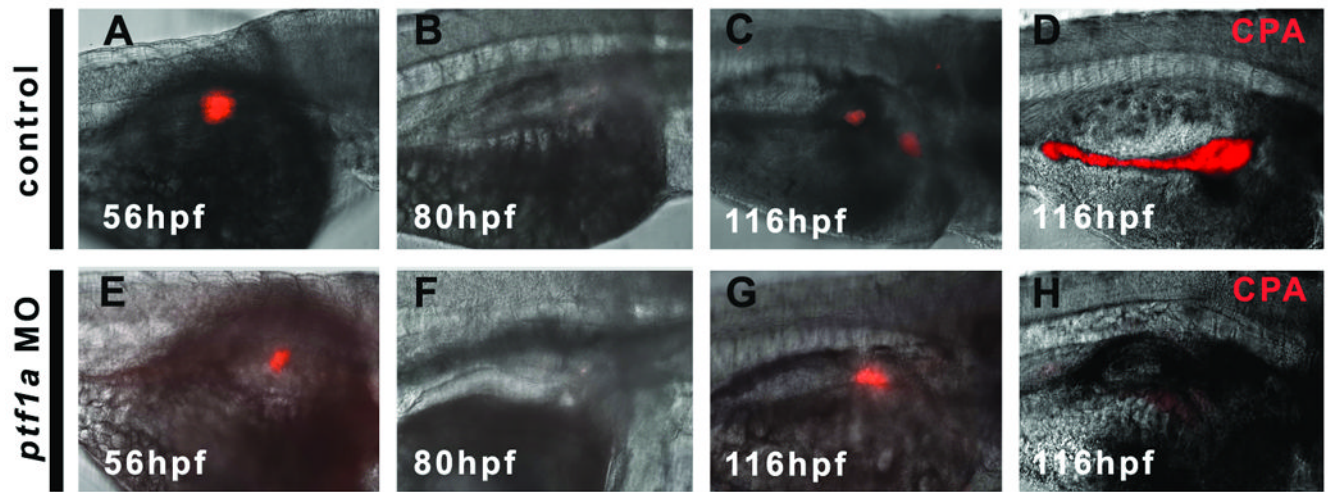


**Fig. 4.**

Expression of *insulin* but not *glucagon* or Somatostatin, is lost following prodrug dependent  $\beta$  cell ablation. (A – C) Marker analysis on *ins:nfsB-mCherry* untreated control embryos or embryos treated with Met from 56hpf to 80hpf (D to F). In situ hybridization with riboprobes to detect the following transcripts: *insulin* (*ins*) (A, D); *glucagon* (*gcg*) (B, E). Immunofluorescence detection of Somatostatin protein, using confocal microscopy, (C, F). Presence of the NTR-mCherry fusion protein can be seen in the control embryo (C) as red fluorescence, but is lost following ablation (F).

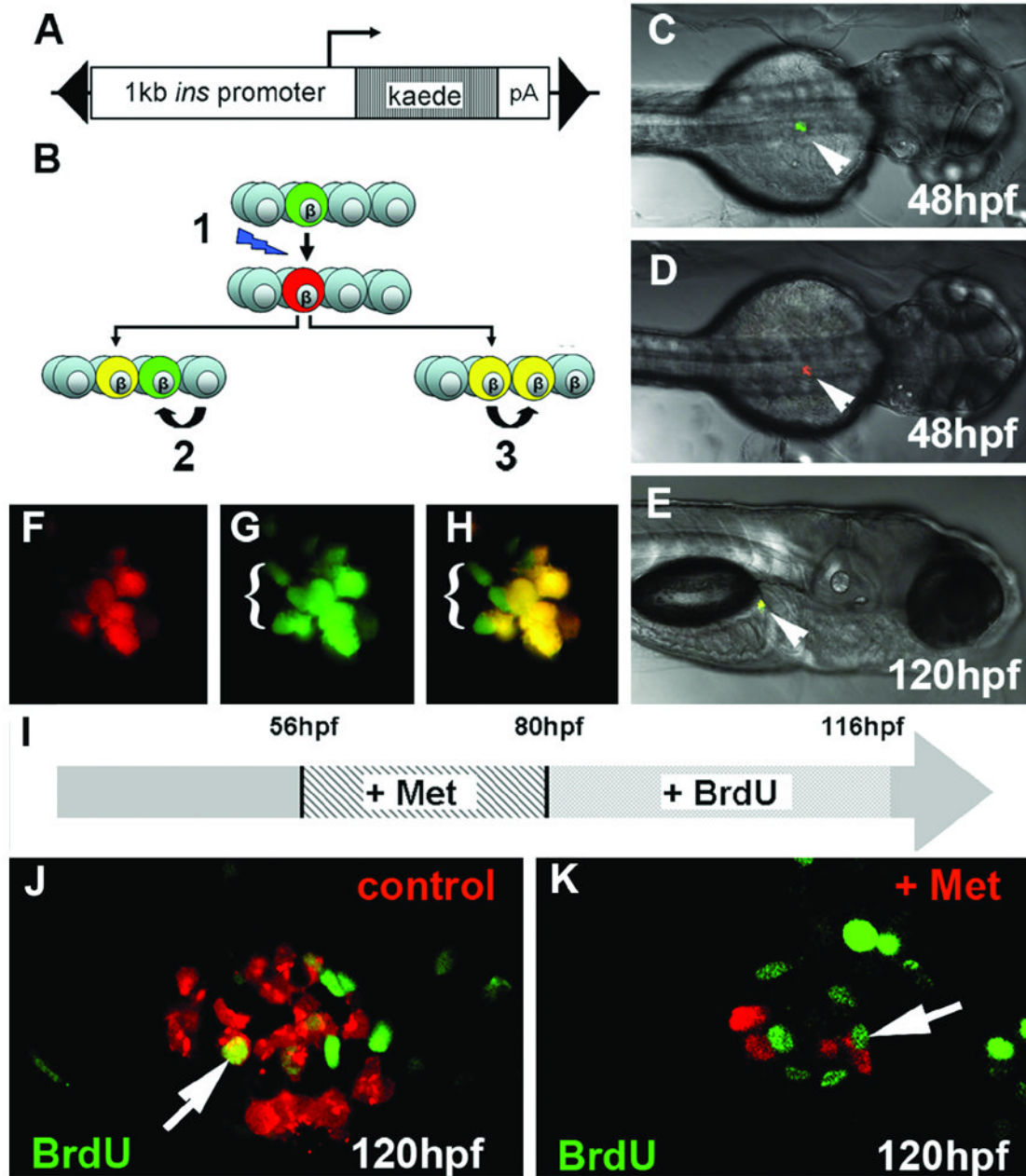


**Fig. 5.**  $\beta$  cell recovery following ablation. (A) Schematic timeline of prodrug treatment. (B – D) Untreated *ins:nfsB-mCherry* embryos at stages indicated. *Ins:nfsB-mCherry* embryos before prodrug incubation (E), following 24hrs of ablation with prodrug (F) and following 36hrs of recovery in absence of prodrug (G). Return of  $\beta$  cells is indicated by the reoccurrence of NTR-mCherry. Dissected pancreas and adjacent endoderm from a untreated *ins:nfsB-mCherry*, 84hpf embryo (H) and treated and recovered 84hpf embryo (K). Nuclei are blue due to Hoescht counter staining. Images generated from *ins:nfsB-mCherry; ptfla:eGFP* transgenics, hence exocrine pancreas appears green. (H, K) In comparison to controls, treated pancreata look morphologically normal. Close up images of an islet following treatment and recovery in (L), show presence of  $\beta$  cells with a normal appearance but in a reduced number compared to untreated controls (I). (J) Untreated *ins:nfsB-mCherry* in a fry, 8 days post fertilization (dpf), and following prodrug treatment described above (M).



**Fig. 6.**

$\beta$  cell recovery is independent of the exocrine pancreas. (A, E) *Ins:nfsB-mCherry* embryos before prodrug incubation. (B,F) Ablation following 24hrs of prodrug incubation. (C, D, G, H) Following 36hrs of prodrug removal, recovery can be detected. Control embryos (A – D), show normal exocrine pancreas as demonstrated by CPA immunofluorescent detection (pseudo color image D). *Ptf1a* morphant embryos shown in E to H lack an exocrine pancreas as demonstrated by absence of CPA (H), but still recover  $\beta$  cells (G).



**Fig. 7.**

Following ablation, both continued maturation and proliferation may contribute to recovery in  $\beta$  cell numbers. (A) Construct used to generate *ins:Kaede* transgenics, made within a transposable element (black triangles). (B) Model of Kaede labeling, a green  $\beta$  cell is photoconverted at 48hpf (1). Subsequent differentiation of progenitors gives rise to  $\beta$  cells that possess only green Kaede (2) whereas division of  $\beta$  cells already present during photoconversion inherit red Kaede, produce green kaede and hence appears yellow (3). A live *ins:Kaede* F1 embryo viewed before (C) and after photoconversion (D). The  $\beta$  cells of the islet can be detected (arrowhead). (E) Later in development, at 120hpf, fluorescence in both channels is apparent and via confocal, it is clear that there are less cells fluorescing red in (F) than green in (G). (H) In a merged image  $\beta$  cells that fluoresce only green can be detected

indicating that these cells have arisen from non- $\beta$  cells. (I) Schematic of the experiment to test for proliferation following  $\beta$  cell ablation in *ins:nfsB-mCherry* embryos. (J, K) 1 $\mu$ M confocal sections through islet showing BrdU labelling (green nuclei) and  $\beta$  cells (red). In both controls (J) and islets recovering from  $\beta$  cell ablation (K), proliferation of  $\beta$  cells can be detected as double labeled cells (arrows).