In vivo imaging of emerging endocrine cells reveals a requirement for PI3K-regulated motility in pancreatic islet morphogenesis

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Summary Statement: Through in vivo imaging, we show that pancreatic endocrine cells project previously unrecognized finger-like projections to guide the process of clustering into a compacted islet.
Abstract
The three-dimensional architecture of the pancreatic islet is critical for beta cell function, but the process of islet formation remains poorly understood due to the difficulties of imaging internal organs with cellular resolution. Within transparent zebrafish larvae, the developing pancreas is relatively superficial and thus amenable to live imaging approaches. We performed in vivo time lapse and longitudinal imaging studies to follow islet development, visualizing both naturally occurring islet cells and cells arising with an accelerated time course following an induction approach. These studies revealed previously unappreciated fine dynamic protrusions projecting between neighboring and distant endocrine cells. Using pharmacologic compound and toxin interference approaches, and single-cell analysis of morphology and cell dynamics, we determined that endocrine cell motility is regulated by PI3K and G-protein coupled receptor (GPCR) signaling. Linking cell dynamics to islet formation, perturbation of protrusion formation disrupted endocrine cell coalescence, and correlated with decreased islet cell differentiation. These studies identified novel cell behaviors contributing to islet morphogenesis, and suggest a model in which dynamic, exploratory filopodia establish cell-cell contacts which subsequently promote cell clustering.

Introduction
Pancreatic islets are clusters of endocrine cells which produce hormones important for regulation of glucose homeostasis. During pancreas development, a process conserved across vertebrate species, morphogenesis is coordinated with establishment of exocrine, ductal and endocrine islet compartments. In mammalian pancreas, the ‘first transition’ is characterized by epithelial budding and formation of early primitive endocrine cells, while the definitive endocrine cells arise during a second wave of cell expansion and differentiation called the ‘secondary transition’ (Marty-Santos and Cleaver, 2015). During the secondary transition, endocrine precursors differentiate and emerge from the pre-ductal epithelial plexus and progressively assemble into islets (Bankaitis et al., 2015; Pan and Wright, 2011).

In zebrafish, an early-forming principal islet originates from the coalescence of a population of dorsal endoderm cells, and arises prior to formation of the gut tube (Tehrani and Lin, 2011). These early endocrine cells thus form independently from pancreatic ductal tissue, and they contribute minimally to the adult endocrine mass (Hesselson et al., 2009). Secondary islet cells in zebrafish, on the other hand, arise through a genetic program that is highly similar to that of secondary transition endocrine cells in mammals (Beer et al., 2016; Kimmel and Meyer, 2016). They furthermore show analogous behavior to their mammalian counterparts, as they emerge from a branching ductal network and cluster into polyclonal islets (Beer et al., 2016; Ninov et al., 2013).
Genetic studies in model organisms have successfully defined signaling pathways contributing to pancreas and islet cell type differentiation. However, due to the inaccessible location of the pancreas in humans and most animal models, relatively little is known about the cellular dynamics and molecular mechanisms controlling islet morphogenesis. As single endocrine cells as well as clusters have been identified in pancreatic mesenchyme, islet formation has been postulated to involve cell migration in response to external directional cues (Cole et al., 2009; Puri and Hebrok, 2007, Pauerstein, 2017 #1354). However, the finding of islet clusters forming in close proximity to the ductal epithelium (Bader et al., 2016; Kesavan et al., 2014; Pan and Wright, 2011), suggests that cell interactions at close distances may contribute to islet formation. In vitro, endocrine cells have an intrinsic capacity to organize into aggregates that recapitulate many aspects of native islet structure (Woodford and Zandstra, 2012). This further implicates endocrine intercellular communication in islet formation, which could be mediated by secreted factors or cell-cell contacts.

Several pathways have been linked to islet morphogenesis based on fixed tissue and in vitro observations. EGFR signaling was postulated to act through Rac1 to modulate cell-cell contacts important for endocrine cell movements (Greiner et al., 2009; Miettinen et al., 2000). Furthermore, EGF is able to induce migration of pancreas-derived cells in vitro (Hardikar et al., 2003). Cell-cell adhesion was shown to impact islet assembly in mouse, where beta cells overexpressing a dominant negative E-cadherin remained dispersed instead of forming clusters (Dahl et al., 1996).

A limited number of studies have used in vivo imaging to address mechanisms of endocrine cell clustering. Time lapse imaging of endocrine cells in mouse pancreatic explants revealed active movements, dynamic morphologies, and aggregation of cells into clusters (Kesavan et al., 2014; Pauerstein et al., 2015; Puri and Hebrok, 2007). In addition, beta cell expression of a constitutively active Cdc42, which perturbs actin dynamics, interfered with delamination and differentiation, and reduced cell movement (Kesavan et al., 2014). Additional studies showed that blockade of G-protein coupled receptor (GPCR) signaling resulted in a dispersed islet phenotype in mouse pancreas, and disrupted the clustering of principal islet cells in zebrafish (Serafimidis et al., 2011). In a recent report, Semaphorin signaling from the peripheral mesenchyme was suggested to promote directional movement of islet cells (Pauerstein et al., 2017).

In this work, we performed live imaging with novel transgenes to visualize endocrine cell morphologies and movements with high spatial and temporal resolution. We show that islet cells are highly motile and generate fine dynamic protrusions, and we characterize this motility at the single cell level. In probing molecular mediators of motility and assembly, we found that disruption of protrusion formation through inhibition of PI3K is associated with
perturbation of islet assembly and also blocks endocrine cell differentiation. We further demonstrate that blockade of GPCR signaling similarly inhibits cellular motility and disrupts islet formation. Our findings suggest that cell motility, regulated by PI3K and GPCR potentially acting in a common pathway, plays an important role in islet morphogenesis.

Results

Morphology and dynamics of nascent endocrine cells

During zebrafish secondary islet formation, which begins around 5 days post fertilization (dpf), endocrine precursors differentiate from progenitors located in the intrapancreatic duct. Although it initially develops in a deep internal location, after 5 dpf the zebrafish pancreas assumes a planar form with a lateral superficial position, which is accessible to live imaging using fluorescent transgenes (Fig 1A). As development progresses in later larval stages, the pancreas assumes a complex lobular morphology, curving around the gut (Fig. 1B).

From 6 dpf to 8 dpf, secondary islet cells appear with low frequency and beta cells are rarely detected (Fig. S1A-D, Table S1, Moro et al., 2009; Parsons et al., 2009). Cell clusters are detectable around 13-15 dpf and can be visualized in microdissected pancreata expressing cell type-specific transgenes (Fig. 1B-C), and in live samples by confocal microscopy. At these stages, the quantity of cells and islets and their distribution is highly variable between samples (Fig. S1E, F, Table S2). Secondary endocrine cells expressing transgenes driven from the pan-endocrine pax6b promoter (Delporte et al., 2008) are found as single cells, as well as in small and larger clusters (Fig. 1C-F, Fig. S1E, F). In fixed samples, these cells exhibit long cytoplasmic extensions, fine intercellular connections, as well as shorter filopodia (Fig. 1D-F).

The complex cell morphologies observed in fixed samples imply that cell dynamics contribute to islet morphogenesis. To explore cellular behaviors, we imaged secondary islet cells in 2 week old (13-15 dpf) pax6b-promoter-driven transgenics. Secondary islet cells project protrusions (Fig. S1H), which change over time (Fig. S1G, I, J). We observed cells tethered by narrow connections and movement of cells into closer proximity (Fig. 1H, Fig. S1G, I, J, Summarized in Table S3).

Induced islet cells show dynamic protrusions during clustering

In naturally arising endocrine cells, the asynchronous and heterogeneous progression of islet development yields only a minority of samples at a given stage with cells in the process of clustering, and in which movements can be captured during imaging. Furthermore, optical accessibility of developing islets decreases after 8dpf, as overlying tissues increase in thickness and opacity, and organ complexity increases. To permit studies of islet
morphogenesis with conditions favorable for imaging and manipulation, precursor differentiation and secondary islet formation can be accelerated through modulation of regulatory pathways, for example by inhibition of Notch or retinoic acid signaling (Huang et al., 2014; Parsons et al., 2009; Wang et al., 2011).

We applied this approach to follow islet cell clustering by applying Notch inhibitor for 24 hours starting at 4 dpf. We began imaging at 6 dpf, to provide time for cell differentiation and initiation of transgene expression, and followed samples by confocal microscopy for up to 3 days. pax6b:dsRed indicated the entire endocrine cell population, while beta cell morphology was highlighted by the mnx1:memGFP transgene, in which an mnx1 promoter upstream of a membrane-targeted, farnesylated GFP drives expression in neurons and early beta cells (Arkhipova et al., 2012; Flanagan-Steet et al., 2005). To maintain viability over several days, samples were removed from the agarose and allowed to recover between imaging sessions. We noted progressive cell aggregation over time, with distinct clusters evident within 48-72 hours (8-9 dpf). While cluster formation was a consistent finding (n=9/9), cell behaviors were complex and heterogeneous, and the configuration of nascent islets varied between samples (Fig. S2).

To gain further insights into cellular mechanisms, we focused on cells that were loosely associated or in close proximity but not yet clustered. As we observed in naturally occurring endocrine cells, induced endocrine cells showed filopodia, shape changes, and narrow cell-cell connections as they moved closer together (Fig. 2A-C, Fig. 3A-C). Particularly striking was the appearance of long, flexible, and highly dynamic protrusions revealed by the membrane-targetted mnx1:memGFP transgene, that are barely detectable from cytoplasmic dsRed expression (Fig. 2A, Fig. 3). By collecting image sequences at shorter (minutes) and longer (hours) time intervals, we captured movements of single cells and groups of cells along axes demarcated by narrow cell-cell connections (Fig. 2A-C, Fig. S3B; n = 7/11 samples tracked over time). We further observed that as cells coalesce, narrow filopodia transform into broader connections with progressive shifting of cytoplasm (Fig. 3A-B, Fig. S4, Movie 1).

To quantitate cell clustering in complex cell configurations, we calculated the volume of a polygon enclosing the tracked cells to represent the combined distances between cells in one measure. As cells move closer together, the volume of this polygon decreases (Fig. S3A). Tracking studies showed that clustering was not uniform or constant, as examples showed variations of slope in plots of clustering versus time, both within and between samples (Fig. 2D, Fig. 3D).
**krt18:LifeActTom** transgene labels actin cytoskeleton in epithelia and pancreatic duct

To further dissect the process of islet formation, we more closely examined the origin of the newly emerging cells. These cells arise from bipotent duct/endocrine progenitors which are labeled by transgene expression driven from the Notch-responsive Tp1 element (Parsons et al., 2009; Wang et al., 2011), and are organized in a branching network that extends the length of the pancreatic tail (Fig. S5A, B).

The protrusion formation and cell shape changes observed during islet morphogenesis are indicative of dynamic rearrangements of the actin cytoskeleton. In order to better visualize actin-based cytoskeletal dynamics within the duct and emerging endocrine precursors in vivo, we generated transgenic fish containing the epithelial krt18 promoter upstream of a red fluorescent LifeAct-Tandem-dimer-Tomato fusion protein Tg(krt18:LifeActTom). *Keratin 18* (krt18) is expressed in intrapancreatic ducts in zebrafish (Lorent et al., 2010; Yee et al., 2005), and a previously described krt18 promoter was shown to direct reporter gene expression to epidermis and gut (Wang et al., 2006), and the biliary system (Wilkins et al., 2014). The F-actin binding peptide LifeAct labels F-actin filaments without disrupting endogenous actin dynamics (Phng et al., 2013; Riedl et al., 2008).

Imaging of live krt18:LifeActTom embryos showed labeled surface epidermis at the 15 somite stage, with enhanced signal at cell-cell boundaries (Fig. S5C, D). To localize krt18 promoter-directed expression within the pancreas, we examined krt18:LifeActTom in combination with previously characterized pancreas cell-type specific transgenes. Within the exocrine pancreas region defined by expression of Ptf1a:GFP (Pisharath et al., 2007), a branching network of LifeActTom-expressing cells extend from the principal islet (PI) posteriorly into the pancreatic tail (Fig. S5E, F). *LifeActTom* expression colocalized with duct-specific Tp1:GFP expression (Parsons et al., 2009), and delineated the long processes that interconnect these cells (Fig S5G,H). Expression of krt18:LifeActTom was also observed in blood vessels, consistent with previous reports (Schaffeld et al., 2002; Yee et al., 2005) (Fig S5E). krt18:LifeActTom transgene signal colocalized with Phalloidin staining in cell membranes of the gut epithelium (Fig S5I, J), and with enhanced Phalloidin signal in the pancreatic duct (Navis and Bagnat, 2015) (Fig. S5K-L). krt18:LifeActTom further colocalized with 2F11 antibody staining, which targets the calcium binding protein Annexin A4 (Anxa4; (Zhang et al., 2014), and in zebrafish labels the pancreatic duct and emerging endocrine cells (Matsuda et al., 2013; Zhang et al., 2014) (Fig. S5M-N).
Nascent endocrine cells form dynamic protrusions

Having established that the *krt18:LifeActTom* transgene indicated actin distribution in the pancreatic duct of living embryos, we next examined nascent beta cells emerging from, and in proximity to, the pancreatic duct. In pancreas of Notch inhibitor-treated, *krt18:LifeActTom;mnx1:memGFP* transgenic larvae at 6 dpf, we could distinguish LifeActTom+/memGFP+ early beta cells still within the duct that were relatively round and showed diffuse signal from both GFP and LifeactTdT (Fig. 4A). Beta cells emerging from the duct extended fine actin-rich protrusions directed away from the duct as well as along the duct, connecting to nearby emerging cells (Fig. 4A-D). An enrichment of actin signal was detected at the point where the cell remains attached to the duct (Fig. 4B-D). LifeActTom+/memGFP+ cells detached from the duct also projected actin-rich protrusions, which made contact with nearby cells (Fig. 4E-G). These studies implicate actin-based cytoskeletal rearrangements not only in delamination, consistent with previous studies (Kesavan et al., 2014), but also in performing important functions in cells that have left the duct and are beginning to cluster.

As fine protrusions can serve various functions depending on their morphologic properties (Jacquemet et al., 2015), we analyzed filopodial dynamics in single cells. *mnx1:memGFP*-positive early beta cells, located in proximity to the duct defined by *krt18:LifeActTom* expression, were examined with spinning disc confocal microscopy, with image z-stack capture at time intervals ranging from 12s to 20s. These studies revealed highly dynamic, small diameter (<2μm) protrusions, that displayed undulations and branching (Fig. 4H, Movie 2). The majority (75%) had a maximal extension of less than 9μm, but filopodia in some cases (3/41 examined) reached lengths of >15μm (Fig. 4J). Mean velocities of extension (3.2μm/min) and retraction (2.9μm/min) were similar (unpaired t-test, p=0.3), with maximum speeds of greater than 6μm/min (Fig. 4I). Filopodial length and extension/retraction velocities resemble those seen in vivo in endothelial cells (Yu et al., 2015) and in vitro in neuronal growth cones (Mallavarapu and Mitchison, 1999). Overall, these analyses revealed dynamic, flexible protrusions produced by clustering endocrine cells, which show characteristics of exploratory filopodia with function in environmental sensing and cell-cell recognition.

To demonstrate that similar protrusions are found in secondary endocrine cells arising during the normal course of development, we first imaged *mnx1:memGFP*-expressing cells in uninduced samples at 8 dpf, which are only rarely detectable (n=7/45 larvae). In 4/7 samples, the signal was too weak to distinguish cell morphology. In 2/3 samples containing cells with sufficiently strong signal, fine dynamic protrusions were detected (Fig. S6B-D). At 2 weeks
(13-15 dpf), more cells show mnx1:memGFP+ expression (Table S1), and dynamic protrusions could be observed from cells in isolation and already in clusters (Fig. S6F-G; 6/14 samples imaged).

The above studies highlighted protrusions in mnx1:memGFP-positive beta cells and pax6b-transgene expressing endocrine cells. To show that dynamic morphologies are also characteristic of non-beta endocrine cells, we performed time lapse imaging of glucagon (gcga)-expressing alpha cells using gcga:GFP transgenics (Zecchin et al., 2007) at 7 dpf following endocrine cell induction. Gcga:GFP cells displayed protrusions and active changes in cell shape (n=4/4 time lapse movies, Fig S6H).

**PI3K regulates protrusion formation in aggregating endocrine cells**

Phosphoinositide-3-kinase (PI3K) has a critical and highly conserved role in cell motility in many cell types, controlling the location of actin polymerization, and thereby the orientation and shape of protrusions (Leslie et al., 2008). To examine the contribution of PI3K to islet cell dynamics, mnx1:memGFP;kt18:LifeActTom larvae were first treated from 4-5 dpf with Notch inhibitor, then at 7 dpf with the irreversible PI3K inhibitor Wortmannin (WORT) and observed by time lapse microscopy for cell morphology and protrusion dynamics. Control mnx1:memGFP+ cells in the vicinity of the pancreatic duct (Fig. 5A) extended and retracted processes both towards and away from neighboring cells (Fig. 5C, top, Movie 3). By contrast, in samples treated with Wortmannin for 3-4 hours prior to imaging, GFP+ cells produced less protrusions and there were few contacts between neighboring cells (Fig. 5B and 5C, bottom, Movie 4).

To more precisely define the effect of PI3K inhibition on cellular morphology and dynamics, we applied quantitative measures of cell shape to sequential frames within time lapse image series captured at 6 dpf, focusing on isolated single cells (Fig. 5D, S7A). For these studies, we applied WORT and in addition used the reversible PI3K inhibitor Ly294002 (LY). We used the parameter solidity as a quantitative indicator of the extent of filopodia formation (Xue et al., 2014), that is amenable to efficient and unbiased image analysis approaches (Barry et al., 2015). Solidity, the ratio of cell area to convex hull area, has a maximum of one for a regular round object and this value decreases towards 0 in proportion to protrusion formation (Fig. S8A). In single cells traced over time, cell morphologies, as revealed by cell solidity, circularity and area, differed between control and treatment groups (Fig S8B, Table S4). The high area, low solidity phenotype of individual cells in control samples was maintained over the time of imaging, suggesting that sustained periods of active motile behaviors are a feature of islet cell coalescence. Since individual cells were followed for variable times due to technical issues of tissue movement and signal bleaching, we combined all timepoints from
all cells for statistical analysis (Fig. 5E-G). Overall, following PI3K inhibition, beta cells in the pancreatic tail were more compact, with decreased cell area and higher circularity as compared to controls (Fig. 5E,F). Consistent with our observation of decreased protrusion formation following PI3K inhibition, solidity was significantly higher in treated cells compared to controls (Fig. 5G).

To assess effects of PI3K inhibition on overall membrane motility, we examined expansion and retraction of cell membranes using an analysis approach that defines the cell body excluding fine protrusions (Tsygankov et al., 2014) (Fig. S7B). When overall membrane motility was considered, there was no reduction in the PI3K-inhibitor treated groups as compared to controls (Fig. S7C). These studies showed that global membrane motility was maintained when PI3K was inhibited, and suggest that PI3K preferentially regulates fine protrusions.

**Quantitative assessment of islet cell clustering**

Our studies above indicate that islet formation, both naturally occurring and when accelerated by Notch inhibition, occurs in an asynchronous, non-stereotypical fashion. To assist in identifying molecular pathways acting in islet formation, it was necessary to be able to make global assessments and quantify perturbations. Due to the small size of the zebrafish, and the accessible location of the pancreas during early larval stages, one can readily image the entire pancreas in the living animal (Fig. 1A). Efficient and consistent processing of many samples is facilitated by capturing the entire pancreas in one image, which enables imaging sufficient numbers of samples (at the same stage) for meaningful quantitative comparisons.

To establish a quantitative method to assess islet formation, we induced islets at 4 dpf, then imaged the whole pancreas at 6 dpf, after endocrine cells can be detected, and at 8 dpf, a time when clusters have formed (Fig. S9A). Emerging secondary islet cells were detected through nuclear Tp1:H2BmCherry transgene expression (Ninov et al., 2012), combined with pax6b:GFP to indicate newly generated endocrine cells (Delporte et al., 2008). At 6 dpf, newly differentiated double positive GFP+/H2BmCherry+ cells were distributed predominantly as single cells (Fig. S9B-E, n=7 larvae). 48 hours later, at 8 dpf, the GFP+ cells retained the long-lived H2BmCherry in their nuclei but were arranged in larger clusters (Fig. S9F-I, n=7 larvae), consistent with our previous observations (Fig. S2). Quantitation of secondary islet 3-dimensional volumes showed a significant increase in the size of islets between 6 dpf and 8 dpf (Fig. S6E, I, J). Islet volumes at 8 dpf thus represent a robust quantitative phenotype, despite the overall heterogeneity of islet morphogenesis.

We then asked whether cell division contributes to the increase in cell cluster size, by comparing endocrine cell number in the pancreatic tail at 6 dpf and 8 dpf. Using the
H2BmCherry nuclear label to facilitate counting of differentiated endocrine cells, we determined that the number of GFP+/H2BmCherry+ secondary islet cells did not change significantly between 6 dpf and 8 dpf (Fig. S9K). This result is consistent with previous findings that differentiation towards an endocrine fate is associated with cell cycle exit (Bechard et al., 2016; Ninov et al., 2012). This implied that cell proliferation contributed minimally, if at all, to the increase in cluster size.

**PI3K impacts islet cell coalescence**

Having validated a quantitative assay for examining islet cell clustering, we used this approach to provide evidence for the hypothesis that PI3K-regulated cell motility is required for islet cell aggregation. We applied a combination of Notch inhibitor Ly411575 and retinoic acid inhibitor DEAB to robustly induce a population of secondary islet cells at 4 dpf (Huang et al., 2014), with minimal toxicity. In addition to performing manual image analysis, we optimized imaging parameters and developed an analysis pipeline for automated detection of secondary islets (Fig. 6A-B, S10A). In pax6b:GFP;ela:mCherry double transgenics, ela:mCherry delineated the exocrine pancreas, thus defining the region that contained secondary islets. For validation of our automated analysis in comparison with manual measurement, we applied the small molecule PI3K inhibitor Ly294002. Consistent with PI3K-regulated motility having a role in islet cell coalescence, treatment of pax6b:GFP;ela:mCherry double transgenics with Ly294002 in our assembly assay significantly decreased islet volumes as determine by manual ImageJ analysis (Fig. S10B-D), and closely matching results were obtained using the automated algorithm (Fig. 6C).

**PI3K inhibition impacts differentiation but not cell number**

PI3K is a central regulator of cell survival and proliferation, in addition to its roles in cell motility (Leslie et al., 2008). Therefore, it was necessary to confirm that equivalent numbers of secondary islet cells were maintained under control and Ly294002 treatment conditions, to rule out confounding influences from cell proliferation or cell death. For this, we counted the individual GFP+ endocrine cells in the pancreatic tail in control and Ly294002-treated larvae at 8 dpf, following islet induction treatment as performed for islet volume analyses. We found no significant difference in GFP+ cells within the pancreatic tail at 8 dpf (average of 32±5 cells in control samples, n=13; 29±8 cells in LY-treated samples, n=9; p=0.35, unpaired t-test, Fig. 6D). This supports our hypothesis that PI3K is influencing islet morphogenesis and not impacting cell number.

At 6 dpf following endocrine cell induction, endocrine hormones insulin and glucagon are barely detectable (Fig S11A,C,D), while expression is increased at 7 dpf in nascent clusters (Fig S11B,E), thus showing a temporal association between the progression of clustering
and cell differentiation in our system. We then determined whether PI3K inhibition, with its effect on islet assembly, also had an effect on cell differentiation. To count beta cells, we used a transgenic line based on the rapidly maturing, nuclear-localized mKO2-zCdt1 fusion protein (Sugiyama et al., 2009), expressed under control of a 1.2kB insulin promoter (Huang et al., 2001; Moro et al., 2009). *ins:mKO2* transgenic larvae were treated to induce islets, followed by addition of Ly294002 (LY) or DMSO alone as a control (CTL). Supporting the hypothesis that islet assembly promotes beta cell differentiation, the number of *ins:mKO2*+ beta cells in Ly294002-treated larvae was significantly reduced compared to controls at 8 dpf (17±4 cells in controls, *n*=16; 5±2 cells in Ly294002-treated samples, *n*=14; *p*<0.0001, unpaired t-test; Fig. 6E-G). To determine whether other endocrine cell types were affected, we performed antibody staining for Ins in combination with antibodies against glucagon (Gcg) and somatostatin (Sst). We found that the combined number of Gcg+ and Sst+ cells was reduced to a similar degree to that of Ins+ cells (Fig. S11F-J). This supports a disruption of differentiation rather than a fate change of Ins-expressing cells into a different endocrine cell type.

**Ductal progenitor plexus is reestablished following islet induction**

While PI3K inhibition changed morphologies of endocrine cells, activity of PI3K inhibition on adjacent structures may also impact islet cell clustering. To avoid potential confounding effects of islet induction on duct morphology, we examined the effect of PI3K inhibitor treatment alone. *krt18:LifeActTom;Tp1:GFP* double transgenics were treated from 6 dpf to 7 dpf with Ly294002. Imaging of native fluorescence in living larvae did not show any alteration in duct morphology (Fig. S12).

We then examined how islet induction treatment affects the notch-responsive progenitor cell network. Following endocrine cell induction, as many duct-associated progenitor cells convert to endocrine cells and begin to cluster, ductal structure appears disorganized, with decreased branching (Fig. S13A, B). To observe the later consequences, following treatment at 4 dpf, islet-induced transgenic larvae were grown in parallel with control samples. At 14 dpf, larvae were fixed and immunostained for pancreatic lineage transgenic markers. In *ela:GFP;pax6bDsRed* transgenics, exocrine pancreas appeared similar between control and induced samples (Fig. S13C, D). Secondary islets could be detected in control samples based on pax6b:dsRed-expression, while treated samples, as expected, showed increased secondary islets (*n*=3, Fig. S13C, D). In 14 dpf *Tp1:GFP;ela:mCherry* transgenics that had been treated for endocrine cell induction at 4 dpf, a ductal network, similar to that of untreated controls, was detected (*n*=8, Fig. S13E, F). Thus, while endocrine cell induction alters the architecture of the progenitor plexus, it appears restored during subsequent development.
EGFR blockade and Rac1 inhibition do not affect islet assembly

PI3K can be activated by tyrosine kinase receptors, and has downstream effects on cytoskeleton-regulating Rho-family GTPases (Artemenko et al., 2014; Shewan et al., 2011). EGF signaling, potentially acting through Rac1, was suggested to regulate islet morphogenesis (Greiner et al., 2009; Miettinen et al., 2000). To examine whether these factors act in a pathway with PI3K to influence islet cell aggregation, we utilized Tyrphostin AG 1478 and NSC 23766 to inhibit EGFR and Rac1, respectively (Gao et al., 2004; Goishi et al., 2003). Following islet induction from 4-5 dpf, inhibitors were applied for 48 hours (from 6 dpf to 8 dpf), and our automated analysis method was applied. We used a well-tolerated lower dose (100% survival) and a higher dose where toxicity was seen at the end of 48 hours (Table S7). EGFR or Rac1 inhibition did not impact islet size, at both doses tested (Fig. S14A, B), suggesting that EGFR-signaling and Rac1 activity do not influence islet cell clustering. As we focused on later islet assembly events, we do not exclude a role for these proteins in islet cell delamination in our system. We also cannot rule out compensation for Rac1 inhibition by other RhoGTPase family members.

GPCR signaling blockade perturbs islet formation

PI3K can also be activated by GPCR signaling (Artemenko et al., 2014), and previous studies implicated GPCR signaling in islet morphogenesis using mouse explants and transient misexpression in early zebrafish principal islet beta cells (Serafimidis et al., 2011). To test whether blockade of GPCR signaling results in a secondary islet assembly phenotype similar to PI3K inhibition, we generated transgenic fish expressing the G protein-inhibiting pertussis toxin (PTX) under control of a heat-shock inducible promoter, in a vector that co-expresses LifeActTom, and a heart-specific cmlc2:GFP cassette as an indicator of transgenesis (Fig. 7A, top). Functionality of this construct was confirmed by performing a heat shock at 50% epiboly and examining transgenic embryos at 24 hpf. Robust ubiquitous LifeActTom expression was associated with dramatically reduced expression of cardiac cmlc2:GFP, and shortening and curvature of the body axis (Fig. S15A), consistent with the previously described role of GPCR signaling in early embryogenesis and cardiac development (Pauli et al., 2014; Scott et al., 2007).

To examine the effects of GPCR blockade on islet formation, we induced secondary islets in hsp70:LifeactTom-PTX;pax6b:GFP double transgenic larvae at 4 dpf, and then applied a heat shock at 6 dpf and 7 dpf (Fig. 7B). Heat-shocked samples containing an hsp70:LifeactTom transgene or pax6b:GFP alone served as controls. Quantitation of islet volumes at 8 dpf showed that cell clustering was significantly reduced in PTX-expressing
embryos as compared to controls (Fig. 7C, S15B).

Given the similar impact of PI3K inhibition and GPCR blockade on islet cell aggregation, we examined whether protrusion formation and cell motility was affected in endocrine cells of PTX-expressing larvae. To test this, hsp70:LifeactTom-PTX;mnx1:memEGFP embryos induced by Notch inhibition were heat shocked at 7 dpf, then examined 4-6 hours later by time lapse microscopy. Similar to our findings following application of PI3K inhibitors, PTX-expressing cells appeared rounder with reduced protrusion formation, while control cells showed active extension and retraction of fine protrusions (Fig. 7D-E, S16A, B, movie 5, movie 6). Quantitation of morphology parameters in time lapse image series of single cells (Fig. S16A-B) corroborated that PTX-expressing cells had higher solidity and significantly reduced area (Fig. 7F,G, S16C, D).

To examine the impact of PTX expression on endocrine cell differentiation, we determined insulin- and glucagon-expressing cell number using ins:GFP (Pisharath et al., 2007) and gcga:GFP transgenics. Transgene expressing cells in the pancreatic tail were counted at 8 dpf, following islet cell induction and heat shock treatments as performed for assessing islet assembly (Fig. 7B). There was a minor but significant reduction in both Ins- and Gcg-expressing cells (Fig. S16E-H).

**Discussion**

In this work we show that endocrine cells form highly dynamic fine protrusions, and we provide evidence that this protrusion formation is important for islet assembly. Moreover, we define the morphologic properties of these protrusions, establishing analogy to other tissues in which filopodia act as exploratory structures to facilitate cell-cell recognition and initiate tissue coalescence. Furthermore, the above studies define roles for GPCR signaling and PI3K in regulating the endocrine cell dynamics that occur during islet assembly.

Previous studies (Moro et al., 2009; Parsons et al., 2009) and this current work document the slow accumulation of new islet cells beginning around 5-6 dpf, with cell clusters being detectable by 13-14 dpf. To develop a system in which islet morphogenesis can be readily and consistently detected within a relatively short time frame, we applied an induction treatment based on endogenous signalling pathways that act during development. In this way, clustering endocrine cells can be visualized with high resolution, in sufficient numbers for quantitative analyses, and the animals are young enough to take up compounds supplied in the incubation media to probe modulators of islet clustering.
Several lines of evidence support our hypothesis that processes detected in induced islets are relevant for normal islet clustering. Single instances of endocrine cell protrusions in fixed samples, and cell movements and coalescence in live imaging, have been previously shown in uninduced zebrafish larvae (Kimmel et al., 2011; Ninov et al., 2012; Ninov et al., 2013). In the current work, we built on these prior studies to present a detailed analysis of cell dynamics during islet morphogenesis within living animals. In mice, the deep internal location of the pancreas precludes in vivo imaging, so explant culture systems are used. Similar to our observations in zebrafish, clustering endocrine cells in explants make protrusions and show dynamic morphologies coincident with cluster formation (Bechard et al., 2016; Pauerstein et al., 2015; Puri and Hebrok, 2007; Kesavan, 2014 #1087). The considerable number of cells induced to cluster in our model creates a situation similar to that in mouse, where large numbers of cells differentiate during the secondary transition (Marty-Santos and Cleaver, 2015). It was possible but difficult to visualize filopodial dynamics in naturally arising endocrine cells in zebrafish, as only rare samples showed sufficiently strong fluorescent signal. Overall, cell behaviors during islet morphogenesis are similar in naturally occuring and induced cells in zebrafish, and appear furthermore to be conserved between zebrafish and mammals. Future studies can define additional pathways and corroborate similarity of molecular regulation.

In many systems, filopodia play an important role as exploratory sensors in mediating cell-cell recognition and initiating cell-cell adhesion (Mattila and Lappalainen, 2008). This has been described in Drosophila dorsal closure (Jacinto et al., 2000), zebrafish blood vessel anastomosis (Phng et al., 2013), mammalian blastula compaction (Fierro-Gonzalez et al., 2013), and recently in mouse neural tube closure (Rolo et al., 2016). In both Drosophila and vertebrate models, it has been proposed that filopodial contacts can transmit a mechanical pulling force to draw cells together (Fierro-Gonzalez et al., 2013; Jacinto et al., 2000; Millard and Martin, 2008). The endocrine cell filopodia we described show similarities to those of neuronal growth cones and vascular endothelial cells (Mallavarapu and Mitchison, 1999; Yu et al., 2015). We propose that endocrine islet cell filopodia scan the environment, to identify and establish contacts with neighboring islet cells and initiate coalescence.

In the above studies, we focused on early events following simultaneous induction of a pool
of differentiated cells, which resembles the robust endocrine differentiation during the secondary transition in mouse (Bankaitis et al., 2015). It remains possible that single cell directional migration guides new endocrine cells to established islets later during postnatal growth and in adulthood, when the rate of new cell emergence is dramatically reduced (Ackermann and Gannon, 2007). Movement of islets away from the duct may occur through a combination of exocrine tissue expansion and active directional migration (Pauerstein et al., 2017).

GPCRs are key signal transducers impacting cell morphology and motility (Cotton and Claing, 2009). Previous studies found that GPCR signaling plays a role in islet morphogenesis using mouse in vivo and explant models, as well as by examining principal islet formation in zebrafish (Serafimidis et al., 2011). Consistent with this earlier work, we found that disruption of GPCR signaling by expression of PTX disrupted clustering of secondary islet cells, which are analogous to the mammalian secondary transition cells. In our work, we further demonstrate changes in cell motility resulting from inhibition of GPCR signaling, providing a potential mechanistic link between this signaling pathway and morphogenetic consequences. Serafimidis (2011) found no change in endocrine cell specification following PTX treatment, based on c-peptide and glucagon expression, while we detected a minor decrease in insulin and glucagon expressing cells. This discrepancy could be due to technical differences in the experimental approaches. Taken together, these results suggest that PI3K activity and GPCR signaling in endocrine cells affect differentiation to varying degrees, conceivably through discrete mechanisms.

Upon activation, GPCRs signal through heterotrimeric G-proteins, whereby both Gα and Gβγ components interact with downstream signaling pathways (Cotton and Claing, 2009; Syrovatkina et al., 2016). Membrane-associated PI3K can be activated by GPCR signaling (Cotton and Claing, 2009; Zhao and Vogt, 2008), locally generating phosphoinositides (PIs), which in turn modulate the actin cytoskeleton in discrete subcellular domains (Shewan et al., 2011). Signaling lipids have been specifically implicated for directing filopodia that scan the environment and initiate cell-cell contact, for example in wound healing (Li et al., 2013; Pickering et al., 2013). In future studies it will be important to define the relative roles of Gα and Gβγ in islet morphogenesis, and to identify additional signals that coordinate cytoskeletal
dynamics, and mediate cell-cell recognition and clustering. Our studies do not exclude the possibility that differentiation impacts clustering, or that insulin itself serves as a signal contributing to islet assembly.

Screening of chemically active compounds for their biological effects in zebrafish embryos and larvae is a rapidly expanding field, which has been recently applied to identify modulators of beta cell differentiation (Rovira et al., 2011; Wang et al., 2015). Here, we described an assay for islet assembly with an automated image analysis pipeline that can be applied for compound screening, as it involves minimal manipulation, with transgenes conveniently providing a phenotypic readout in the living animal.

Generation of replacement islets from stem or other progenitor cells is a major goal for the realization of regenerative therapies for diabetes. Cell-cell interactions that occur during formation of the three-dimensional islet are crucial for beta cell function in vitro or in vivo. In this study, live imaging approaches reveal previously unappreciated protrusion dynamics of endocrine cells during islet coalescence. Through pharmacologic approaches combined with a novel quantitative assay, we established that PI3K directs actin-based cell motility, and acts along with GPCR signaling to regulate islet morphogenesis. Improving our understanding of islet morphogenesis can be applied for optimizing production of functional beta cells from undifferentiated progenitors in vitro and for directing in vivo differentiated endocrine cells to form islets.

Materials and Methods

Zebralfish transgenic lines and maintenance

Zebralfish were maintained using standard protocols. Embryos were grown in 0.0015% PTU to reduce pigmentation. Larvae to be studied at 2 weeks were kept in petri dishes until 5 dpf, then transferred to our fish facility until the time of harvest. A list of transgenic lines used in this study is found in Table S6. Component elements were assembled using the Gateway system (Kwan et al., 2007) and transgenic lines were generated using standard techniques (for details, see Supplemental Materials and Methods).

This study was approved by the Austrian Bundesministerium für Wissenschaft und Forschung GZ BMWF-66.008/0007-II/3b/2012 and GZ BMWF-66.008/0009-WF/II/3b/2014. All
procedures were carried out in accordance with approved guidelines.

**Compound treatments and heat shock**

For time lapse analyses, secondary islets were induced using 10 µM Ly411575 from 4-5 dpf. For cell morphology studies, larvae were treated for 3-4 hours with 100 nM Wortmannin or 50 µM Ly294002. For the islet assembly assay, embryos were treated with 10 µM Ly411575 plus 25 µM DEAB (Huang et al., 2014). Embryos were treated in E3 media (5 mM NaCl; 0.17 mM KCl; 0.33 mM MgSO4; 0.33 mM CaCl2; pH 7.5 in ddH2O) supplemented with 1% DMSO and 1x antibiotic/antimycotic solution (Sigma). The following compounds were used: Ly411575 (Sigma, SML0506), Wortmannin (Sigma, W1628), Ly294002 (Antibodies-Online, ABIN412265), Tyrphostin AG 1478 (Sigma, T4182), NCS23766 (Tocris, 2161). Stock solutions were prepared in DMSO, with the exception that NCS23766 was prepared in ddH2O. Wortmannin was stored aliquotted at -80ºC, for the remaining compounds, aliquots were stored at -20ºC. For experimental doses, we were guided by previously published studies (Sabha et al., 2016; Yoo et al., 2010), and additional testing in our system. Dilution studies were performed by treating pools of 10-15 embryos in E3 media containing varying compound concentrations, to determine an optimal dose for 48 hours of treatment (Table S7, Fig. S17). Samples were observed for lethality, or changes in morphology or movement. Embryos carrying *hsp70:LifeActTom-PTX* or *hsp70:LifeActTom* were heat shocked at 6 dpf and 7 dpf for 20 minutes at 38ºC in a shaking H2O bath.

**Antibody staining**

Larvae were harvested at 7 dpf, fixed for 1 to 2 hours at room temperature in 4% PFA/1%DMSO in PBS, then washed 3 x 5 minutes with 1 x PBS/0.1% Tween. To improve access of antibodies to internal structures, the ventral skin was cut open. Larvae were incubated in blocking buffer containing 1% DMSO, 1% sheep serum, 1% BSA and 1% Triton X-100 in 1 x PBS for at least 60 min at room temperature. Samples were then incubated overnight at 4ºC with primary antibody, washed and then reblocked and incubated in secondary antibody overnight at 4ºC. The primary antibodies used were: mouse anti-GFP (Roche, #11814460001, 1:100 dilution); mouse anti-Glucagon (Sigma #G2654, 1:100 dilution); mouse anti-2F11 (Abcam #ab71286, 1:100 dilution); rabbit anti-Somatostatin (Dako #A0566, 1:200); guinea pig anti-Insulin (Dako #A0564, 1:200 dilution), rabbit anti-GFP
(antikörper #ABIN110592, 1:200), Rabbit anti-dsRed (Clontech #632496, 1:200 dilution).

Secondary antibodies were labeled with Alexa Fluor (Invitrogen) and diluted 1:1000. Nuclei were labeled by incubation overnight at 4°C in 100 ng/ml DAPI/1%DMSO in PBS. Phalloidin staining (Alexa Fluor 488 Phalloidin, ThermoFisher A12379) was performed as described (Goody et al., 2012).

**Microscopy**

Live samples were mounted in 1.2% low melt agarose and overlaid with egg water or E3 media containing 0.003% tricaine. Brightfield and low magnification fluorescent images were captured on a Leica DM6000B. Imaging of fixed and live samples was performed using the Leica Sp5 with a 63x glycerol immersion objective or a Zeiss Axio Observer.Z1 equipped with a CSU-X1 spinning disc confocal using 25x, 40x, or 63x water immersion lenses. Time lapse imaging of cell morphology was performed on a Leica Sp5 using a 40x air objective. Time lapse images of cell protrusion dynamics were acquired using a microlens-enhanced Nipkow disk-based confocal system UltraVIEW RS and a 40x water objective. Confocal imaging for islet assembly studies were performed using a Zeiss LSM5 with a 20x dipping lens or a Leica Sp5 laser scanning confocal microscope with a 20x air objective. The HyD detector was used for optimal detection of the GFP signal on the Leica Sp5. For single cell imaging, cell location was confirmed by first imaging mnx1:memGFP in combination with krt18:LifeActTom, then the GFP channel was captured alone to maximize imaging speed. Details of acquisition settings for time lapse studies are found in Table S8.

**Image Analysis**

Confocal image stacks were processed using ImageJ. A median filter was used to reduce speckle noise, contrast adjustment and background subtraction were uniformly applied. Sample shift in time lapse series was corrected using StackReg (http://bigwww.epfl.ch/thevenaz/stackreg/ (Thevenaz et al., 1998)) and PoorMan3Dreg (developed by Michael Liebling, http://sybil.ece.ucsb.edu/pages/poorman3dreg/index.html) plugins of ImageJ. Sample shift occuring between acquisition of channels was corrected on z-projections using Photoshop. 3D visualizations were prepared using Imaris. Cell counts based on nuclear transgene expression were performed using the Spot Detection function of Imaris (Bitplane AG, Zurich) with a spot diameter of 5µm by 3D visualization of a confocal z-
stack spanning the region. Objects were filtered based on signal intensity at the center. Cell counting based on immunohistochemistry with DAPI staining was achieved using the “Point Picker” Plugin of ImageJ. Cell counting results are representative of two independent experiments.

**Islet Quantitation**

For islet size determination using Imaris, the Surfaces function was applied. Images were processed by smoothing (0.5µm) and local background subtraction, then contour surfaces were generated that enclosed the pax6b:GFP+ fluorescent signal in the pancreatic tail, and volumes were determined. Signal threshold and object size filtering were applied consistently to all images. For counting GFP+/RFP+ cells, the previously determined surfaces were applied to mask signals external to the secondary islets. The masked RFP+ nuclei were counted as previously described. 3D rendering was performed using the “Quick 3D” function of Imaris. 3D islet volume analysis procedures using the Particle Analyser Plugin (Doube et al., 2010) of ImageJ are detailed in Supplementary Methods. Image acquisition and analysis parameters were optimized within each experiment. Reported differences between control and treated groups were consistent in 2 or more independent experiments.

**Automated Islet Quantitation**

For the automated detection of islet volumes, a custom feature detection pipeline was developed that is adequate for batch processing large numbers of images. It was implemented in the Quocmesh library (AG Rumpf, Institute for Numerical Simulation, University of Bonn), using C++ for image processing, and Linux shell scripts for file management and automation. Our custom script requires an initial user interaction step to delineate the boundary between the pancreatic head region and tail, then subsequent delineation of the pancreas based on ela:mCherry expression and volume quantitation of pax6b:GFP+ secondary islets proceeds without further intervention (Fig. 6B). Imaging settings and processing parameters were adjusted for each experiment for optimal detection of true objects. The chosen settings were applied uniformly. (Details of the processing pipeline are contained in Supplementary Methods.) When processing is completed, a table is generated containing islet volumes, and tiled image montages show pre- and post-processing images for an overview and visual assessment of the analysis. Results shown
are representative of at least 2 independent experiments.

**Statistical Analysis**

Data were analyzed and graphs were produced using Prism (GraphPad). Statistical tests were applied as indicated in the Results and Figure Legends.

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**Competing interests**

The authors declare no competing or financial interests.

**Author Contributions**

J.F., M.H., A.W., R.A.K. performed experiments. T.W., A.W. generated transgenic lines. J.A.I. developed the automated islet analysis method. J.F., R.A.K., J.A.I. analyzed and interpreted data. R.A.K. conceptualized and coordinated the study, obtained funding, wrote and revised the manuscript. D.M. provided intellectual input, supervision, and edited the manuscript. All authors reviewed and approved of the final manuscript.

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Fig 1. Endocrine cells display complex morphologies during secondary islet assembly. (A) Maximal projection of confocal stack of the pancreas at 5 dpf, imaged in a living larva. Fluorescent transgenes label the exocrine (ela:GFP, green) and endocrine (pax6b:dsRed, red) compartments. Scale bar = 100 μm. (B) Maximal projection of confocal stack of fixed and microdissected pancreas from 14 dpf ela:GFP;pax6b:dsRed transgenic larva. Scale bar = 100 μm. This image was assembled by stitching together images of partially overlapping regions, using the Pairwise Stitching Plugin for ImageJ (Preibisch et al., 2009). (C) Close-up of pax6b:dsRed+ secondary islets at 14 dpf (in gray, from sample shown in B, white box). Scale bar = 50 μm. (D-G) Confocal z-stack projections of secondary islet cells and clusters from samples as in B, showing pax6b:dsRed transgene expression (gray). (D) Scale bar = 25 μm. (E-G) Scale bar = 10 μm. Yellow arrows highlight cell protrusions. (H) Confocal image series (maximum projections) of endocrine cells in the posterior pancreas of a pax6b:dsRed transgenic larva beginning at 14 dpf, with subsequent images acquired at the times indicated. Outlines (blue, yellow and purple) indicate individual cells that move into closer proximity to each other and to a pre-existing secondary islet (si). Arrows indicate fine cell-cell connections. Inset, cell-cell connections become visible with contrast enhancement (blue arrow). Scale bar = 10 μm. Nonlinear gamma adjustment was applied to highlight fine protrusions and cell-cell connections.
Fig 2. Clustering endocrine cells extend dynamic protrusions. (A-C) Image series acquired by confocal microscopy showing pancreatic endocrine cells in *pax6b:dsRed;mnx1:memGFP* transgenics at 7 dpf, following Notch inhibitor treatment from 4 dpf to 5 dpf. Shown are maximum intensity projections (A, B, left) and 3D representations of tracked cells (colored spheres), rotated to best visualize individual cells (A, B, right, C). *mnx1:memGFP* transgene expression delineates fine, dynamic protrusions and fine cell-cell connections. D. Progression of cell clustering over time. Clustering is quantitated as the volume of a convex 3D polygon enclosing the cell centers (See Fig. S3A), plotted against time for the samples shown in A (blue), B (red), C (green). Volume decreases over time as cells come into closer proximity. (For details see Supplementary Methods.)
Fig 3. Dynamics of cell coalescence. (A) Confocal time lapse series of Notch-inhibitor treated *pax6b:dsRed; mnx1:memGFP* transgenic at 7 dpf showing coalescence of 2 cells (orange and green spheres). 3 additional cells are tracked. (B) Z-projections corresponding to the images in (A), with RFP rendered in grey. (C) 2-channel image, close-up view of the GFP+ cell shown outlined in (B, green). A subset of images from a series collected at 18 min intervals are shown. Scale bar = 10 μm. (D) Quantitation of clustering (as in Fig. 2D) for the tracked cells indicated in (A).
Fig 4. **Emerging endocrine cells extend protrusions.** Confocal image stacks of differentiating pancreatic endocrine cells in living 6 dpf *krt18:LifeActTom;mnx1:memGFP* transgenics following Notch inhibitor treatment from 4 dpf to 5 dpf. Shown are maximum intensity projection (A, E-G) and single z-slices (B-D). (A) Rounded GFP+/LifeActTom+ early beta cell found within the duct (arrowhead), cell leaving the duct has a narrow protrusion (arrow). (B-D) Close-up view of region from A (yellow box) merged (B) and single channels (C,D). GFP+/LifeActTom+ early beta cells extend actin-rich processes (arrows). Scale bar = 10 µm. (E-G) Thin, actin-rich processes (arrows) project and connect two LifeActTom+ cells (arrowhead) in close proximity to the duct (pd). The GFP+ cell is a nascent beta cell while the GFP- cell likely represents a differentiating non-beta endocrine cell. Scale bar = 10 µm. (H) MIPs at indicated time points of time lapse series acquired by spinning disc microscopy of Notch inhibitor-treated larva analyzed at 6 dpf. Nonlinear gamma adjustment was applied to enhance low contrast signals. Dynamic extension and retraction of fine protrusions (arrowheads) were detected. Scale bar = 10 µm. (I) Quantitation of instantaneous retraction and extension rates analyzed from images captured as in (H), plotted as box-whisker plot, whiskers represent 10-90%. (J) Maximal filopodia lengths from image series as in (H), whiskers represent 10-90%, outliers are indicated by dots. The mean is indicated by ‘+’. Sample details are indicated in the Supplementary Methods.
Fig 5. PI3K inhibition impacts cell morphologies and dynamics. (A,B) Left, MIPs of confocal image stacks of early beta cells in control (A, CTL) and Wortmannin-treated (B, WORT) 7 dpf krt18:LifeActTom;mnx1:memGFP transgenics following islet induction at 4 dpf. Right panels contain single z-planes from the indicated regions of the image stack (box) with the duct indicated (white outline). (C) Selected time points from time lapse image series of
samples from A and B. Arrows indicate protrusions. Scale bar = 10μm. (C,top) Asterisk indicates cell that moves closer to cluster. (D) Single images from time lapse series, showing individual nascent beta cells in mnx1:memGFP transgenics treated to induce secondary islets from 4-5 dpf, followed by treatment at 6 dpf as indicated (WORT, 100nM wortmannin, LY, 50μm Ly294002 or CTL, DMSO), for 3-4 hours prior to imaging (top). Scale bar = 5μm. The lower panel shows the cell outline used for quantitative morphology analysis. (E) Cell area, (F) circularity, and (G) solidity, measured in individual frames of time lapse series (see Fig. S3). (CTL: 14 embryos, 118 time points; WORT: 14 embryos, 111 time points; LY: 13 embryos, 162 time points). (Additional sample information is found in Table S4).
**Fig 6. PI3K inhibition interferes with islet assembly assay.** (A) Secondary islet analysis is based on identification and 3-dimensional segmentation of exocrine pancreas as labeled by *ela:mCherry* expression. (B) With our automated script, the user defines the posterior pancreas based on a composite image of the middle slice of *ela:mCherry* (red channel) and a z-projection of the *pax6b:GFP* image stack (left, white line). The custom software automatically delineates the whole pancreas and secondary islets (right). (C) Quantitation of secondary islet size of the samples analyzed in Fig. S10B-D, using the automated method. In box-whisker plots, box extends from the 25th to 75th percentile, whiskers indicate 5th and 95th percentile, line at median, '+' indicates mean. ***p<0.001, Mann-Whitney Test (one-
tailed). (CTL, n=10 larvae, 133 objects, LY, n=8 larvae, 80 objects.) (D) pax6b:GFP+ cells in
the posterior pancreas of embryos treated as in (C). Graphed is mean±SD. (CTL n=13; LY
n=9; unpaired t-test, p=0.35) (E) ins:mKO2+ cells at 8 dpf in samples treated as in (C).
(Controls, n=16; LY-treated, n=14.) Graphed is mean±SD, unpaired t-test, ***p<0.0001. (F,
G) Representative images of 8 dpf pax6b:GFP;ins:mKO2 larvae. Control (CTL, F), or treated
from 6 dpf to 8 dpf with 15uM Ly294002 (LY, G). Overview images (F, G, top) were
assembled by stitching together images of partially overlapping regions, using the Pairwise
Stitching Plugin for ImageJ (Preibisch et al., 2009). Scale bar = 50μm. (F, G, bottom) Single
slices from z-stacks of higher magnification images. Scale bar = 10μm.
**Fig 7. GPCR inhibition disrupts islet assembly** (A) Transgene (*hsp70:LifeActTom-PTX*) used to inducibly express pertussis toxin (PTX, top). The transgene *hsp70:LifeActTom* served as a control (bottom). (B) Schematic of islet assembly experiment with heat shock induction of PTX. (C) Islet volume quantitation of heat shock treated controls (n=12, 99 objects), compared to larvae with induced expression of LifeActTom (n=14, 120 objects), or LifeActTom-PTX (n=6, 61 objects). Images were captured on a Zeiss LSM5 and volume quantitation performed using ImageJ (minimum object size 100µm³). *p<0.05, **p<0.01, Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test. (Results are representative of 2 independent experiments.) (D, E) Selected confocal projections from time lapse series of control (D) and PTX-expressing (E) transgenic larvae at 7 dpf, 4 or more hours following a heat shock. Quantitation of cell solidity (F) and area (G) measured in individual frames of time lapse series with images acquired at 4 minute intervals (as shown in Fig. S16A, B). ***p<0.0001, Mann-Whitney test, two-tailed. Data is combined from a total of 8 control and 8 PTX-expressing larvae analyzed in 3 separate experiments. Control samples include non-heat shocked *hsp70:PTX* transgenics, and LifeActTom+, heat-shocked samples. (Additional sample information is found in Table S5).
Supplementary Methods

DNA Constructs and Generation of transgenic lines

To generate \textit{krt18:LifeActTom}, 3.9kb upstream of \textit{keratin 18} was amplified from genomic DNA of 24 hpf embryos and cloned into the 5’ Gateway entry vector (\textit{p5E-krt18Pr}). The following primers were used:

\textbf{For:} AGGACATCTGCCCTCCAGCAC

\textbf{Rev:} GTCGCTGGTGTAAGTGAGCAGACG

The Tandem-dimer-Tomato (Tom) fluorophore was PCR amplified from \textit{pCAG-2A-H2B-Tomato} (gift of H. Lickert), fused downstream of the LifeAct peptide in \textit{pGEM-T-LifeAct} (gift of R. Aufschnaiter), then cloned into the Gateway middle entry vector (\textit{pME-LifeActTom}). The final construct, \textit{krt18:LifeActTom}, was generated by a Gateway reaction (Kwan et al., 2007). For generating \textit{hsp70:LifeActTom} by Gateway cloning, the \textit{hsp70} promoter in \textit{p5E-hsp70} (Kwan et al., 2007) was combined with \textit{pME-LifeActTom} in the \textit{pDestTol2pA} destination vector. \textit{hsp70:LifeActTom-PTX} was assembled by Gateway cloning in the \textit{pDestTol2CG2} destination vector (Kwan et al., 2007), which contains the \textit{cmic2:GFP} transgenesis marker. A middle entry vector (\textit{pME-LifeActTom-PTX}) was created containing the coding sequence for \textit{PTX} (gift of A. Gavales) downstream of \textit{LifeActTom}, with an intervening 2A sequence to drive separate expression of the two proteins. The \textit{ela:mCherry} line was generated by Gateway cloning of the following entry vectors: \textit{p5E-elastase} promoter, containing 1.9kB of the \textit{elastase3l} promoter (Wan et al., 2006), \textit{pME-mCherry} (Kwan et al., 2007), \textit{p3E-polyA} (Kwan et al., 2007) into the \textit{pDestTol2CG2} destination vector (Kwan et al., 2007). The \textit{ins:mKO2} construct was generated by cloning the \textit{ins} promoter (Moro et al., 2009, gift of F. Argenton) upstream of \textit{mKO2-zCdt1} (Sugiyama et al., 2009, gift of A. Miyawaki) in the \textit{pT2KXIGΔin} backbone.

Plasmid DNA was injected in combination with transposase mRNA into one-cell stage mitfa embryos. Embryos (sorted for fluorescent signal where possible) were raised to adulthood and crossed to identify germ-line transmitting founders. For each construct, several transgenic lines showed similar expression patterns, the line with consistent strong expression was maintained and used for further experiments.

Time Lapse Imaging

Time lapse studies were initiated with a z-stack that extended beyond the sample in both
directions, to account for potential sample movement. Data was not included for analysis in cases in which the cell shifted out of view. When possible, the sample was re-positioned and imaging re-started. This causes the occasional appearance of irregular time intervals in our time lapse image sequences. A proportion of image series could not be analyzed due to poor quality (low signal or loss of signal over time) or difficulty in distinguishing individual cells. In some samples, signal recovery permitted resumption of analysis at subsequent time points, with times of image acquisition as indicated in the figures.

As older fish (>5dpf) show decreased viability following continuous imaging for more than 2-3h, imaging studies extending >3h were performed by adapting a 'catch-and-release' approach (Mumm et al., 2006). In brief, samples were mounted in low melt agarose in glass bottom plates (maximum 2 per plate), overlaid with tricaine and directly imaged. The x,y coordinates of the imaged region were recorded relative to the primary islet to enable identification of the position at later times. After imaging, samples were carefully removed from the agarose using fine forceps, rinsed in egg water, and returned to the incubator in individual 3cm plates until the next imaging session.

To follow clustering of Notch inhibitor-induced endocrine cells from 6-9 dpf (Fig. S2), 20 samples were imaged in total. 9/9 that were followed to 8 or 9 dpf showed cluster formation. 5/8 samples imaged to 7 dpf had formed clusters, while the remaining 3 showed cell movements but had not formed clusters. 3 samples were not analyzed as they did not survive beyond the first time point. Regions for extended imaging in uninduced 13-15 dpf samples were selected to contain single cells within 2-3 cell diameters of a larger cluster or of other cells. Already formed clusters or isolated single cells were not followed.

**Cell Tracking**

For analysis of time lapse series, image stacks were first cropped and registered using ImageJ. Images for extended time series were manually aligned. Individual cells followed over time in z-projections were highlighted by a color overlay using Photoshop. Cells were manually tracked using 3D visualization in Imaris, with identity based on relative positions
and relation to nearby structures. In some series, cells could be followed based on GFP in addition to dsRed expression. A subset of dsRed+ cells became GFP+ over time, reflecting progressive differentiation of endocrine progenitor into beta cells. To quantitate cell clustering, cell coordinates (x,y,z) at each time point were determined using Imaris and exported to Matlab. Volume of a polygon that contains the tracked cells was calculated using the ‘convex hull’ function of Matlab. Polygon representations were generated in Matlab.

**Filopodia Tracking**

For filopodia tracking, the image stack was processed using ImageJ. The image was first cropped to contain a single cell. Contrast enhancement and background subtraction were performed, and gamma adjustment was applied to enhance the weak signal of fine protrusions. Filopodia length was measured in each frame using the ‘Neuron Growth’ plugin for ImageJ (Fanti et al., 2011). As recommended (Fanti et al., 2011), images were resized twofold to increase pixel density and improve detection of boundary features. Detection was performed using the automatic mode where possible, with manual correction applied as necessary. Cell morphology and motility were analyzed on 2D projections, recognizing that information along the z-axis will go undetected. From a lateral view, the pancreas extends primarily along the x-y dimension. Z-projections were necessary in order to have sufficient signal intensity to detect boundaries and fine protrusions in the x-y dimension. Of 13 time lapse movies acquired in 2 independent experiments, 5 had cells with robust membrane signal, and maintained signal intensity that was sufficient for filopodial tracking analysis. In all, 41 filopodia were analyzed.

**Quantitation of cell morphology and membrane dynamics**

For analysis of morphology and membrane dynamics in time series, PI3K-inhibitor treated and control samples at 6 dpf were imaged at 3-minute intervals with 1024x1024 pixel resolution. For analysis of cell morphology in *hsp:LifeActTom-PTX* transgenics and controls, heat-shocked samples at 7 dpf were imaged at 4-minute intervals with 512x512 pixel resolution to minimize bleaching, and images were resized to double the pixel density. Images were smoothed by a median filter, and gamma adjustment was applied. Cell morphology analysis was performed using the ImageJ plugin ADAPT (Barry et al., 2015) on image series cropped to contain a single cell. Parameters were adjusted empirically for optimized detection. Fine protrusions not recognized automatically were outlined manually.
Cell circularity is calculated as $4\pi \frac{\text{area}}{\text{perimeter}^2}$. Solidity is the area divided by the area of the convex hull (Fig. S8A). Membrane dynamics were determined using CellGeo (Tsygankov et al., 2014), using data sets of cell boundaries computed by the ADAPT Plugin. Total cell membrane dynamic activity (protrusion + retraction) between successive video frames was normalized to cell perimeter. The boundary velocity threshold was set to 5.

**Secondary islet quantitation**

In control experiments, islet formation was most robust and consistent when more cells were induced. In a previous report, maximal islet cell induction was achieved with 50µM Ly411575 for up to 3 days (Ninov et al., 2012). For our islet assembly assay, we applied an induction method using lower doses of notch inhibitor and retinoic acid inhibitor for 24h, as samples are subjected to an additional 48h of inhibitor treatments. Combining low-dose inducing treatments acting on 2 different pathways minimized systemic toxicity, and represents the milieu that controls physiological islet cell differentiation (Huang et al., 2014; Kimmel and Meyer, 2016). Results shown for islet assembly assays are representative of at least 2 independent experiments.

To quantitate inhibitor effects on secondary islet formation, 3D object analysis was performed using the Particle Analyzer of ImageJ (Doube et al., 2010). Image stacks were prepared for analysis by preprocessing steps using ImageJ, in which the pancreas is manually outlined based on mCherry expression in the exocrine pancreas, to define the location of the secondary islets, and exclude other GFP+ cells in the region (Fig. S10B-C, for details see Supplemental Protocol 1, below). For experiments using heat-shock inducible PTX expression, in preprocessing steps the pancreas is manually outlined based on a corresponding brightfield image, followed by analysis using the Particle Analyzer (see Supplemental Protocol 2, below).

**Automated islet quantitation**

The process for automated analysis of islet volumes involved the following steps: (1) The user is presented with an image combining the middle slice of the red channel (exocrine pancreas) and a maximum-intensity projection of the green channel (endocrine islets), and required to mark a line separating the pancreatic head from the tail region. (2) Both channels are blended with a histogram equalized version of themselves, to achieve contrast enhancement. (3) The red channel (pancreas) is segmented using the Chan-Vese model.
(Chan and Vese, 2001), with an additional term added for boundary avoidance. (4) The image for volume detection is prepared by first excluding points outside the segmented pancreas or anterior to the pancreatic tail, then the intensity and gradient of the green image are combined. To reduce the sensitivity to noise, the image gradients are computed by applying an anisotropic Gaussian filter. (5) Object boundaries are determined by thresholding the image of the previous step and triangulating with standard level set techniques (Sethian, 1999). (6) An edge connectivity measure is applied to split minimally connected objects, and hidden components (ie, completely contained inside others) are discarded. (7) Finally, the volume and surface area of each component is computed. Minimum object size is 50µm³. In addition, we apply the assumption that islets have a certain regularity of shape, and components with isoperimetric ratio (Area/Volume^(2/3)) above a defined threshold were considered noise and discarded. (Details of program code used for analysis are available upon request.)
Supplemental Protocol 1

Software Tools required:
ImageJ - https://imagej.nih.gov/ij/download.html
StackReg PlugIn – http://bigwww.epfl.ch/thevenaz/stackreg (Thevenaz et al., 1998)
Particle Analyser PlugIn– http://bonej.org/particles (Doube et al., 2010)

File requirements: 2 channel image, endocrine pancreas/exocrine pancreas
• Open image
• If necessary depending on file type and import method:
  [Image > Properties > Assign pixel size (width, height, depth)]
• Split Channels: Image > Color > Split channels
• Select channel with islets
• Smooth image: Process > Filters > Median (radius = 1)
• Enhance Contrast: Process > Enhance Contrast (saturated=0.4, equalize, process all slices)
• If gut movements caused shift during image acquisition: apply StackReg (Rigid Body)

[Additional pre-processing, such as background subtraction, can further improve islet detection (apply uniformly to data set).]
• Perform Z projection of islets: Image > Stack > Z-project (Max Intensity)
• Select image stack of el a:mCherry* exocrine pancreas
• Smooth image: Process > Filters > Median (radius = 2, process all slices)
• Perform Z projection of el a:mCherry image: Image > Stack > Z-project (Max Intensity)
• Set tool to ‘polygon’ and outline pancreas as a closed object
• Select projection of pancreatic islets
• Apply pancreas outline to islets : Edit > Selection > Restore Selection
• Adjust selection to include only posterior pancreas
• Select stack of pancreatic islets
• Apply polygon to define posterior islets: Edit > Selection > Restore Selection
• Clear outside – Edit > Clear outside (Stack)
• Set threshold: Image > Adjust > Threshold (apply uniformly across experimental data set)
• Volume analysis: “Particle Analyser” (min = 100(50)*; max=100000; other settings default)
• Save results table.

→ * Value depends on imaging conditions, can decrease to 50 if robust signal with high signal-to-noise ratio.
Supplemental Protocol 2
Software Tools required:
ImageJ - https://imagej.nih.gov/ij/download.html
StackReg Plugin – http://bigwww.epfl.ch/thevenaz/stackreg (Thevenaz et al., 1998)
Particle Analyser Plugin– http://bonej.org/particles (Douve et al., 2010)

File requirements: image stack of endocrine pancreas and corresponding bright field
• Open image
• If necessary depending on file type:
  [Image > Properties > Assign pixel size (width, height, depth)]
• Split Channels: Image > Color > Split channels
• Select channel with islets
• Smooth image: Process > Filters > Median (radius = 1)
• If gut movements caused shift during image acquisition: apply StackReg (Rigid Body)
• Perform Z projection of islets: Image > Stack > Z-project (Max Intensity)
• Select brightfield image – choose z-slice where pancreas can be best identified
• Set tool to ‘polygon’ and outline pancreas as a closed object
• Select projection of pancreatic islets
• Apply pancreas outline to islets : Edit > Selection > Restore Selection
• Adjust selection to include only posterior pancreas
• Select stack of pancreatic islets
• Apply polygon to define posterior islets: Edit > Selection > Restore Selection
• Clear outside – Edit > Clear outside (Apply to all slices)
• Set threshold: Image > Adjust > Threshold (apply uniformly across experimental data set)
• Volume analysis: “Particle Analyser” (min = 100*; max=100000; other settings default)
• Save results table.
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Supplementary Figures

Figure S1. Morphology and dynamics of naturally occurring secondary islet cells. Related to figure 1.

(A) Maximal projection of confocal stack of pax6b:dsRed transgenic at 8 dpf. Scale bar = 50µm. (B) Close-up view of endocrine cell (in A, arrow), which displays a long protrusion (B, arrow). (C, D) Close-up views of secondary endocrine cells in samples as in A. Arrows indicate cell protrusions. (B-D) Scale bars = 10µm. (E, F) Maximal projections of confocal stacks of pax6b:dsRed; ela:mCherry transgenic at 15 dpf. Larvae show variable islet development, ranging from small islets (arrow, E) and single cells (arrowhead, E), to larger clusters (arrows, F). Scale bar = 50µm. (G) Islet cells at times indicated in 13 dpf pax6b:GFP transgenic. 3D view (left) showing tracked cells (colored spheres), maximal projection (right) with cells pseudocolored to highlight cell movements. Single time point (H) and image series at times indicated (I-J) from 15 dpf pax6b:GFP transgenics. Single cells show protrusions (H, I) and move closer to existing secondary islets (I, J). Increasing contrast reveals cell protrusions (J, right, inset). PI, principal islet; si, secondary islet. (G-J) Scale bar = 10µm. (For sample details see Tables S1, S2, S3.)
Fig S2. Induced endocrine cells cluster over ≥72 hours.

Islet morphogenesis was followed in pax6b:dsRed (left) and pax6b:dsRed;mnx1:memGFP (center, right) transgenics beginning at 6dpf, by a modified catch-and-release approach (see Supplementary Methods), following Notch inhibitor treatment at 4 dpf for 24 hours. Selected cells are labeled by pseudocoloring to facilitate identification in subsequent images. Scale bars = 10µm. Sample details are described in Supplementary Methods.
Fig S3. Protrusion formation coincides with endocrine cell movements and clustering. Related to Figure 2.

(A, top) Tracked cells (colored spheres) shown alone, from the time series shown in Fig 2B. (A, bottom) Enclosing polygon as calculated using Matlab. Polygon volume reflects the spread of cells in three-dimensional space, and thus provides an index of cell clustering. (B) Image series of pancreatic endocrine cells in pax6b:dsRed;mnx1:memGFP transgenics following Notch inhibitor treatment from 4 dpf to 5 dpf, and imaged at 6 dpf. 3D representations with cells tracked (colored spheres) using Imaris. Movement of cells follows the appearance of a fine intercellular tether (arrow). Scale bars = 10µm. *Indicates neurite projections from mnx1:memGFP transgene expressed in overlying neurons.
**Fig S4. Dynamics of cell coalescence. Related to Figure 3.**

Z-projections from time-lapse image series of pancreatic endocrine cells in pax6b:dsRed;mnx1:memGFP transgenics following Notch inhibitor treatment at 4 dpf. Sample was imaged at 7 dpf, with images acquired every 9 minutes. (A) The indicated cell (arrow) forms a connection to a nearby cluster which strengthens over time (bottom frame, arrowhead). (B) Further time points of the sample shown in (A), projections of a subset of z-slices to highlight cell shape changes that accompany coalescence of a single cell with a cluster. (B') dsRed channel shown alone for clarity. Scale bars = 10µm.
Figure S5. Pancreatic duct morphology delineated with krt18:LifeActTom transgene.

(A) Maximal projection of confocal z-stack showing pancreatic duct morphology at 7 dpf delineated by expression of Tp1:H2BmCherry (red, nuclear) and Tp1:GFP (green, cytoplasmic), immunostained for GFP. Nuclei (gray) are stained by DAPI. Pancreas is delineated by yellow outline. (A’) Image as in (A) with DAPI signal removed. (B) Single z-slice of sample shown in (A). (C) Widefield view showing krt18:LifeActTom transgene expression in surface epithelium in 15-somite stage embryo (right), with corresponding brightfield view (left). (D) Maximum intensity projection of confocal stack of surface epithelium of embryo as in (C), showing actin accumulation at cell-cell junctions. Scale bar = 25µm. (E-F) Projection of confocal stack through the pancreas in krt18:LifeActTom;ptf1a:GFP larva at 6 dpf. (E’-F’) krt18:LifeActTom expression, with GFP channel removed. (E’) Pancreas as defined by ptf1a:GFP, is outlined in white. LifeActTom is also expressed in some blood vessels (bv). PI = principal islet. (E) Scale bar = 50µm. (F) Scale bar = 15µm. (G-G’) Projected substack within the pancreas of krt18:LifeActTom;Tp1:GFP larva at 6dpf. Scale bar = 15µm. (H-H”) Single z-plane of pancreas as in (G). Merged image (H) and corresponding single channel images (H’, H”). (I)...
Projection of confocal stack of 6 dpf krt18:LifeActTom larva, co-labeled with Phalloidin-488 (Phall-488, green). (J) Single slice merged image (top) and single channels (middle, bottom), of the region boxed in (I), showing actin labeling of apical membranes within the developing gut epithelium. Scale bar = 10µm. (K) krt18:LifeActTom labels intrapancreatic duct, shown is a projected z-stack, overlay with brightfield image. (L) Pancreatic expression of krt18:LifeActTom overlaps with actin-rich duct structures highlighted by Phall-488. Merged image (L) and corresponding single channel images (L', L''). (M) 6 dpf krt18:LifeActTom larva labeled with anti-dsRed antibody and Phall-488, nuclei are labeled with DAPI. (M') Same sample as in (M), immunostained to indicate localization of Phall-488 in relation to 2F11 antibody. (N, N', N'') Single channel, single z-plane images of the region indicated in M (white box).
Figure S6. Protrusions in naturally occurring secondary islet cells.

(A) Maximal projection of confocal stack of *mnx1:memGFP;ela:mCherry* transgenic at 8 dpf. *mnx1:memGFP*-positive cells are rare at this stage (see Table S1). (A') GFP signal shown alone for clarity, a single cell is indicated (yellow box). Scale bar = 50µm. This image was assembled by stitching together images of partially overlapping regions, using the Pairwise Stitching Plugin for ImageJ (Preibisch et al., 2009). (B) Close-up view of endocrine cells showing long protrusions, from larva as in (A). Scale bar = 10µm. (C, D) Time lapse series of cells as in sample (A), at the times indicated in minutes (C) and min:sec (D). Extension and retraction of fine protrusions can be observed. (E) At 15 dpf, *mnx1:memGFP*-positive cells are more frequently observed, and small clusters can be detected (white box). Scale bar = 50µm. (F) Time lapse series of cells from sample in (E), which display fine dynamic protrusions. Nonlinear gamma adjustment was applied to enhance weak signals of
protrusions. Scale bar = 10µm. (G) Time lapse series of cell as in sample (E), at the times indicated in minutes (m). Arrow indicates protrusion. (H) Confocal projections from time lapse series of 7 dpf gcga:GFP transgenic that was treated with Notch inhibitor at 4 dpf. Nonlinear gamma adjustment was applied to enhance weak contrast signals. Arrows indicate dynamic protrusions. Scale bar = 10µm.
**Figure S7. Single-cell analysis of endocrine cell dynamics. Related to Figure 5.**

(A) Time series of representative single cells, in mxn1:memGFP transgenics treated to induce secondary islets from 4-5 dpf, followed by treatment at 6 dpf as indicated (WORT, 100nM wortmannin, LY, 50µM Ly294002 or CTL, DMSO), for 3-4 hours prior to imaging. (B) Membrane protrusion analysis indicates regions of expansion (white) and retraction (black) around the cell perimeter between adjacent frames in time lapse series of control, WORT, and LY treated embryos, imaged as in (A). (C) Protrusive Activity (area of expansion + area of retraction) for each cell over time, analyzed as in (B), normalized to cell perimeter. (n.s., not significant; Kruskal-Wallis followed by Dunn’s Multiple Comparison test).
Figure S8. Single cell plots of cell morphology time series. Related to Figure 5.

(A) Schematic illustration showing the impact of cell protrusions on the morphology parameter solidity. Broad protrusions (upper right, arrowhead) increase both cell and convex hull area. Narrow protrusions (lower right, arrowhead) increase convex hull area while minimally influencing cell area, thus more significantly impacting solidity as compared to broad protrusions. For cells treated and imaged as in Fig S8A, parameters of area (B), circularity (C), and solidity (D) plotted for each cell versus time (x-axis), under control (left), WORT treated (center), and Ly294002 treated (right) conditions.
Figure S9. Quantitative assessment of islet assembly.

(A) Schematic illustration of the progression of islet assembly (left) and experimental design (right). (B-I) 3D-projections of pax6b:GFP;Tp1:H2BmCherry larva generated using Imaris. Dispersed mCherry+/GFP+ cells at 6 dpf (B, arrows, D-E) and clusters at 8 dpf (F, arrows, H, I). (E, I) Secondary islets in the posterior pancreas (blue surfaces), identified using Imaris. Nearby GFP+/mCherry- cells (I, arrow) belong to the gut enteroendocrine system. Scale bar = 50 μm. (J) Volumes of secondary islets, as analyzed using Imaris (blue surfaces in E, I), at 6 dpf (n=7) and 8 dpf (n=7) (p<0.0001, Mann-Whitney test, one-tailed). (K) GFP+/H2BmCherry+ cells at 6 dpf and 8 dpf in samples as in (B) and (F) (p=0.25, t-test, two-tailed). The same samples were imaged at 6 dpf and 8 dpf (n=7). Results of all analyses are representative of 2 independent experiments.
Figure S10. Image analysis to assess islet assembly. Related to Figure 6.

(A) Experiment design for pharmacological modulation of islet assembly. (B, C) Image analysis for quantitation of secondary islet size in pax6b:GFP;ela:mCherry larvae showing a subset of controls (B) and Ly294002-treated samples (C). Images were obtained on a Leica Sp5 and ImageJ was used for image processing (For details, see Supplementary Methods). Shown is a projection of pancreas with external signals removed (left). GFP signal alone representing endocrine pancreas (right). (D) Quantitation of secondary islet size in pax6b:GFP;ela:mCherry larvae following treatment from 6 dpf to 8 dpf with 15µM Ly294002 (LY), versus controls (CTL). Samples were imaged on a Leica Sp5 and analyzed with ImageJ Particle Analyser (minimum object size 50µm³). **p<0.01, Mann-Whitney Test (one-tailed).(CTL, n=10 larvae, 133 objects, LY, n=8 larvae, 80 objects.)
Figure S11. Expression of endocrine hormones in induced secondary islets.

Immunohistochemistry of pax6:dsRed larvae at 6 dpf (A, C, D) and 7 dpf (B, E) treated to induce secondary islets, labeled with anti-dsRed (red), anti-ins (cyan) and anti-gcg (green) antibodies. Pancreas is outlined in white (A,B). Inset in (A, white box), single z-plane view of principal islet (PI) shows robust staining with anti-ins and anti-gcg. (A,B) Scale bar = 25µm
(C-E) Scale bar = 10µm. (F,G) Endocrine hormone expression detected by antibody staining at 8 dpf, following islet induction in controls (CTL) and Ly294002-treated (LY) samples. Nuclei are counterstained with DAPI. Scale bar = 10µm. (H), (I) Relative numbers of cells from samples as in (F,G), expressing Ins (H) and Gcg (I), as compared to controls. Box plot graphs maximum to minimum, line at mean, n=4 per group, **p <0.01 (t-test). (J) Ratio of ins to gcg/sst expressing cells, plotted is the mean ± S.D.
Figure S12. PI3K inhibition does not alter duct morphology.

Live-imaging of krt18:LiveActTom;Tp1:GFP transgenics at 7dpf, in controls (CTL, A,B) and following treatment with 15µM Ly294002 from 6 dpf to 7 dpf (Ly, C,D).  (A, C) Confocal projections of image stack. (B, D) Single z-plane close-up views. Scale bar = 25µm.
Figure S13. Islet induction transiently affects duct morphology.

(A, B) Confocal image stacks of pancreas at 7 dpf from control and islet-induced samples transgenic for *Tp1:GFP* and *pax6:dsRed*. Samples were immunostained with anti-GFP and anti-2F11. Scale bar = 25µm. (C, D) Confocal image stacks of pancreas at 14 dpf from control and islet-induced samples transgenic for *ela:GFP* and *pax6:dsRed*, immunostained with anti-dsRed. Scale bar = 100µm. (E, F) Confocal image stacks of pancreas at 14 dpf of representative control and islet-induced samples transgenic for *ela:mCherry* and *Tp1:GFP*, immunostained with anti-GFP. Scale bar = 25µm. pi, principal islet.
Figure S14. Impact of EGFR and Rac1 inhibition on islet assembly.

(A) Representative images from islet assembly assay and analysis (as in Fig. 6B), showing automated detection of secondary islets in control (DMSO-treated) samples and larvae treated with EGFR inhibitor (Tyr) and Rac1 inhibitor (NSC) at the indicated concentrations. (B) Quantitation of secondary islets using the automated method, graphed after log transformation to display the full range of values. Boxes extends from the 25th to 75th percentile, whiskers indicate 5th and 95th percentile, line indicates the median, mean is indicated by ‘+’. Islet volumes in treated samples are not significantly different from DMSO-treated controls. (p=0.6599, ANOVA followed by Dunnett’s Multiple Comparison Test). Number of larvae per group as indicated. CTL, 111 objects; Tyr (0.5µM), 115 objects; Tyr (1.0µM), 93 objects; NCS(75µM), 153 objects; NCS(100µM), 55 objects.
Figure S15. Heat shock induction of PTX expression impacts islet assembly. Related to Figure 7.

(A) hsp70:LifeActTom-PTX embryos untreated (top), or heat shocked for 30 minutes at 50% epiboly (bottom), examined at 24 hpf. Induction of PTX leads to reduced body axis length (left) and perturbed cardiac development, as evidenced by reduced cmhc2:GFP expression (center). LifeActTom, and by inference PTX, is ubiquitously induced following a heat shock (right). (B) Representative images of transgene expression in larvae quantitated in (7C), transgenic for pax6b:GFP alone (top), or pax6b:GFP in combination with hsp70:LifeActTom (middle) or hsp70:LifeActTom-PTX (bottom). Green channel alone (pax6b:GFP), posterior islets subjected to quantitative analysis are outlined (center, white line). Projection of 3D objects as identified by ImageJ plugin Particle Analyser (right).
Figure S16. PTX expression impacts cell morphology. Related to Figure 7.

(A, B) Selected confocal projections, containing single cells, from time lapse series of control (A, CTL) and PTX expressing (B, PTX) transgenic larvae at 7 dpf, 4 or more hours following a heat shock. (C, D) For treated cells as in (A) and (B), morphology parameters of area (C), and solidity (D) plotted for each cell versus time (x-axis). (Samples as analyzed in Fig. 7F, G, Table S5 contains details of cells analyzed.) (E, F) Hormone-positive cells indicated by expression of ins:GFP (E) and gcga:GFP (F) transgenes in LifeActTom-expressing control, and LifeActTom-PTX induced samples, treated as in Fig. 7B. (G, H) Quantitation of cell number, expressed in relation to the controls. Box plot graphs maximum to minimum, line at mean. *p<0.05, t-test. (G) CTL, n=26; PTX, n=11; (H) CTL, n=12; PTX, n=12. Samples are combined from 2 independent experiments.
Figure S17. Toxicity curves for Tyrphostin and NSC23766.

Zebrafish larvae (5 dpf) were treated with the indicated concentrations of Tyrphostin (A) and NSC23766 (B) and observed for survival for 48h (for details see Materials and Methods and Table S7).

Supplementary Tables

Table S1. Frequency of naturally occurring secondary islet and beta cells.

| # of cells | 8dpf pax6b:GFP (n=25) | 8dpf mnx1:memGFP (n=45) | 2wk mnx1:memGFP (n=33) |
|------------|------------------------|-------------------------|------------------------|
| 0          | 9 (36%)                | 38 (84%)                | 11 (33%)               |
| 1          | 7 (28%)                | 4 (9%)                  | 4 (12%)                |
| 2          | 4 (16%)                | 3 (7%)                  | 7 (21%)                |
| 3          | 3 (12%)                |                         | 3 (9%)                 |
| 4          | 0                      |                         | 3 (9%)                 |
| 5          | 2 (8%)                 |                         | 2 (6%)                 |
| 6          |                        |                         | 2 (6%)                 |
| 7          |                        |                         | 1 (3%)                 |

Samples with transgenes indicating endocrine- (pax6b-promoter) and early beta-cells (mnx1-promoter) were examined by confocal microscopy and the number of transgene-positive cells in the pancreatic tail were counted.
Table S2. Quantitation of secondary islets at 2 weeks.

| Maximum Cluster Size | None | Small | Medium | Large |
|----------------------|------|-------|--------|-------|
| %                    | 2.2% | 32.3% | 37.8%  | 28.2% |

Samples transgenic for pax6b:GFP (n=53) or pax6b:dsRed (n=42) were examined by confocal microscopy at 2 weeks (14-15dpf). Secondary islets in the pancreatic tail were counted and categorized as small (1-3 cells), medium (4-6 cells) or large (>7 cells).

Table S3. Cell morphologies and dynamics in naturally occurring secondary islet cells.

| Type               | Description                  | Frequency (%) |
|--------------------|------------------------------|---------------|
| static             | Cells in cluster with protrusions | 6 (14%)      |
| static             | Isolated cells with protrusions | 13 (31%)     |
| static             | Cell-Cell connection          | 9 (21%)       |
| dynamic            | Protrusion changes over time  | 12 (29%)      |
| dynamic            | Cell position changes over time | 5 (12%)     |

Samples transgenic for pax6b:dsRed or pax6b:GFP were imaged by confocal microscopy at 13-15 dpf (as shown in Fig. 1H, S1). Configurations selected for imaging contained single isolated cells in proximity to each other or close to larger clusters (see Materials and Methods). Of 106 samples examined, 42 were imaged and followed as possible. "Static" refers to observations seen at a single time point, dynamic features occurred over >1 time point. Samples may be entered into >1 category.
### Table S4. Cells imaged and analyzed in control and treatment groups (Related to Figs. 5, S7, S8).

| CTL | WORTMANNIN | LY294002 |
|-----|------------|----------|
| Cell # | # Frames | Cell # | # Frames | Cell # | # Frames |
| 1 | 3 | 1 | 7 | 1 | 13 |
| 2 | 4 | 2 | 7 | 2 | 13 |
| 3 | 4 | 3 | 7 | 3 | 13 |
| 4 | 3 | 4 | 7 | 4 | 15 |
| 5 | 12 | 5 | 8 | 5 | 6 |
| 6 | 16 | 6 | 8 | 6 | 15 |
| 7 | 10 | 7 | 7 | 7 | 9 |
| 8 | 15 | 8 | 13 | 8 | 15 |
| 9 | 13 | 9 | 5 | 9 | 15 |
| 10 | 4 | 10 | 10 | 10 | 12 |
| 11 | 4 | 11 | 10 | 11 | 12 |
| 12 | 11 | 12 | 10 | 12 | 12 |
| 13 | 8 | 13 | 10 | 13 | 12 |
| 14 | 11 | 14 | 7 | |

### Table S5. Cells imaged and analyzed for control and PTX-induced groups (Related to Figs. 7, S16).

| CTL | PTX |
|-----|-----|
| Cell # | # Frames | Cell # | # Frames |
| 1 | 12 | 1 | 6 |
| 2 | 11 | 2 | 8 |
| 3 | 11 | 3 | 10 |
| 4 | 11 | 4 | 11 |
| 5 | 11 | 5 | 11 |
| 6 | 11 | 6 | 11 |
| 7 | 11 | 7 | 11 |
| 8 | 2 | 8 | 4 |
| 9 | 2 | 9 | 7 |
| 10 | 7 | 10 | 11 |
| 11 | 7 | 11 | 10 |
| 12 | 6 | 12 | 11 |
| 13 | 12 | 13 | 11 |
| Transgenic Line                      | Abbreviation      | Cell type/location                      | Reference                  |
|-------------------------------------|-------------------|-----------------------------------------|----------------------------|
| Tg(ela3l:EGFP)gz2                   | ela:GFP           | Exocrine, cytoplasm                      | (Wan et al., 2006)         |
| Tg(ins:Eco.NfsB-EGFP)ml11           | ins:GFP           | Beta cells, cytoplasm                    | (Pisharath et al., 2007)   |
| Tg(Tp1bglob:EGFP)um14               | Tp1:GFP           | Notch-responsive cells, cytoplasm        | (Parsons et al., 2009)     |
| Tg(Tp1:H2B-mCherry)S939             | Tp1:H2BmCherry    | Notch-responsive cells, nuclear          | (Ninov et al., 2012)       |
| TgBAC(NeuroD:EGFP)nl1               | neurod:EGFP       | Early endocrine, cytoplasm               | (Obholzer et al., 2008)    |
| Tg(P0-pax6b:DsRed)ulg302            | pax6b:DsRed       | Endocrine, cytoplasm                     | (Delporte et al., 2008)    |
| Tg(P0-pax6b:GFP)ulg515              | pax6b:GFP         | Endocrine, cytoplasm                     | (Delporte et al., 2008)    |
| Tg(gcga:GFP)ia1                     | gcga:GFP          | Alpha cells, cytoplasm                   | (Zecchin et al., 2007)     |
| Tg(mxnx1:memGFP)ml4                 | mnx1:memGFP       | Early beta cells, membrane               | (Flanagan-Stee et al., 2005)|
| Tg(ptf1a:eGFP)jh1                   | ptf1a:GFP         | Exocrine, cytoplasm                      | (Pisharath et al., 2007)   |
| ela3l:mCherry                       | ela:mCherry       | Exocrine, cytoplasm                      | this work                  |
| ins:mKO2                            |                   | Beta cells, nuclear                      | this work                  |
| krt18:LifeActTom                    |                   | epithelia/duct, actin filaments          | this work                  |
| hsp70:LifeActTom                    |                   | heat shock inducible, actin filaments    | this work                  |
| hsp70:LifeActTom-PTX                |                   | heat shock inducible, PTX + actin filaments | this work |
Table S7. Toxicity assays for compounds used in this study.

| Compound      | Concentration | Survival          | Changes in morphology and/or physiology                        |
|---------------|---------------|-------------------|---------------------------------------------------------------|
| DMSO          | (control)     | 80-100% survived  | no change                                                     |
| Wortmannin    | 2 nM          | all survived      | no change                                                     |
| Wortmannin    | 5 nM          | all survived      | no change                                                     |
| Wortmannin    | 10 nM         | 80-100% survived  | reduced swimming, abdominal edema                             |
| Wortmannin    | 15 nM         | all dead          |                                                              |
| Wortmannin    | 20 nM         | all dead          |                                                              |
| Ly294002      | 10 µM         | 67-100% survived  | no change                                                     |
| Ly294002      | 15 µM         | 70-100% survived  | no change                                                     |
| Ly294002      | 20 µM         | all dead          |                                                              |
| Ly294002      | 50 µM         | all dead          |                                                              |
| NSC23766      | 50 µM         | all survived      | no change                                                     |
| NSC23766      | 75 µM         | all survived      | no change                                                     |
| NSC23766      | 100 µM        | 50% survived      | curved body, decreased movement                               |
| NSC23766      | 160 µM        | 0% survived       |                                                              |
| Tyrphostin    | 0.25 µM       | 92% survived      | no change                                                     |
| Tyrphostin    | 0.5 µM        | 100% survived     | no change                                                     |
| Tyrphostin    | 0.75 µM       | 75% survived      | no change                                                     |
| Tyrphostin    | 1.0 µM        | 67% survived      | no change, reduced swimming                                   |
| Tyrphostin    | 1.5 µM        | 33% survived      | abdominal and pericardial edema, no swimming                  |
| Tyrphostin    | 2.0 µM        | all dead          |                                                              |

Concentrations used are shown in **bold**. For details refer to Materials and Methods.

Table S8. Parameters of time-lapse imaging experiments.

| Experiment | z-interval | z-range | t-interval |
|------------|------------|---------|------------|
| Fig. 3     | 0.8µm      | 64.8µm  | 18 min     |
| Fig. 4H    | 1.5µm      | 32µm    | 19 sec     |
| Fig. 5C (top) | 1.5µm     | 41-54µm | 4 min      |
| Fig. 5C (bottom) | 1.5µm   | 45µm    | 4 min      |
| Fig. 7D    | 1.5µm      | 71µm    | 4 min      |
| Fig. 7E    | 1.5µm      | 58.5µm  | 4 min      |
| Fig. S4    | 0.8µm      | 54.4µm  | 9 min      |
| Fig. S6C   | 0.4µm      | 34.2µm  | 3 min      |
| Fig. S6D   | 0.4µm      | 36.9µm  | 20 sec     |
| Fig. S6F   | 0.4µm      | 63µm    | 3 min      |
| Fig. S6G   | 0.4µm      | 36.9µm  | 3 min      |
| Fig. S6H   | 1.4µm      | 68µm    | 3 min      |
Supplementary Movies

**Movie 1.** Time lapse of induced islet cells in *mnx1:memGFP; pax6b:dsRed* transgenic larvae at 7 dpf. (Corresponds to Fig. S4)

**Movie 2.** Time lapse of *mnx1:memGFP*-expressing early beta cell, imaged by spinning disc confocal microscopy at 6 dpf. (Corresponds to Fig. 4H)
Movie 3. Time lapse of mnx1:memGFP-expressing early beta cells following induction by Notch inhibition from 4-5 dpf, imaged at 7 dpf. (Corresponds to Fig. 5C, top)

Movie 4. Time lapse of mnx1:memGFP-expressing early beta cells, following induction by Notch inhibition from 4-5 dpf, then treatment with Wortmannin for 3 hours prior to imaging at 7 dpf. (Corresponds to Fig. 5C, bottom)
**Movie 5.** Time lapse series of *mnx1:memGFP;hsp:LifeActTom* transgenic larvae at 7 dpf, at 4 hours or more following a heat shock. Shown are confocal projections, GFP channel alone. (Corresponds to Fig. 7D)

**Movie 6.** Time lapse series of *mnx1:memGFP;hsp:LifeActTom-PTX* transgenic larvae at 7 dpf, at 4 hours or more following a heat shock. Shown are confocal projections, GFP channel alone. (Corresponds to Fig. 7E)