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Veen, T.P.M. van

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Author: Veen, T.P.M. van

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Chapter 4

Glycerophosphodiesterase GDE2/GDPD5 affects pancreas differentiation in zebrafish

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Michiel van Veen^{2&}, Laurie A. Mans^{1&}, Elisa Matas-Rico², Jason van Pelt¹, Anastassis Perrakis³, Wouter H. Moolenaar², and Anna-Pavlina G. Haramis¹

¹ Institute of Biology, Leiden University, Leiden, The Netherlands

² Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands

³ Division of Biochemistry, Netherlands Cancer Institute, Amsterdam, The Netherlands

[&] These authors contributed equally

Abstract

Notch signaling plays an essential role in the proliferation, differentiation and cell fate determination of various tissues, including the developing pancreas. One regulator of the Notch pathway is GDE2 (or GPD5), a transmembrane ecto-phosphodiesterase that cleaves GPI-anchored proteins at the plasma membrane, including a Notch ligand regulator. Here we report that Gdpd5-knockdown in zebrafish embryos leads to developmental defects, particularly, impaired motility and reduced pancreas differentiation, as shown by decreased expression of insulin and other pancreatic markers. Exogenous expression of human GDE2, but not catalytically-dead GDE2, similarly leads to developmental defects. Human GDE2 restores insulin expression in Gdpd5a-depleted zebrafish embryos. Importantly, zebrafish Gdpd5 orthologues localize to the plasma membrane and show catalytic activity against GPI-anchored GPC6. Thus, our data reveal functional conservation between zebrafish Gdpd5 and human GDE2, and suggest that strict regulation of GDE2 expression and catalytic activity is critical for correct embryonic patterning. In particular, our data uncover a role for GDE2 in regulating pancreas differentiation.

Introduction

A better understanding of pancreatic homeostasis is essential to identify new ways to increase insulin-producing beta cell number and to enhance their function, in order to tackle diabetes, a rising epidemic.

The Notch cell-to-cell signaling axis plays an important role in proliferation, differentiation and cell-fate decisions in the developing pancreas and other tissues [241, 242]. Notch receptor signaling is activated by transmembrane Notch ligands in adjacent cells [243]. Following ligand binding, Notch is cleaved by the metalloprotease TACE (factor-a-converting enzyme) [243]. Truncated Notch is in turn cleaved by the a secretase complex, leading to intracellular release of NICD (Notch intracellular domain) [243] and activation of Notch target genes [243].

Different levels of Notch can elicit distinct fates [241, 242]. Notch signaling maintains progenitor cells in an undifferentiated proliferative state and regulates timing of differentiation, not only in the embryonic pancreas but also in developing motor neurons [109, 244]. Loss of Notch signaling in mice [245] and zebrafish [244, 246] results in aberrant/excessive differentiation of pancreatic progenitors to endocrine cells, at the expense of the later-appearing exocrine cells [242]. Modulation of Notch signaling may thus affect the balance between proliferation and differentiation of pancreatic progenitor cells.

A recently identified modulator of Notch activity is GDE2 (aka GDPD5), a multipass membrane glycoprotein with an extracellular glycerophosphodiesterase (GDPD) domain. The GDE2 catalytic domain cleaves a subset of glycosylphosphatidylinositol (GPI)-anchored proteins, including a Notch ligand regulator (RECK) [110] and certain heparan sulfate proteoglycans, the glypicans (GPCs) [113, 137]. Through RECK cleavage, GDE2 sheds and inactivates the Notch ligand Delta-like 1 in the developing spinal cord, leading to Notch inactivation in adjacent progenitor cells [109, 110]. Thus, GDE2 promotes motor neuron differentiation by downregulating Notch signaling during spinal cord development. Mice lacking GDE2 exhibit selective losses of limb-innervating motor neuron pools leading to neurodegeneration [109, 113]. In addition, GDE2 can promote neuronal differentiation in a cell-autonomous manner, namely through GPI-anchor cleavage of glypican-6 (GPC6), which correlates with improved clinical outcome in neuroblastoma [247]. However, a possible role for GDE2 in regulating the fate of non-neuronal cell types has not been investigated to date.

Here, we examine the role of Gde2 during embryonic development, focusing on pancreas differentiation in zebrafish. Zebrafish is an excellent model to conduct developmental studies owing to the availability of large numbers of readily-accessible, transparent embryos. Importantly, mammalian and zebrafish pancreas share many morphological and physiological similarities [242], while important genes in mammalian islet development are functionally conserved in zebrafish [242]. The conservation of signaling pathways and mechanisms of

pancreas development suggests that results found in zebrafish will advance our understanding of pancreas development in humans.

We show that Gdpd5 knockdown affects the differentiation of specific endocrine and exocrine progenitors. Furthermore, exogenous expression of human GDE2 restores insulin expression in Gdpd5a-depleted zebrafish embryos. Expression of human GDE2 but not catalytically-dead GDE2 in zebrafish embryos leads to developmental defects, in a dose-dependent manner. Importantly, zebrafish Gdpd5 orthologues localize to the plasma membrane and show catalytic activity against GPC6. These results highlight the functional conservation of GDE2 and suggest that tight regulation of Gdpd5a levels and catalytic activity are important for correct embryonic patterning.

Results

Analysis of zebrafish Gde2/Gdpd5

GDE2, encoded by *GDPD5*, is a six-transmembrane-domain protein with an extracellular phosphodiesterase domain and intracellular N- and C-termini (Fig. 1A). GDE2 catalytic activity has long been elusive, but recent studies have shown that GDE2 cleaves GPI-anchored proteins at the plasma membrane to drive neuronal differentiation and survival, in either a cell-autonomous or non-cell-autonomous manner [110, 113, 137] (Fig. 1A). Human *GDPD5* has two orthologues in the zebrafish genome, *gdpd5a* and *gdpd5b*. *Gdpd5a* (glycerophosphodiester phosphodiesterase domain containing 5a) gives rise to three alternatively spliced isoforms; Isoforms *gdpd5a*-001 and *gdpd5a*-002 are included within the long isoform *gdpd5a*-201, which encodes for 595-aa protein. Phylogenetic analysis shows that the long isoform of zebrafish *gdpd5a* is closely related to human, rodent and chicken *GDPD5*/*Gdpd5* (Fig. 1B). The predicted catalytic domains of human GDE2 and zebrafish Gdpd5a share 53% identity and 81% similarity (Fig. 1C). *Gdpd5b* (ENSDARG00000076962) encodes a 566-aa protein that is characterized by a relatively short, truncated C-terminal tail (SFig. 1).

Gdpd5 depletion leads to developmental defects

To investigate the function of Gdpd5 during zebrafish embryonic development, we knocked down its function by using antisense morpholino oligonucleotides (MO) against *gdpd5a* and *gdpd5b* mRNAs. The MO against *gdpd5a* mRNA targets the 5'UTR of the long isoform, *gdpd5a*-201, and blocks translation of the message [248]. Since the other isoforms are included in *gdpd5a*-201, we reasoned that this MO would block translation of all isoforms.

Zebrafish embryos were injected with various amounts of *gdpd5a*MO at the 1-2 cell stage, and development of the injected embryos was followed up to 5 days post-fertilization (dpf). Injections with low amounts (< 8,4 ng/ul) MO did not result in morphological abnormalities. Following injection of 8,4 ng/ul (1 mM) *gdpd5a*MO, injected embryos

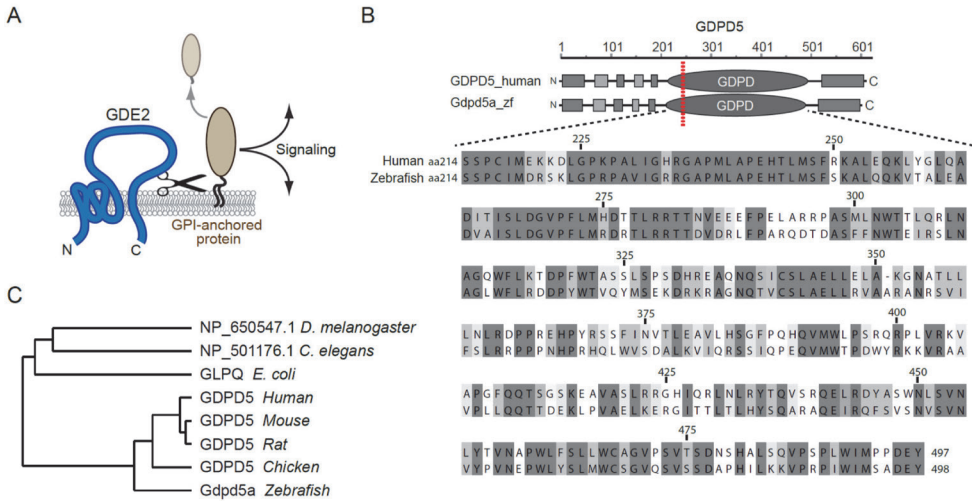


Figure 1. Sequence analysis of zebrafish Gdpd5a

(A) GDE2 cleaves GPI anchored proteins at the plasma membrane resulting in their shedding with consequent activation of signalling pathways, either in the same cell or in adjacent cells. (B) Amino acid sequence comparison between human GDPD5 and zebrafish Gdpd5a. The red line depicts key catalytic residue H²³³ in GDE2 [137]. Sequence comparison of the ecto-domain using Clustal Omega, shows identical residues in dark grey and similar residues in light grey. The catalytic domains share 52% sequence identity and 82% similarity. (C) Phylogenetic analysis showing that zGdpd5a is closely related to mammalian GDPD5. Protein sequences used: hGDPD5 (NP_110419.5), mGDPD5 (NP_958740.2), rGDPD5 (XP_006223480.1), cGDPD5 (NP_001032348.1), zGDPD5 (XP_005157799.1), *D. melanogaster* GDPD5 (NP_650547), *C. elegans* GDPD5 (NP_501176), *E. coli* GDPD5 (NP_416742.1).

exhibited developmental defects. Specifically, the majority of *gdpd5a*MO-injected embryos exhibited heart abnormalities accompanied by heart oedema, as well as a shorter and bent body axis (Fig. 2). Furthermore, while at 3 dpf almost all non-injected controls have hatched from their chorions, the majority of *gdpd5a*MO-injected embryos were still in their chorions, indicating inability to hatch (SFig. 2). At 5 dpf, very few of the *gdpd5a*MO-injected larvae had hatched. The Gdpd5a-depleted larvae that hatched (either on their own or following manual dechoriation) showed severe motility defects, either swimming inability or trembling (data not shown), consistent with *Gdpd5* knockout causing behavioral motor deficits in mice [113]. To investigate the function of Gdpd5b in zebrafish embryonic development, we injected a morpholino targeting the 5' UTR of the zebrafish *gdpd5b* mRNA. Embryos injected with *gdpd5b*MO at the one-cell stage, exhibited dose-dependent developmental defects at 3 dpf. Specifically, injection of 2ng/ul (0,25 mM) *gdpd5b*MO led to slight body curvature defects and heart oedemas (SFig. 3B). Following injection of 4 ng/ul (0,5 mM) *gdpd5b*MO, injected

embryos showed more severe axis defects, small eyes, and larger heart oedemas (SFig. 3C). Finally, embryos injected with 8 ng/ul (1mM) *gdpd5b*MO showed very severe antero-posterior axis truncations, cyclopia, and very large heart oedemas (SFig. 3D). In contrast to *gdpd5a* morphants however, the majority of *gdpd5b*MO-injected embryos hatched normally and did not show motor defects. These results indicate that the two zebrafish Gdpd5 homologs have both shared and distinct functions during zebrafish development.

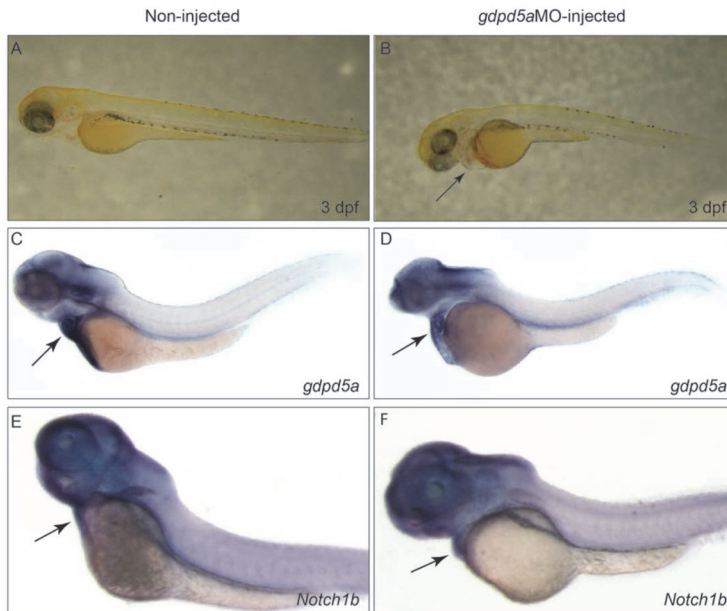


Figure 2. Gdpd5a depletion causes developmental defects

(A) Non-injected embryo at 3 dpf. Arrow indicates looped heart. (B) Representative example of a *gdpd5a*MO-injected embryo at 3 dpf. *Gdpd5a*MO-injected embryos show a bent and shorter body axis, heart oedemas (arrow) and unlooped heart. Whole-mount *in situ* hybridization (WISH) for expression of *gdpd5a* and *notch1b* in non-injected wt (C, E) and *gdpd5a*MO-injected embryos (D, F) at 3 dpf. Lateral views. (C) wt embryo at 3 dpf stained for *gdpd5a*. *Gdpd5a* expression is detected in the brain, strongly in the heart (arrow) as well as the intestine. (D) *Gdpd5a*MO-injected embryo at 3 dpf stained for *gdpd5a*. *Gdpd5a* is expressed at comparable levels as in non-injected controls, except for reduced expression in the heart (arrow). (E) wt embryo at 3 dpf stained for *notch1b*. Strong expression in the head and heart (arrow) is detected (F) *Gdpd5a*MO-injected embryo at 3 dpf stained for *notch1b*. The expression pattern of *notch1b* is similar to that of the non-injected controls.

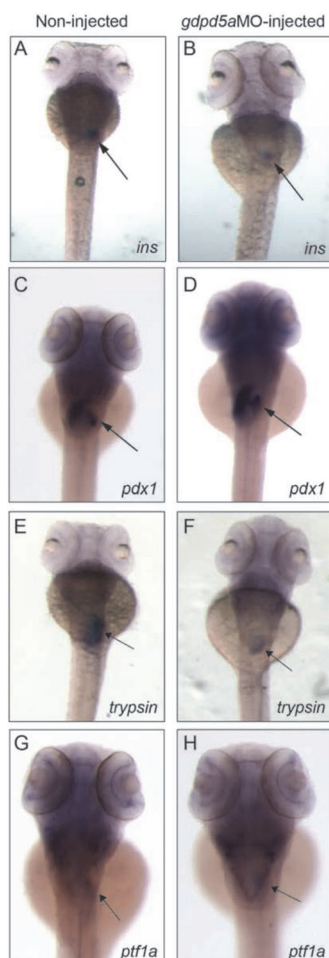


Figure 3. Expression of *insulin* and *trypsin*, but not *pdx1* or *ptf1a*, is reduced upon *Gdpd5a* knockdown

WISH for expression of the endocrine pancreas markers, *insulin* (*ins*) and *pancreatic and duodenal homeobox 1* (*pdx1*) and the exocrine pancreas markers, *trypsin* and *ptf1a* in non-injected wt (A, C, E, G) and *gdpd5a*MO-injected embryos at 3 dpf (B, D, F, H). Dorsal views. (A) wt embryo at 3 dpf stained for *insulin*. Strong expression is detected in the endocrine pancreas (arrow). (B) *Gdpd5a*MO-injected embryo at 3 dpf. *ins* expression is dramatically reduced. (C) wt embryo at 3 dpf stained for *pdx1*. *Pdx1* is expressed in the intestine and endocrine pancreas (arrow). (D) *Pdx1* expression in the intestine and endocrine pancreas is very similar in *gdpd5a*MO-injected embryos at 3 dpf. (E) wt embryo at 3 dpf stained for *trypsin*. Strong expression is detected in the exocrine pancreas (arrow). (F) *Gdpd5a*MO-injected embryo at 3 dpf. *Trypsin* expression is dramatically reduced. (G) wt embryo at 3 dpf stained for *ptf1a*. *Ptf1a* expression is detected in the brain and the exocrine pancreas (arrow). (H) *Gdpd5a*MO-injected embryo at 3 dpf. *Ptf1a* is expressed at comparable levels in the brain and exocrine pancreas (arrow) as in non-injected controls.

Expression studies

We next assayed *gdpd5a* and *notch1b* expression in *gdpd5a*MO-injected embryos at 3 dpf. We analysed expression of *gdpd5a* by *in situ* hybridization using an antisense riboprobe against *gdpd5a*-201. *Gdpd5a* mRNA was expressed in the brain and intestine, and at high levels in the heart and associated blood vessels in larvae at 3 dpf (Fig. 2C). This localization pattern suggests a role for *gdpd5a* during differentiation of these organs. The MO against *gdpd5a* is targeted to the 5' UTR including the ATG start codon blocking translation, and is not expected to influence mRNA levels. Nonetheless, we detected reduced expression of *gdpd5a* mRNA in *gdpd5a*MO-injected embryos, particularly in the heart (Fig. 2D), suggesting that the MO may affect the stability of the *gdpd5a* mRNA. We also assayed *notch1b* expression in *gdpd5a* morphants. *Notch1b* is expressed highly in brain and heart of 3 dpf un-injected embryos (Fig. 2E). *Notch1b* expression was comparable in *gdpd5a* morphants (Fig. 2F).

Pancreas differentiation is affected upon Gdpd5 depletion

Given the role of Notch1b signaling in pancreas differentiation and the purported role of Gdpd5 in modulating Notch activity, we examined how Gdpd5a depletion affects pancreas differentiation in zebrafish embryos. The pancreas in zebrafish develops from the posterior foregut endoderm; the dorsal bud which produces endocrine cells emerges after 24 hours post-fertilization (hpf), and the ventral bud emerges at 32 hpf and produces mostly exocrine and some endocrine cells [249]. At around 72 hpf the pancreas is fully developed [242]. At 14 hpf the pancreatic progenitor cells express different levels of *pdx1* (Pancreatic and duodenal homeobox 1) [242]. Cells expressing high levels of *pdx1* give rise to the endocrine cells, while cells with lower *pdx1* expression give rise to exocrine and intestinal cells. The first expression of *insulin* begins at 15 hpf when the insulin-producing cells begin to migrate towards the midline (Tehrani and Lin, 2011). In the mature pancreas, insulin is expressed exclusively in the beta cells.

To investigate the role of Gdpd5a in pancreas differentiation we studied the expression of markers of endocrine and exocrine pancreas components in wt and *gdpd5a* morphants at 3 dpf. In *gdpd5a*MO-injected embryos *insulin* (*ins*) expression was greatly reduced (Fig. 3A). *Pdx1* was expressed in the head, intestine and endocrine pancreas at 3 dpf (Fig. 3C and SFig. 4A), with no differences in *pdx1* expression between non-injected and *gdpd5a*MO-injected embryos (Fig. 3D and SFig. 4B). We therefore conclude that the defect in insulin expression is specific.

We next examined exocrine pancreas development in Gdpd5a-depleted embryos. *Trypsin* and *pancreas-specific transcription factor 1a* (*ptf1a*) mark the exocrine pancreas. As shown in Fig. 3E, *trypsin* expression was dramatically reduced in *gdpd5a*MO-injected embryos compared to un-injected controls. *Ptf1a* was strongly expressed in the head and the exocrine pancreas in un-injected controls at 3 dpf (Fig. 3G and SFig. 4C). The *ptf1a* expression pattern was not altered in *gdpd5a*MO-injected embryos (Fig 3H and SFig. 4D).

To investigate whether *Gdpd5b* depletion would also affect pancreas differentiation, we assayed *insulin* and *trypsin* expression in embryos at 3 dpf, following injection of 2 ng/ul *gdpd5b*MO at the one-cell stage. As mentioned, this concentration of *gdpd5b*MO leads to only mild developmental defects. We found that upon *gdpd5b*MO injection, expression of both *insulin* and *trypsin* was reduced in *Gdpd5b* morphants at 3 dpf (SFig. 5). Specifically, the *insulin* expression domain was smaller and appeared split in *gdpd5b*MO-injected embryos (SFig. 5B, D). Furthermore, the *trypsin* expression domain was smaller and irregularly shaped in *gdpd5b*MO-injected embryos (SFig. 5F, H). These results suggest that the two zebrafish *Gdpd5* genes share common functions in pancreas differentiation.

Since *Gdpd5a*-depleted embryos exhibit mobility defects and impaired pancreas differentiation, and *Gdpd5* knockout in mice leads to motor neuron defects [109], we examined expression of *islet1* in *Gdpd5a* morphants. The transcription factor *Islet1* is involved in both motor neuron differentiation and pancreas differentiation in mice and zebrafish [250] [251]. *Islet1* was expressed in the brain, spinal motor neurons and the pancreas in wt embryos at 3 dpf (Fig. 4A, C). In *Gdpd5a* morphants, *islet1* was expressed at high levels in the brain and pancreas (Fig. 4B, D). However, in the ventral spinal cord, *islet1* expression was reduced upon *Gdpd5a* knockdown (Fig. 4B).

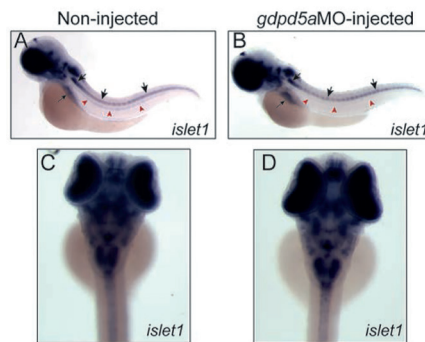


Figure 4. *Islet1* expression upon *Gdpd5* knockdown

WISH hybridization for *islet1* expression on non-injected (A, C), or *gdpd5*MO-injected embryos (B, D) at 3 dpf. (A, B) Lateral views, anterior to the left. (A) wt embryo at 3 dpf, *Islet1* is expressed in the brain, spinal cord (arrowheads) and the pancreas (arrow). (B) *Islet1* is expressed at similar levels in *gdpd5*MO-injected embryo. (C, D) Dorsal views of the head region. (C) wt embryo at 3 dpf. *Islet1* is expressed at distinct domains in the brain. (D) *Gdpd5*MO-injected embryos at 3 dpf show a staining pattern similar to that in non-injected controls.

Expression of human GDE2 in zebrafish leads to developmental defects

To investigate the functional conservation between human GDE2/GDPD5 and zebrafish Gdpd5, we injected mRNA encoding human GDE2 (hGDE2) tagged with HA in zebrafish embryos at the one-cell stage. Expression of hGDE2-HA mRNA led to developmental abnormalities in a dose-dependent manner (Fig. 5B, C). Specifically, hGDE2 mRNA-injected larvae showed malformations in the body axis and defects in the vasculature at 4 dpf (Fig. 5C). Higher doses resulted in more severe disruption of the axis, of the vasculature, blood accumulation and heart oedemas (Fig. 5C). Importantly, expression of catalytically-dead human GDE2 (GDE2^{His233Ala} or GDE2^{His233Ala/His275Ala}) [137] did not induce morphological abnormalities (Fig. 5D). We verified that wt hGDE2, hGDE2^{His233Ala} and hGDE2^{His233Ala/His275Ala} are expressed in zebrafish at comparable levels (Fig. 5E). These data indicate that

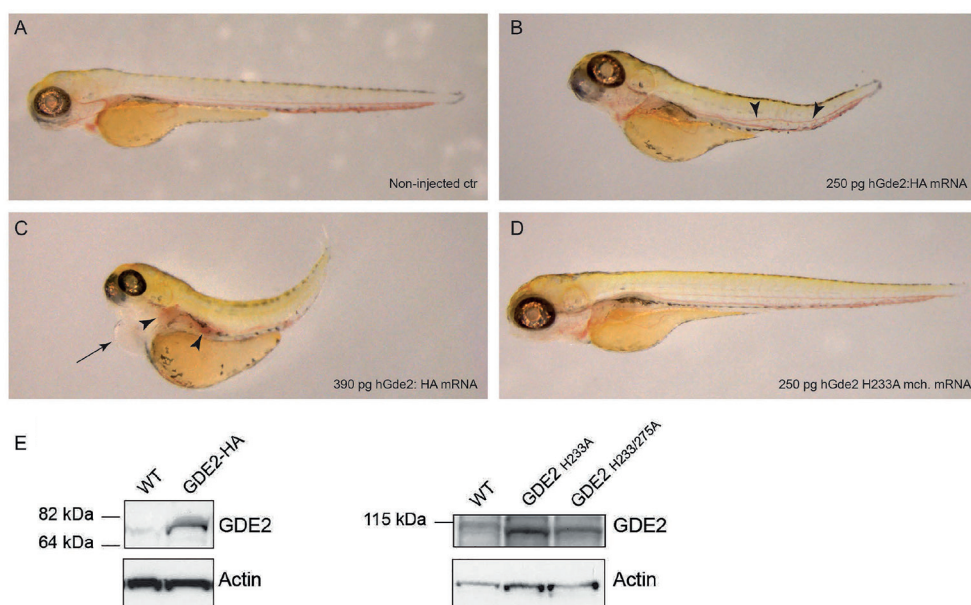


Figure 5. Expression of human GDE2 leads to developmental defects, depending on GDE2 catalytic activity

Representative pictures of zebrafish larvae at 4 dpf that have been injected at the one-cell stage with mRNAs encoding human GDE2:HA (B, C), or catalytically-dead hGDE2^{H233A} mRNAs (D). Lateral views, anterior to the left. (A) Non-injected larva at 4 dpf. (B) Larva injected with 250 pg of wt hGDE2:HA mRNA. The body axis is shorter with an upward curvature, and defects in the organization of posterior vasculature can be observed (arrowheads). (C) Larva injected with 390 pg of hGDE2:HA. The defects are stronger, the body axis is severely shortened, there is blood accumulation and disruption of the posterior vasculature (arrowheads), as well as heart oedemas (arrow). (D) Larva injected with 250 pg of the catalytically-dead hGDE2^{H233A}; mCherry mRNA. No obvious morphological abnormalities are observed. (E) Western-blot analysis of zebrafish larvae at 4 dpf, expressing HA-tagged wt human GDE2, or mCherry tagged GDE2 catalytic dead mutant, GDE2^{H233A}. Actin is used as a loading control.

the malformations observed depend on the catalytic activity of GDE2, and they confirm functional conservation between human and zebrafish GDE2.

We next examined whether exogenously provided human GDE2 could rescue the defects in pancreas differentiation induced by *Gdpd5a* knock-down. We assayed for *insulin* expression in embryos that have been injected with either 1mM *gdpd5a*MO, 300pg hGDE2-HA mRNA, or co-injected with 1mM *gdpd5a*MO and 300pg hGDE2-HA mRNA. *Insulin* expression was dramatically reduced in *Gdpd5a* morphants at 3 dpf as we previously observed (Fig 6B). Expression of hGDE2-HA mRNA did not affect *insulin* expression (Fig. 6C). Notably, *insulin* expression was restored in embryos that have been co-injected with *gdpd5a*MO and hGDE2-HA mRNA (Fig. 6D). These results further validate functional conservation between zebrafish and human GDE2 in pancreas differentiation.

To further validate whether the conservation extends at the biochemical level, we examined zebrafish *Gdpd5* enzymatic activity using cell-based assays. We co-expressed Glypican-6 (GPC6) and zebrafish constructs or human GDE2, confirmed that zebrafish *Gdpd5* localized to the plasma membrane (Figure 7A), and examined the presence of GPC6 in the conditioned medium by immunoblotting, as described by [247]. We used intact cells treated with bacterial phospholipase C (PI-PLC) as positive control. As shown in Figure 7B, we found GPC6 released into the medium from cells transfected with zebrafish *Gdpd5*, although the amount of GPC6 detected into the medium appears to be smaller than that generated by hGDE2 cleavage.

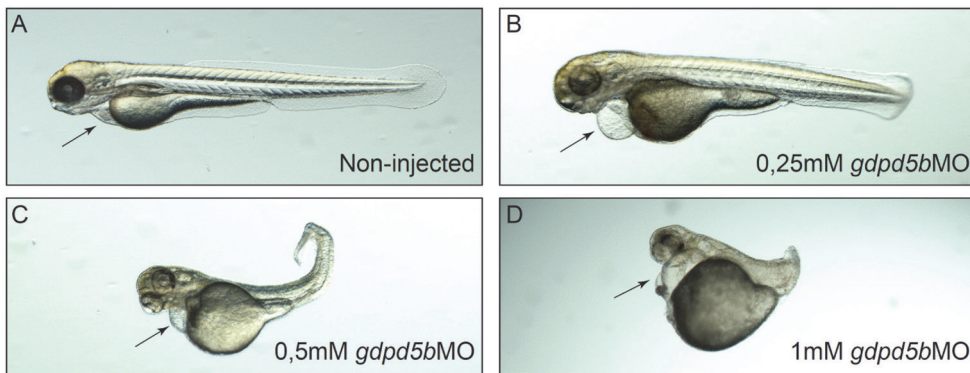


Figure 6. Human GDE2 mRNA restores *insulin* expression in *Gdpd5a* morphants.

Representative pictures of zebrafish embryos at 3 dpf stained for *insulin* (*ins*) expression that have been either non-injected (A), *gdpd5a*MO-injected (B), hGDE2 mRNA-injected (C) or co-injected with *gdpd5a*MO + hGDE2 mRNA (D), at the one-cell stage. Dorsal views, anterior to the right. (A) Non-injected control embryo at 3 dpf Strong *ins* expression is detected in the endocrine pancreas (arrow). (B) *Gdpd5*MO-injected 3 dpf embryo. *ins* expression is dramatically reduced (arrow). (C) 3 dpf embryo injected with 300 pg hGDE2 mRNA. Arrow indicates *ins* expression. (D) 3 dpf embryo that was co-injected with 1mM *gdpd5a*MO and 300 pg hGDE2 mRNA at the one-cell stage. *Ins* expression is restored (arrow).

Taken together, these results demonstrate that zebrafish Gdpd5 can cleave GPC6 and show that activity over GPC6 is conserved between hGDE2 and zebrafish GDPD5a.

Discussion

In this study we have identified a previously unknown role for glycerophosphodiesterase Gde2 (Gdpd5) in non-neuronal cell types, particularly the pancreas. Here, we focused on the possible role of Gdpd5 during pancreas development in zebrafish and show that Gdpd5 knockdown leads to defects in the differentiation of specific endocrine and exocrine progenitors.

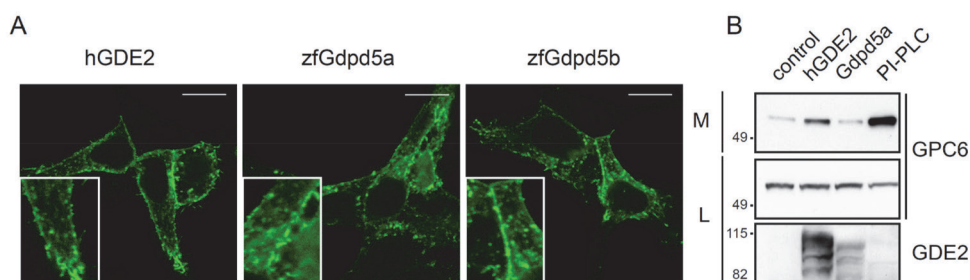


Figure 7. Zebrafish Gdpd5 orthologues localize to the plasma membrane and promote GPC6 release (A) Confocal images of HEK293 cells transiently transfected with human GDE2, zebrafish Gdpd5a or Gdpd5b (GFP-tagged), as indicated. Insets show high-power images of cells depicting that Gdpd5a and Gdpd5b localize to the plasma membrane, similarly to human GDE2-GFP. Scale bars, 10 μm. (B) HEK293 cells co-transfected with GPC6-HA and hGDE2-GFP or zebrafish Gdpd5-GFP, or transfected with GPC6 alone as control. The expression levels of the transfected proteins in cell lysates and conditioned media were assessed by Western-blot analysis. GPC6 expression is visualized by antibodies to HA, and both hGDE2 and Gdpd5 using anti-GFP antibody. Cells transfected with GPC6 and treated with PI-PLC were used as positive control.

Gdpd5-knockdown leads to dramatic reduction in expression of pancreatic markers *trypsin* (which marks exocrine cells) and *insulin*. Other markers of the endocrine pancreas, such as the transcription factor *pdx1* are not affected upon Gdpd5 depletion, indicating that Gdpd5 specifically affects *insulin* expression rather than the development of the entire endocrine pancreas. Our results therefore strongly suggest that Gdpd5 affects terminal differentiation of insulin-producing beta cells. With regard to the exocrine pancreas, expression of *ptfla* is not affected by Gdpd5a knockdown. *Trypsin* expression, however, is strongly reduced. Gdpd5b knockdown also leads to defects in *insulin* and *trypsin* expression, indicating that both zebrafish GDPD5 homologs have important functions in pancreas development. Interestingly, GDPD5 is also expressed in the human pancreas [252] [253]. However, a role for

GDPD5 in pancreas differentiation has not previously been reported. It is of note that ectopic activation of Notch1 signaling inhibits acinar cell differentiation but not initial commitment to the exocrine lineage [246]. We therefore suggest that *gdpd5a* knockdown affects acinar cell differentiation likely through upregulation of Notch signaling. Consistent with this, *islet1*, a marker of early pancreas development, is not affected upon *gdpd5* knockdown, indicating that Gdpd5 affects differentiation of specific subtypes of pancreatic cells.

Given that GDPD5 promotes motor neuron differentiation in chicken embryos and mice [108] [109], while Gdpd5a morphants show severe mobility defects, it was unexpected to find *islet1* expression at relatively normal levels in the brain and spinal cord motor neurons in the morphants. A possible explanation could be that motor neurons are specified normally in the Gdpd5a-depleted zebrafish but undergo impaired terminal differentiation downstream of *islet1*. In addition, protein downregulation achieved by morpholino injections is not complete. Gdpd5 knockdown in zebrafish induces developmental defects including a short and curved body axis and heart defects. We observed reduced *gdpd5a* expression in the heart of Gdpd5a morphants, which may lead to increased Notch1 activity in the heart and account for the abnormalities. In chicken embryos and in mice, GDE2/GDPD5 downregulates Notch signaling in motor neuron progenitors. It was previously shown that GDE2 promotes neuroblastoma cell differentiation [137]. In adult mice, GDE2 prevents neurodegeneration by promoting motor neuron survival [113]. Mechanistically, GDE2 does so by specifically cleaving GPI-anchored Notch ligand regulator RECK as well as glypicans at the plasma membrane [137] [113]. In zebrafish, Glypicans are highly conserved and affect various signaling pathways including the Wnt and Bmp signaling axis [254, 255]. Interestingly, Gpc4 depletion in zebrafish results in strongly reduced cardiomyocyte proliferation [255]. Similarly, perturbations in Notch signaling affect cardiomyocyte proliferation during heart development [256] and regeneration [257]. We find that zebrafish Gde2 localizes to the cell surface, where it cleaves and releases GPC6, similarly to human. Thus, the biochemical properties of GDE2 are conserved between zebrafish and humans. Future studies should reveal if the observed heart malformations after perturbed Gde2 expression are associated with impaired Gpc4 or Notch signaling.

Exogenous expression of human GDE2 mRNA restores *insulin* expression in Gdpd5a-depleted zebrafish embryos indicating functional conservation between human and zebrafish Gde2. Notably, over-expression of human GDE2 leads to developmental abnormalities in zebrafish, namely body axis malformations and vasculature and heart defects, which strictly depend on GDE2 catalytic activity. Interestingly, zebrafish Gdpd5 orthologues localize to the plasma membrane of human cells and show activity against GPI-anchored GPC6. These results further highlight the functional conservation between human and zebrafish Gdpd5, and indicate that GDE2 expression levels must be tightly regulated for proper development. Because of the functional conservation between human and zebrafish Gdpd5, the use of zebrafish is an effective model to elucidate the precise signaling axis of GDE2 in pancreas development with potential therapeutic value to diabetes.

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Experimental Procedures

Zebrafish strains and genotyping methods

Adult zebrafish were maintained at 28°C in compliance with the local animal welfare regulations. Their culture was approved by the local animal welfare committee (DEC) of the University of Leiden and all protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Embryos were staged according to [258].

mRNA and Morpholino injections

For mRNA injections, GDE2 constructs previously described in [137] were used. Briefly, a GDE2 image clone was used as template for PCR amplification and BamH1 and EcoRV were used to clone human full-length GDE2 into pcDNA3 vector containing a C-terminal mCherry or HA tag. The vectors were linearized with XbaI. Capped mRNA was synthesised using the SP6 mMessage mMachine kit (Ambion). mRNA (200-500 pg) encoding either wt hGDE2:HA or hGDE2^{H233A} was injected into one-cell stage zebrafish embryos. Translation-blocking morpholinos (MOs) directed against *gdpd5a* (5' GTTTCACCATAGTCAGGCCACAGC 3'), or *gdpd5b* (5'-TGCTTCACCATGCTCCTTCAATCCA-3') were obtained from Gene-Tools (Oregon, USA). Embryos were injected at the 1-2-cell stage with 0,25 to 1mM of MOs.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridizations were carried out according to standard protocols [259]. Riboprobes against *notch1b*, *ins*, *trypsin* have been previously described. Plasmids encoding *pdx1* and *ptfa1* were a kind gift from Dr. Rubén Marín Juez. The probe against *islet1* was generated from cDNA clone MGC: 73031, IMAGE: 4144017 (Source Bioscience Lifesciences, Germany), which was linearized with NotI and transcribed using SP6 RNA Polymerase. To generate a riboprobe against *gdpd5a*, an 870 bp fragment was amplified from zebrafish cDNA using the following primers: Forward: 5' CAGGTTGTAACCTCTGGCGGT 3', Reverse: 5' TGGGACGAGGCACTTTCTTC 3'. The fragment was cloned into the PGEMT vector, was linearized with XhoI and was transcribed using SP6 RNA polymerase.

Western blot analyses

Approximately 20 larvae/sample were lysed (3 μ l per larva) in ice-cold standard RIPA buffer supplemented with proteinase inhibitors. Lysates were dounced for 5 min and homogenized using an insulin syringe, followed by centrifugation at 13.000 rpm for 15 min at 4 °C to pellet nuclei and cell debris. The protein concentration was measured using a standard BCA protein assay kit (Pierce). Lysates containing 4x Bolt LDS sample buffer, supplemented with DTT were boiled for 5 min. 12 – 30 μ g was loaded onto 12% Bis-Tris SDS-PAGE precast gel (Nu-Page Invitrogen) and transferred to nitrocellulose membranes. Nonspecific protein binding was blocked using 5% skimmed milk in TBST. Primary antibodies were incubated overnight at 4°C followed by 1hr incubation with HRP-conjugated secondary antibodies (DAKO, Glostrup, Denmark) and detection using ECL Westernblot reagent (GE Healthcare). Antibodies used were: home-made rabbit anti-GDE2 [137], mouse anti-beta-actin (1:10.000, Sigma, #A5441).

GDE activity assays

GDE activity assays were carried out in HEK293 cells, as previously described [137]. In brief, HEK293 cells were seeded on polyethyleneimine-coated 6-well plates and co-transfected with expression vectors for human GDE2, or zebrafish Gdpd5a/5b (GFP-tagged) together with GPC6-HA. Bacterial PI-PLC was used as positive control. The amount of GPC6 in the medium and cell lysates was analyzed by western blotting. GPC6 expression was assessed using anti-HA antibody (3F10, Roche), zebrafish Gdpd5 and hGDE2 with home-made anti-GFP antibody.

Microscopy

HEK293 cells were cultured on 24 mm glass coverslips and transiently transfected with human GDE2- or zebrafish Gdpd5a-GFP, Gdpd5b-GFP using Eugene 6 reagent (Invitrogen). After 24 hr, the cells were washed with PBS, fixed with 4% PFA, mounted with Immuno-Mount™ (Thermo Scientific) and visualized on a LEICA TCS-SP5 confocal microscope.

Expression vectors

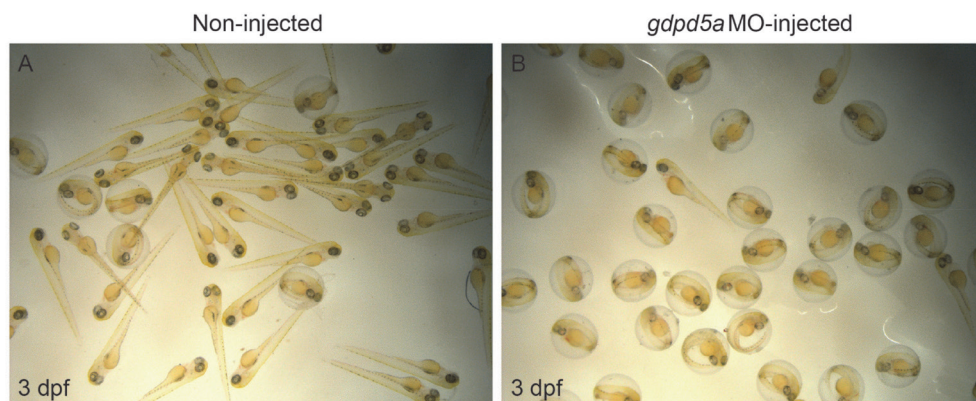
Human GDE2 cDNA was subcloned as described (Matas-Rico et al., 2016). Zebrafish Gdpd5a and Gdpd5b were PCR amplified from synthetic g-Blocks gene fragments (Integrated DNA Technologies Inc., USA) and cloned into pcDNA3-GFP digested with BamHI/EcoRV using SLiCE (Seamless Ligation Cloning Extract)[260] cloning. Primers used: *gdpd5a*, Forward: 5' AAAGCTTAAGCTTGGTACCGAGCTCGGATCCATGGTGAAACACCAGCCG 3', Reverse: 5' GCTCCTCGCCCTTGCTCACCATGATATCTAGTTTGGCGGTATGTGCG 3'; *gdpd5b*, Forward: 5' AAAGCTTAAGCTTGGTACCGAGCTCGGATCCATGGTGAAGC 3', and Reverse: CTCCTCGCCCTTGCTCACCATGATATCATTGGTGTAGTAGTTAAA TCCATTTTCTG 3'.

Supplementary figures



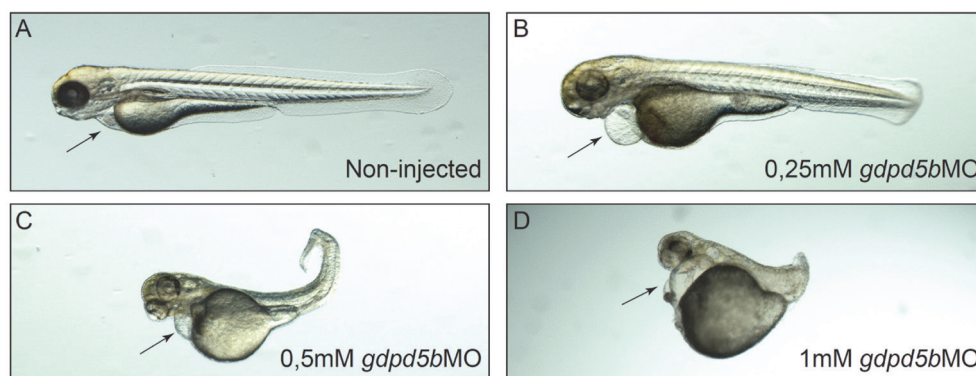
Supplementary Figure 1. Comparison of zebrafish Gdpd5a and Gdpd5b

Protein sequence comparison between zebrafish Gdpd5a and Gdpd5b, using Clustal Omega. Identical aminoacids are shaded in grey. Gdpd5b is characterized by a truncation at the C-terminal cytoplasmic tail. The key catalytic residue (H233 in human GDE2) is indicated by the red line.



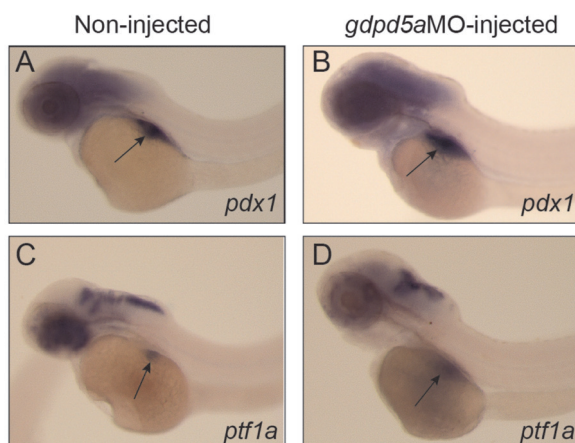
Supplementary Figure 2. The majority of *gdpd5a*MO-injected larvae do not hatch

(A) Population of non-injected zebrafish larvae at 3 dpf. Almost all the embryos have hatched from their chorions. (B) Population of *gdpd5a*MO-injected embryos at 3 dpf. The majority of the morphants are still in their chorions and very few have hatched. In addition, Gdpd5a-depleted embryos do not swim away upon poking.



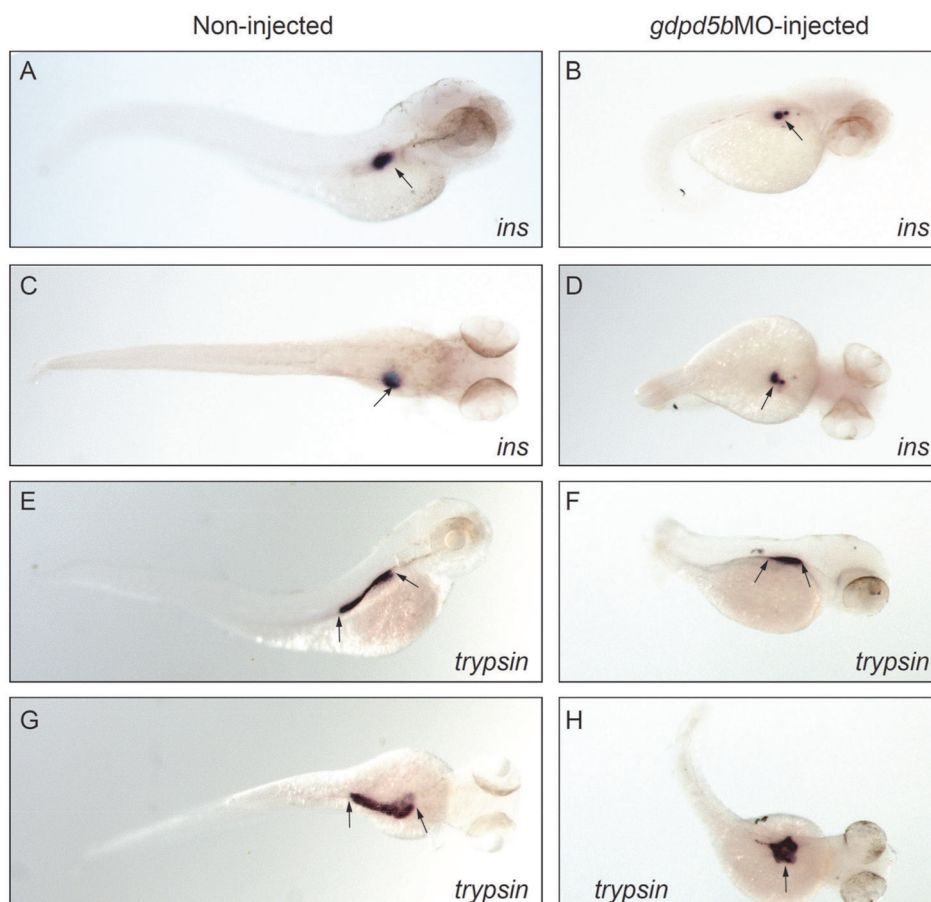
Supplementary Figure 3. Gdpd5b depletion causes developmental defects

Representative pictures of zebrafish embryos at 3 dpf micro-injected with different amounts of *gdpd5b*MO at the one-cell stage. (A) Un-injected embryo at 3 dpf. Arrow indicates looped heart. (B) Embryo injected with 2 ng/ul (0.25mM) *gdpd5b*MO. Arrow indicates heart oedema and unlooped heart. Note the slightly shorter antero-posterior body axis. (C) Embryo injected with 4 ng/ul (0.5mM) *gdpd5b*MO. Arrow indicates heart oedema and unlooped heart. Note the shorter and kinked body axis. (D) 3 dpf embryo injected with 8 ng/ul (1mM) *gdpd5b*MO. Note the severe body truncations, defective eye development, large heart oedema and unlooped heart (arrow).



Supplementary Figure 4. The expression of *pdx1* and *ptf1a* is not affected in *Gdpd5a* morphants

WISH for expression of the endocrine pancreas marker *pdx1* and exocrine pancreas marker *ptf1a* and in non-injected wt (A,C) and *gdpd5a*MO-injected embryos at 3dpf (B,D). Lateral views, anterior to the left. (A) wt embryo at 3dpf stained for *pdx1*. *Pdx1* is expressed in the brain and the endocrine pancreas (arrow). (B) *Gdpd5a*MO-injected embryo at 3dpf. Strong *pdx1* expression is detected in the endocrine pancreas (arrow). (C) wt embryo at 3dpf stained for *ptf1a*. *Ptf1a* expression is detected in the brain and the exocrine pancreas (arrow). (D) *Gdpd5a*MO-injected embryo at 3dpf. *Ptf1a* is expressed at comparable levels in the brain and the exocrine pancreas (arrow) as in non-injected controls.



Supplementary Figure 5. Expression of *insulin* and *trypsin* is reduced upon *Gdpd5b* knockdown

WISH for expression of *insulin* (*ins*) (A-D) and *trypsin* (E-G) in non-injected wt (A, C, E, G) and 2ng/ul *gdpd5b*MO-injected embryos (B, D, F, H) at 3 dpf. (A, B, E, F) lateral views, and (C, D, G, H) dorsal views, anterior to the right. (A) Lateral view of a 3 dpf non-injected embryo stained for *insulin*. Strong expression is detected in the endocrine pancreas (arrow). (B) *Gdpd5b*MO-injected embryo at 3 dpf. *ins* expression is reduced and the expression domain appears split (arrow). (C) Dorsal view of a 3 dpf wt embryo, arrow depicts *insulin* expression. (D) Dorsal view of a *gdpd5b*MO-injected embryo, the *insulin* expression domain is smaller and split (arrow). (E) Lateral view of non-injected 3 dpf embryo stained for *trypsin*. The arrows demarcate the *trypsin* expression domain. (F) Lateral view of a *gdpd5b*MO-injected embryo at 3 dpf. The *trypsin* expression domain is smaller and irregularly shaped (arrows). (G) Dorsal view of non-injected 3 dpf embryo, arrows demarcate *trypsin* expression. (H) Dorsal view of a *Gdpd5b* morphant, the *trypsin* expression domain is reduced (arrow).