International Journal of Biochemistry and Cell Biology 94 (2018) 71–78

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Glycerophosphodiesterase GDE2/GDPD5 affects pancreas differentiation in zebrafish

Michiel van Veen[b,1], Laurie A. Mans[a,1], Elisa Matas-Rico[b], Jason van Pelt[a], Anastassis Perrakis[c], Wouter H. Moolenaar[b], Anna-Pavlina G. Haramis[a,*]

[a] Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE, Leiden, The Netherlands
[b] Division of Cell Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] Division of Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ARTICLE INFO

Keywords:
Glycerophosphodiesterase
GDE2
GDPD5
Zebrafish
Pancreas development
Insulin

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 GDPD5), 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 where they 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.

1. 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 (Ninov et al., 2012; Tehrani and Lin, 2011). Notch receptor signaling is activated by transmembrane Notch ligands in adjacent cells (Wang et al., 2011). Following ligand binding, Notch is cleaved by the metalloprotease TACE (factor-a-converting enzyme) (Wang et al., 2011). Truncated Notch is in turn cleaved by the a secretase complex, leading to intracellular release of NICD (Notch intracellular domain) (Wang et al., 2011) and consequent activation of Notch target genes (Wang et al., 2011).

Different levels of Notch can elicit distinct fates (Ninov et al., 2012; Tehrani and Lin, 2011). 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 (Sabharwal et al., 2011; Zecchin et al., 2007). Loss of Notch signaling in mice (Apelqvist et al., 1999) and zebrafish (Esni et al., 2004; Zecchin et al., 2007) results in aberrant/excessive differentiation of pancreatic progenitors to endocrine cells, at the expense of the later-appearing exocrine cells (Tehrani and Lin, 2011). 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) (Park et al., 2013) and certain heparan sulfate proteoglycans, namely the glypicans (GPCs) (Cave et al., 2017; Matas-Rico et al., 2016a). 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 (Park et al., 2013; Sabharwal et al., 2011). 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 (Cave et al., 2017; Sabharwal et al., 2011). In addition,

* Corresponding author.
E-mail address: a.haramis@biology.leidenuniv.nl (A.-P.G. Haramis).
[1] These authors contributed equally.

https://doi.org/10.1016/j.biocel.2017.11.015
Received 10 March 2017; Received in revised form 18 October 2017; Accepted 30 November 2017

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 (Matas-Rico et al., 2016a,b). 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 (Tehrani and Lin, 2011), and important genes in mammalian islet development are functionally conserved in zebrafish (Tehrani and Lin, 2011). 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.

2. Results

2.1. 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 (Cave et al., 2017; Matas-Rico et al., 2016a; Park et al., 2013) (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. The predicted catalytic domains of human GDE2 and zebrafish Gdpd5a share 53% identity and 81% similarity (Fig. 1B). Phylogenetic analysis shows that the long isoform of zebrafish *gdpd5a* is closely related to human, rodent and chicken *GDPD5/Gdpd5* (Fig. 1C). *Gdpd5b* (ENSDARG00000076962) encodes a 566-aa protein that is characterized by a relatively short, truncated C-terminal tail (SFig. 1 in the supplementary material).

2.2. 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 (Nasevicius and Ekker, 2000). 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 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. 2B). 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 in the supplementary material). 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 dechorionation)

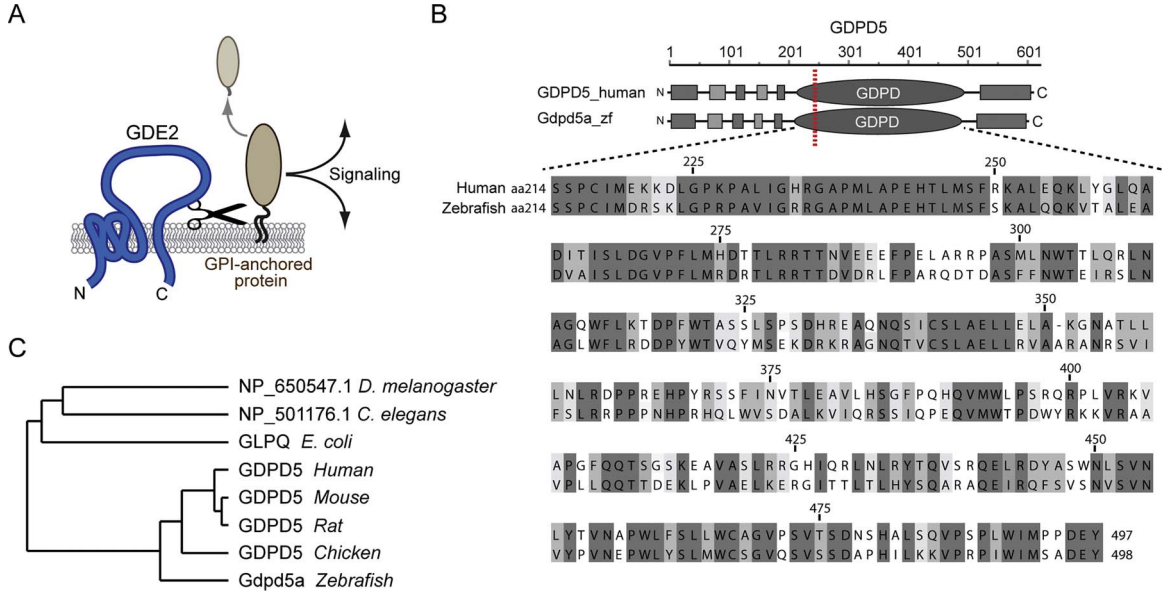

Fig. 1. Sequence analysis of zebrafish Gdpd5a.
(A) GDE2 cleaves GPI anchored proteins at the plasma membrane resulting in their shedding with consequent activation of signaling pathways, either in the same cell or in adjacent cells. **(B)** Amino acid sequence comparison between human GDPD5 and zebrafish Gdpd5a. The red line depicts key catalytic residue H^{233} in GDE2 (Matas-Rico et al., 2016a). 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).

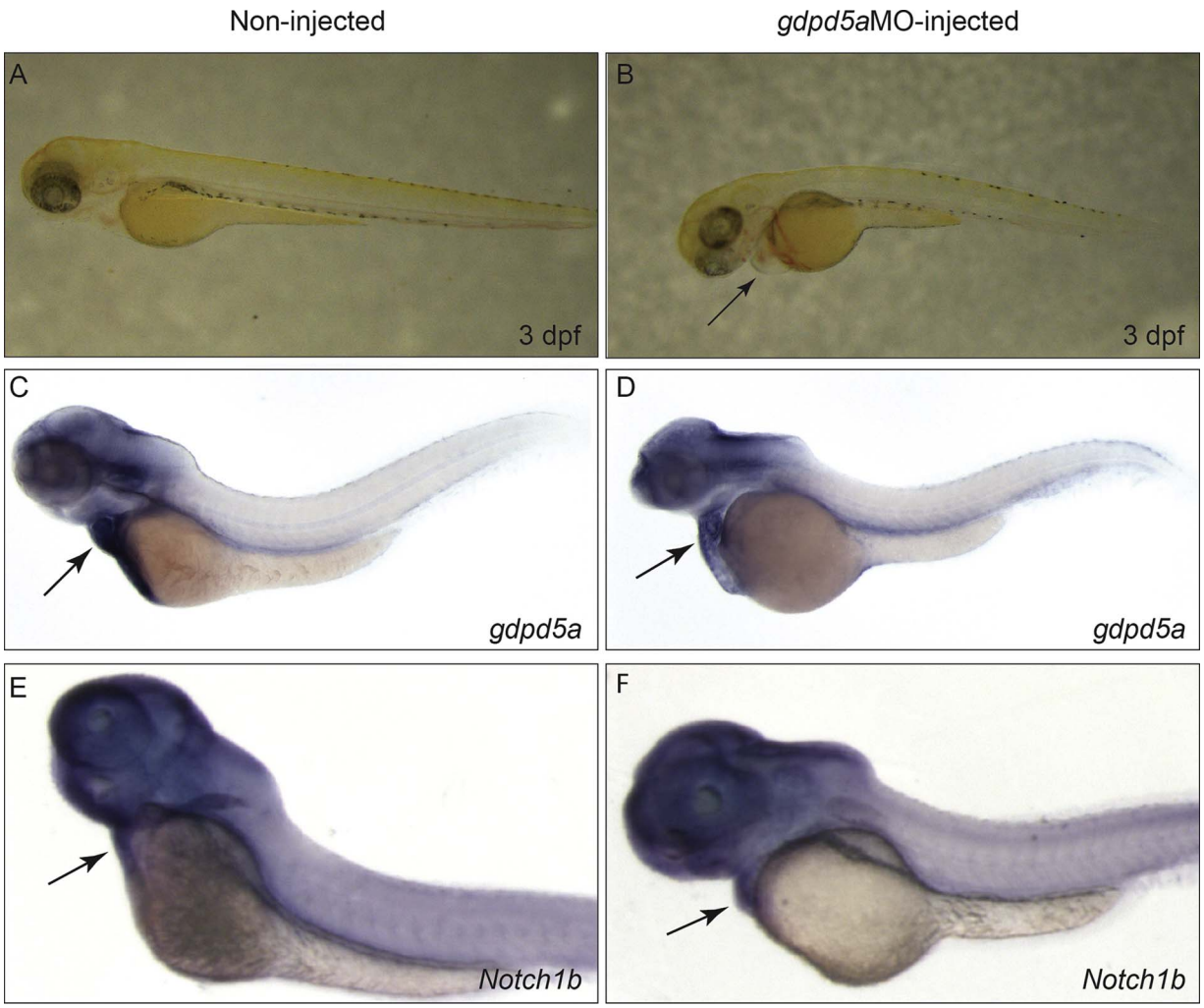

Fig. 2. Gdpd5a depletion causes developmental defects.
(**A)** Non-injected embryo at 3 dpf. Arrow indicates looped heart. (**B)** Representative example of a *gdpd5a*MO-injected embryo at 3 dpf. *Gdpd5a*MO-injected embryos show a bent and shorter body axis, heart oedemas (arrow) and unlooped heart.
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.

showed severe motility defects, either swimming inability or trembling (data not shown), consistent with *Gdpd5* knockout causing behavioral motor deficits in mice (Cave et al., 2017).

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 2 ng/ul (0,25 mM) *gdpd5b*MO led to slight body curvature defects and heart oedemas (SFig. 3B in the supplementary material). 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 in the supplementary material). Finally, embryos injected with 8 ng/ul (1 mM) *gdpd5b*MO showed very severe antero-posterior axis truncations, cyclopia, and very large heart oedemas (SFig. 3D in the supplementary material). 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 overlapping and distinct functions during zebrafish development.

2.3. Expression studies

We next assayed *gdpd5a* and *notch1b* expression in *gdpd5a*MO-injected embryos at 3 dpf. We analyzed 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).

2.4. Pancreas differentiation is affected upon Gdpd5 depletion

Given the role of Notch1b signaling in pancreas differentiation and

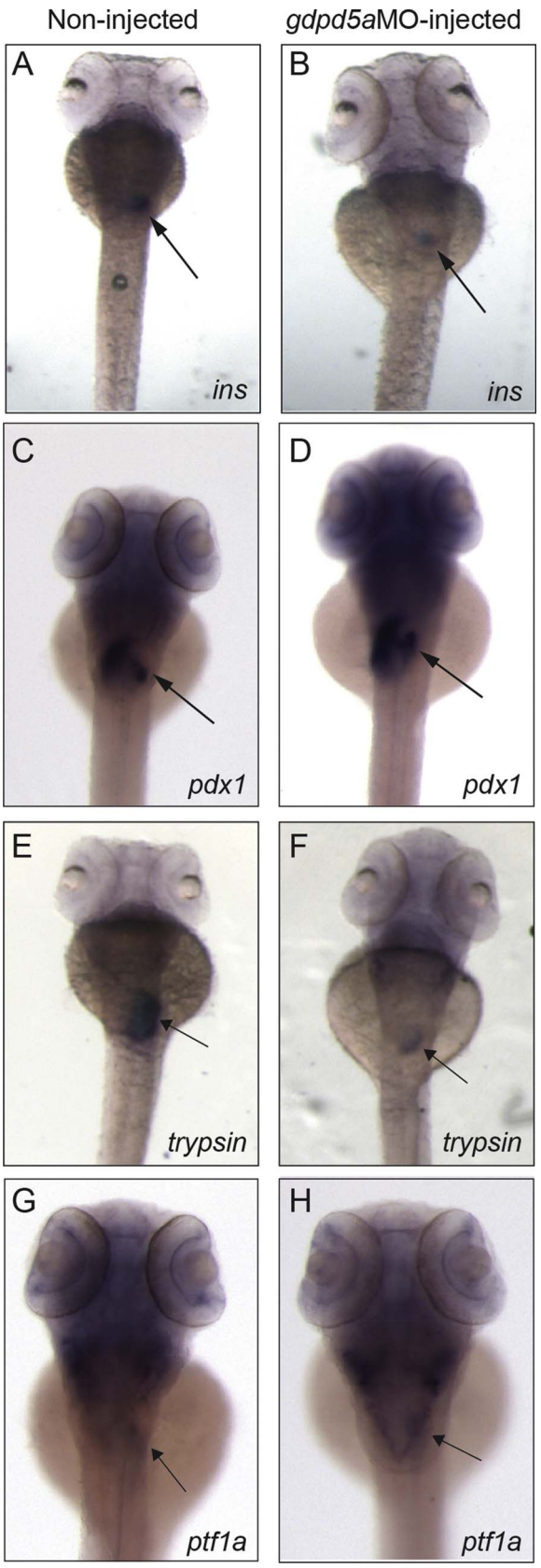

Fig. 3. Expression of *insulin* and *trypsin*, but not *pdx1* or *ptf1a*, is reduced upon Gdpd5a knockdown.
WISH for expression of the endocrine pancreas markers, *insulin (ins)* and *pancreatic and duodenal homeobox 1* (*pdx1)* and the exocrine pancreas markers, *trypsin* and *ptf1a* in non-injected 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)** *Gdpd5*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 *gdpd5*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)** *Gdpd5*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)** *Gdpd5*MO-injected embryo at 3 dpf. *Ptf1a* is expressed at comparable levels in the brain and exocrine pancreas (arrow) as in non-injected controls.

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 h post-fertilization (hpf), and the ventral bud emerges at 32 hpf and produces mostly exocrine and some endocrine cells (Field et al., 2003). At around 72 hpf the pancreas is fully developed (Tehrani and Lin, 2011). At 14 hpf the pancreatic progenitor cells express different levels of *pdx1* (pancreatic and duodenal homeobox 1) (Tehrani and Lin, 2011). 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. 3B). *Pdx1* was expressed in the head, intestine and endocrine pancreas at 3 dpf (Fig. 3C and SFig. 4A in the supplementary material), with no differences in *pdx1* expression between non-injected and *gdpd5a*MO-injected embryos (Fig. 3D and SFig. 4B in the supplementary material). 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. 3F, *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 in the supplementary material). The *ptf1a* expression pattern was not altered in *gdpd5a*MO-injected embryos (Fig. 3H and SFig. 4D in the supplementary material).

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 in the supplementary material). Specifically, the *insulin* expression domain was smaller and appeared split in *gdpd5b*MO-injected embryos (SFig. 5B, D in the supplementary material). Furthermore, the *trypsin* expression domain was smaller and irregularly shaped in *gdpd5b*MO-injected embryos (SFig. 5F, H in the supplementary material). 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 (Sabharwal et al., 2011), 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 (Appel et al., 1995; Dasen and Jessell, 2009). *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

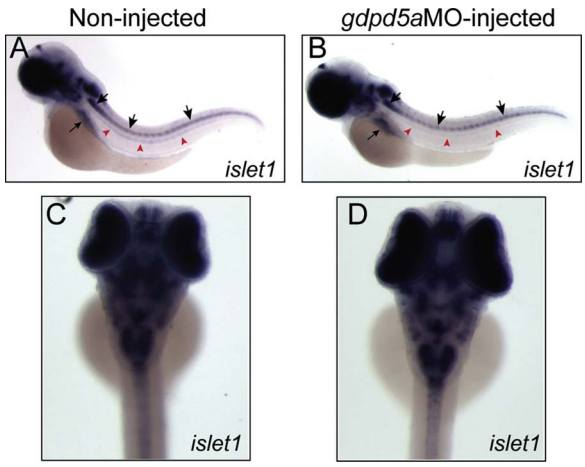

Fig. 4. *Islet1* expression upon Gdpd5 knockdown.
WISH hybridization for *islet1* expression on non-injected **(A, C)**, or *gdpd5a*MO-injected embryos **(B, D)** at 3 dpf. **(A, B)** Lateral views, anterior to the left. **(A)** wt embryo at 3 dpf, *Islet1* is expressed in the brain, spinal cord (arrowheads) and the pancreas (arrow). **(B)** *Islet1* is expressed at similar levels in *gdpd5a*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**) *Gdpd5a*MO-injected embryos at 3 dpf show a staining pattern similar to that in non-injected controls.

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).

2.5. 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$^{His233Ala/His275Ala}$) (Matas-Rico et al., 2016a) 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 the malformations observed depend on the catalytic activity of GDE2, and they indicate 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 knockdown. We assayed for *insulin* expression in embryos that have been injected with either 1 mM *gdpd5a*MO, 300 pg hGDE2-HA mRNA, or co-injected with 1 mM *gdpd5a*MO and 300 pg 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 confirm 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 confirmed that zebrafish Gdpd5 localized to the plasma membrane (Fig. 7A). We co-expressed Glypican-6 (GPC6) and constructs encoding zebrafish or human GDE2, and examined the presence of GPC6 in the conditioned medium by immunoblotting, as described by (Matas-Rico et al., 2016b). We used intact cells treated with bacterial phospholipase C (PI-PLC) as positive control. As shown in Fig. 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. Taken together, these results demonstrate that zebrafish Gdpd5 can cleave GPC6 and show that catalytic activity is conserved between hGDE2 and zebrafish GDPD5a.

3. Discussion

In this study we have identified a previously unknown role for glycerophosphodiesterase Gde2 (Gdpd5) in non-neuronal cell types, particularly the pancreas. We focus 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.

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 *ptf1a* 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 (Uhlen et al., 2015; Lang et al., 2008). 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 (Esni et al., 2004). 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 (Rao and Sockanathan, 2005; Sabharwal et al., 2011), 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 (Matas-Rico et al., 2016a). In adult mice, GDE2 prevents neurodegeneration by promoting motor neuron survival (Cave et al., 2017). Mechanistically, GDE2 does so by specifically cleaving the GPI-anchored Notch ligand regulator RECK as well as glypicans at the plasma membrane (Matas-Rico et al., 2016a; Cave et al., 2017). In vertebrates, Glypicans are highly conserved and affect various signaling pathways including the Wnt and Bmp signaling axes (Gupta and Brand, 2013; Strate et al., 2015). Interestingly, Gpc4 depletion in zebrafish results in strongly reduced cardiomyocyte proliferation (Strate et al., 2015). Similarly, perturbations in Notch signaling affect cardiomyocyte proliferation during heart development (High and Epstein, 2008) and regeneration (Zhao et al., 2014). We find that zebrafish Gdpd5 localizes to the cell surface, where it cleaves and releases GPC6, similarly to

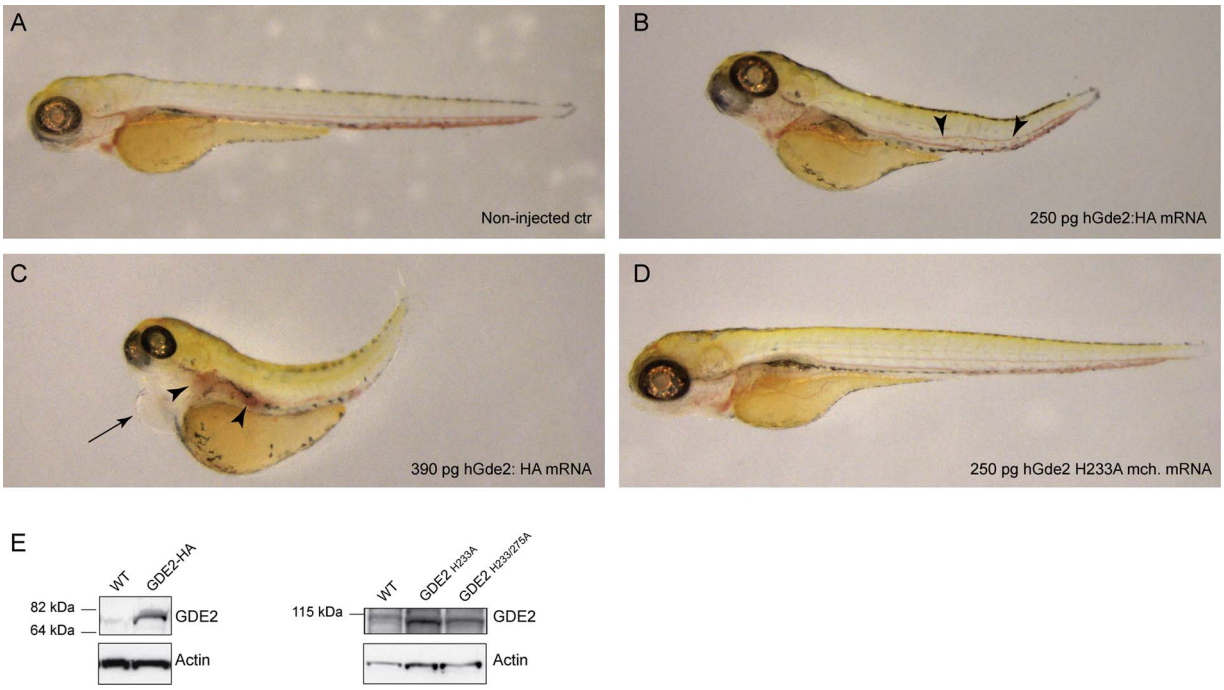

Fig. 5. Expression of human GDE2 leads to developmental defects, depending on GDE2 catalytic activity.
Representative pictures of zebrafish larvae at 4 dpf that have been injected at the one-cell stage with mRNAs encoding human GDE2:HA (B, C), or catalytically-dead hGDE2 ^{H233A} mRNAs (D). Lateral views, 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.

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, or both.

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. 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

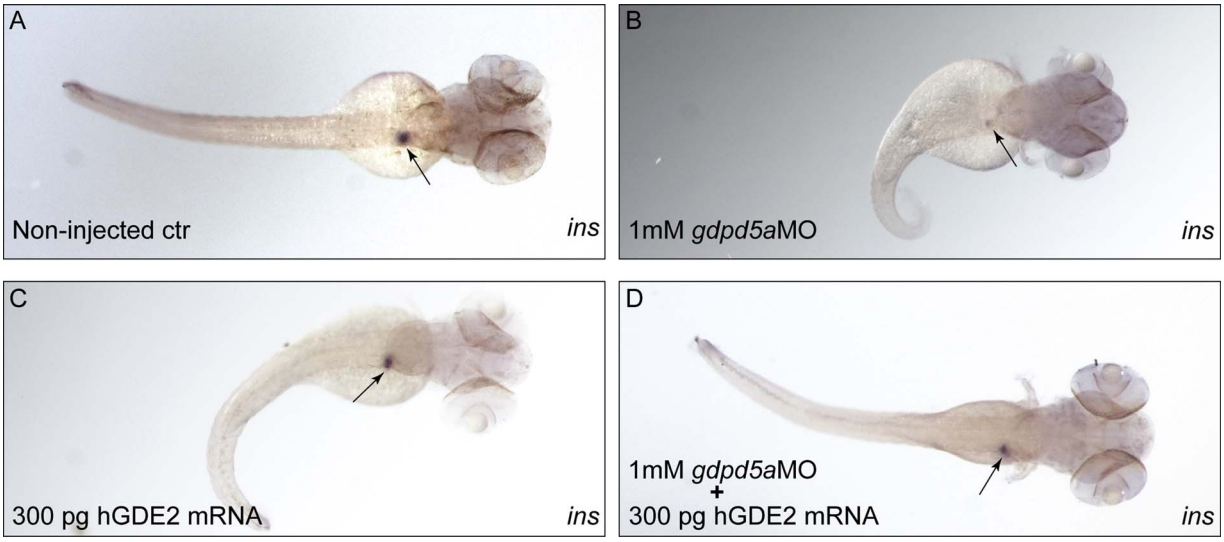

Fig. 6. Human GDE2 mRNA restores *insulin* expression in Gdpd5a morphants.
Representative pictures of zebrafish embryos at 3 dpf stained for *insulin (ins)* expression that have been either non-injected (A), *gdpd5a*MO-injected (B), hGDE2 mRNA-injected (C) or co-injected with *gdpd5a*MO + hGDE2 mRNA (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 1 mM *gdpd5a*MO and 300 pg hGDE2 mRNA at the one-cell stage. *Ins* expression is restored (arrow).

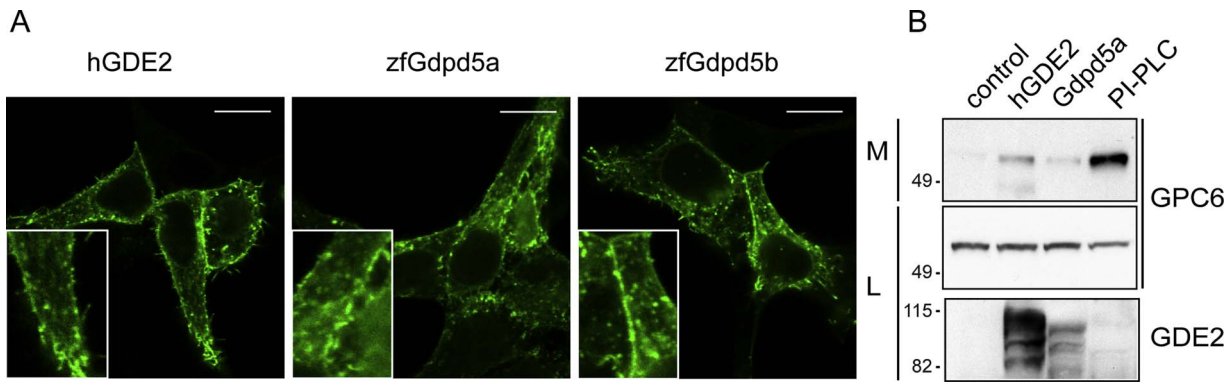

Fig. 7. Zebrafish Gdpd5 orthologues localize to the plasma membrane and promote GPC6 release.
(**A**) Confocal images of HEK293 cells transiently transfected with human GDE2, zebrafish Gdpd5a or Gdpd5b (GFP-tagged), as indicated. Insets show high-power images of cells depicting that Gdpd5a and Gdpd5b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

therapeutic value to diabetes.

4. Materials and methods

4.1. 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 (Kimmel et al., 1995).

4.2. mRNA and morpholino injections

For mRNA injections, GDE2 constructs previously described in (Matas-Rico et al., 2016a) 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 synthesized 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′-TGCTTCACCATG CTCCTTCAATCCA-3′) were obtained from Gene-Tools (Oregon, USA). Embryos were injected at the 1–2-cell stage with 0.25 to 1 mM of MOs.

4.3. Whole-mount in situ hybridization

Whole-mount *in situ* hybridizations were carried out according to standard protocols (Thisse and Thisse, 2008). 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′ CAGGT TGTAACTCTGGCGGT 3′, Reverse: 5′ TGGGACGAGGCACTTTCTTC 3′. The fragment was cloned into the PGEMT vector, was linearized with XhoI and was transcribed using SP6 RNA polymerase.

4.4. Western blot analyses

Approximately 20 larvae/sample were lysed (3 μl 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 4× Bolt LDS sample buffer, supplemented with DTT were boiled for 5 min 12–30 ug 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 (Matas-Rico et al., 2016a), mouse anti-beta-actin (1:10.000, Sigma, #A5441).

4.5. GDE2 activity assays

GDE2 activity assays were carried out in HEK293 cells, as previously described (Matas-Rico et al., 2016a). In brief, HEK293 cells were seeded on polyethyleneimine-coated 6-well plates and co-transfected with expression vectors for human GDE2, or zebrafish Gdpd5a (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.

4.6. Microscopy

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

4.7. Expression vectors

Human GDE2 cDNA was subcloned as described (Matas-Rico et al., 2016a,b). 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)(Zhang et al., 2014) cloning. Primers used: *gdpd5a*,

Forward: 5′ AAACTTAAGCTTGGTACCGAGCTCGGATCCATGGTGAAACACCAGCCG 3′,

Reverse: 5′ GCTCCTCGCCCTTGCTCACCATGATATCTAGTTTGGCGGTATGTCGC 3′;

gdpd5b, Forward: 5′ AAACTTAAGCTTGGTACCGAGCTCGGATCCATGGTGAAGC 3′, and

Reverse: 5′ GCTCCTCGCCCTTGCTCACCATGATATCATTGGTGTAGTAGTTAAATCCATTTTCTG 3′.

Acknowledgements

We thank Dr. Rubén Marín-Juez, (MPI, Bad Neuheim, Germany) for probes, Yvette Stijf-Bultsma (NKI, Amsterdam) for technical assistance, and the animal caretakers at Leiden University for excellent care of the fish. The work was supported by grants from the Dutch Cancer Society (KWF UL 2012-5395) to APGH, KWF 10215/2016-1 to AP, and the Netherlands Organization for Scientific Research (NWO; TOPGO 700.10.354) to WHM and AP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocel.2017.11.015.

References

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., Edlund, H., 1999. Notch signalling controls pancreatic cell differentiation. Nature 400 (6747), 877–881.

Appel, B., Korzh, V., Glasgow, E., Thor, S., Edlund, T., Dawid, I.B., Eisen, J.S., 1995. Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish. Development 121 (12), 4117–4125.

Cave, C., Park, S., Rodriguez, M., Nakamura, M., Hoke, A., Pletnikov, M., Sockanathan, S., 2017. GDE2 is essential for neuronal survival in the postnatal mammalian spinal cord. Mol. Neurodegener. 12 (1), 8.

Dasen, J.S., Jessell, T.M., 2009. Hox networks and the origins of motor neuron diversity. Curr. Top. Dev. Biol. 88, 169–200.

Esni, F., Ghosh, B., Biankin, A.V., Lin, J.W., Albert, M.A., Yu, X., MacDonald, R.J., Civin, C.I., Real, F.X., Pack, M.A., Ball, D.W., Leach, S.D., 2004. Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 131 (17), 4213–4224.

Field, H.A., Dong, P.D., Beis, D., Stainier, D.Y., 2003. Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. Dev. Biol. 261 (1), 197–208.

Gupta, M., Brand, M., 2013. Identification and expression analysis of zebrafish glypicans during embryonic development. PLoS One 8 (11), e80824.

High, F.A., Epstein, J.A., 2008. The multifaceted role of Notch in cardiac development and disease. Nat. Rev. Genet. 9 (1), 49–61.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203 (3), 253–310.

Lang, Q., Zhang, H., Li, J., Yin, H., Zhang, Y., Tang, W., Wan, B., Yu, L., 2008. Cloning and characterization of a human GDPD domain-containing protein GDPD5. Mol. Biol. Rep. 35 (3), 351–359.

Matas-Rico, E., van Veen, M., Leyton-Puig, D., van den Berg, J., Koster, J., Kedziora, K.M., Molenaar, B., Weerts, M.J., de Rink, I., Medema, R.H., Giepmans, B.N., Perrakis, A., Jalink, K., Versteeg, R., Moolenaar, W.H., 2016a. Glycerophosphodiesterase GDE2 promotes neuroblastoma differentiation through glypican release and is a marker of clinical outcome. Cancer Cell 30 (4), 548–562.

Matas-Rico, E., van Veen, M., Moolenaar, W.H., 2016b. Neuronal differentiation through GPI-anchor cleavage. ABBV Cell Cycle 1–2.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. 26 (2), 216–220.

Ninov, N., Borius, M., Stainier, D.Y., 2012. Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. Development 139 (9), 1557–1567.

Park, S., Lee, C., Sabharwal, P., Zhang, M., Meyers, C.L., Sockanathan, S., 2013. GDE2 promotes neurogenesis by glycosylphosphatidylinositol-anchor cleavage of RECK. Science 339 (6117), 324–328.

Rao, M., Sockanathan, S., 2005. Transmembrane protein GDE2 induces motor neuron differentiation in vivo. Science 309 (5744), 2212–2215.

Sabharwal, P., Lee, C., Park, S., Rao, M., Sockanathan, S., 2011. GDE2 regulates subtype-specific motor neuron generation through inhibition of Notch signaling. Neuron 71 (6), 1058–1070.

Strate, I., Tessadori, F., Bakkers, J., 2015. Glypican4 promotes cardiac specification and differentiation by attenuating canonical Wnt and Bmp signaling. Development 142 (10), 1767–1776.

Tehrani, Z., Lin, S., 2011. Endocrine pancreas development in zebrafish. ABBV Cell Cycle 10 (20), 3466–3472.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3 (1), 59–69.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., Ponten, F., 2015. Proteomics: tissue-based map of the human proteome. Science 347 (6220), 1260419.

Wang, Z., Ahmad, A., Li, Y., Azmi, A.S., Miele, L., Sarkar, F.H., 2011. Targeting notch to eradicate pancreatic cancer stem cells for cancer therapy. Anticancer Res. 31 (4), 1105–1113.

Zecchin, E., Filippi, A., Biemar, F., Tiso, N., Pauls, S., Ellertsdottir, E., Gnugge, L., Bortolussi, M., Driever, W., Argenton, F., 2007. Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. Dev. Biol. 301 (1), 192–204.

Zhang, Y., Werling, U., Edelmann, W., 2014. Seamless ligation cloning extract (SLiCE) cloning method. Methods Mol. Biol. 1116, 235–244.

Zhao, L., Borikova, A.L., Ben-Yair, R., Guner-Ataman, B., MacRae, C.A., Lee, R.T., Burns, C.G., Burns, C.E., 2014. Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration. Proc. Natl. Acad. Sci. U. S. A. 111 (4), 1403–1408.