Glycerophosphodiesterase GDE2 Promotes Neuroblastoma Differentiation through Glypican Release and Is a Marker of Clinical Outcome

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SUMMARY

Neuroblastoma is a pediatric embryonal malignancy characterized by impaired neuronal differentiation. A better understanding of neuroblastoma differentiation is essential for developing new therapeutic approaches. GDE2 (encoded by GDPD5) is a six-transmembrane-domain glycerophosphodiesterase that promotes embryonic neurogenesis. We find that high GDPD5 expression is strongly associated with favorable outcome in neuroblastoma. GDE2 induces differentiation of neuroblastoma cells, suppresses cell motility, and opposes RhoA-driven neurite retraction. GDE2 alters the Rac-RhoA activity balance and the expression of multiple differentiation-associated genes. Mechanistically, GDE2 acts by cleaving (in cis) and releasing glycosylphosphatidylinositol-anchored glypican-6, a putative co-receptor. A single point mutation in the ectodomain abolishes GDE2 function. Our results reveal GDE2 as a cell-autonomous inducer of neuroblastoma differentiation with prognostic significance and potential therapeutic value.

INTRODUCTION

Neuroblastoma is a childhood cancer of the developing sympathetic nervous system, and the most common cancer in infancy. It develops from immature neuroblasts in the embryonic neural crest and is characterized by impaired neuronal differentiation (Jiang et al., 2011; Louis and Shohet, 2015; Maris, 2010). It commonly originates in the adrenal gland, but can also develop in nerve tissues elsewhere in the body. Neuroblastoma is a highly heterogeneous disease. In some cases, the tumor undergoes spontaneous regression through mechanisms that remain poorly understood (Brodeur and Bagatell, 2014). In many cases, however, neuroblastoma progresses into a high-risk metastatic disease with very poor prognosis. Remarkably, high-risk neuroblastoma is characterized by relatively few recurrent somatic mutations (Molenaar et al., 2012; Pugh et al., 2013). The paucity of oncogenic mutations, along with an incomplete understanding of neuroblastoma differentiation...
and regression, hampers the development of new targeted therapies.

In general, overall survival of neuroblastoma patients is largely dependent on the degree of neuronal differentiation and, furthermore, inversely correlated with a motile phenotype (Louis and Shohet, 2015; van Nes et al., 2013). Consistent with this, some high-risk neuroblastomas are associated with mutations in Rac-Rho pathway genes that normally regulate F-actin-based neurite outgrowth and remodeling, as well as tumor cell motility, invasion, and metastasis (Molenaar et al., 2012). Unfortunately, treatment options are very limited for high-risk neuroblastoma. One strategy involves differentiation therapy using cis-retinoic acid, but this has led to disappointing results (Ganesan and Schor, 2011; Holzel et al., 2010; Matthay et al., 2009). Thus, there remains an urgent need to identify new therapeutic targets and develop differentiation-inducing protocols (Brodeur et al., 2014). A better understanding of neuroblastoma differentiation at the molecular level is therefore imperative.

In this study we focus on GDE2 (or GDPD5), a transmembrane ecto-phosphodiesterase that promotes embryonic neurogenesis (Rao and Sockanathan, 2005). GDE2/GDPD5, along with GDE3/GDPD2 and GDE6/GDPD4, belongs to a subfamily of glycerophosphodiester phosphodiesterases (GDEs or GDPDs) characterized by six transmembrane domains and a large catalytic ectodomain (Corda et al., 2014; Yanaka, 2007) (Figures 1A and 1B). GDE2 promotes the differentiation of postmitotic motor neurons in vivo by inhibiting Notch signaling in adjacent neural progenitors (Saharwar et al., 2011). GDE3 has been implicated in osteoblast differentiation (Corda et al., 2009; Yanaka et al., 2003), while the function of GDE6 is unknown. Mechanistically, GDE2 acts by cleaving the glycosyolphosphatidylinositol (GPI) anchor of RECK, a Notch ligand regulator, leading to RECK release from the cell surface and subsequent induction of differentiation in contacting neuronal cells (Park et al., 2013). Thus, GDE2 promotes neurogenesis in a non-cell-autonomous manner, requiring cell-cell contact, at least in the developing spinal cord.

In view of these findings, we set out to examine a possible role of GDE2 in regulating neuroblastoma differentiation.

RESULTS

**GDPD5 Expression Levels Strongly Correlate with Clinical Outcome in Neuroblastoma**

We examined how GDPD5 expression relates to overall survival in neuroblastoma, using mRNA expression data of primary tumor samples from three independent patient cohorts (AMC, Oberthuer, and RPM datasets; n = 871 patients). GDPD5 maps to chromosome 11q13, a region often showing loss of heterozygosity in neuroblastoma (Jiang et al., 2011). Kaplan-Meier analysis revealed that high GDPD5 expression is strongly associated with favorable clinical outcome, while low GDPD5 expression correlates with poor outcome in all three patient cohorts (Figure 1C). This marks GDPD5 as a potential tumor suppressor, but no mutations or deletions were detected in tumor samples analyzed by whole-genome sequencing (Molenaar et al., 2012). Furthermore, GDPD5 expression in patients with an 11q deletion was similar to those with a normal chromosome 11, while there was no significant association with MYCN amplification (p = 0.08) or ALK mutation status (p = 0.66, Fisher’s exact test; data not shown).

GDE2 promotes neurogenesis through loss of functional RECK (Park et al., 2013); however, we found no significant correlation between RECK expression and patient survival (data not shown). Of note, GDE2 family members GDE3 and GDE6 (encoded by GDPD2 and GDPD4, respectively; Figure 1A) were not detectably expressed at the mRNA level in the tumors analyzed, and therefore appear not to have clinical significance in neuroblastoma. These results define high GDPD5 expression as a
favorable prognostic marker in neuroblastoma. We went on to examine how GDE2 affects neuroblastoma cell behavior and to explore its signaling functions and pertinent catalytic activity.

**GDE2 Promotes Differentiation of Neuroblastoma Cells in an Autonomous Manner**

We used established models of neuroblastoma differentiation, including SH-SY5Y, IMR-32, Neuro-2A and N1E-115 cells; in the latter cells, Rho/Rac signaling and neurite remodeling pathways have been extensively studied. Overexpression of GDE2 in N1E-115 or Neuro-2A cells resulted in prominent cell spreading and neurite outgrowth (Figures 2A–2C and S1A). Similarly, expression of doxycycline-inducible GDE2 in either SH-SY5Y or IMR-32 cells led to GDE2 overexpression concomitant with increased neurite outgrowth after doxycycline addition (Figures 2D and 2E). GDE2-induced neurite outgrowth was accompanied by upregulation of various established neural differentiation genes, including *NEUROD1*, SNAP25, ENO2, TUBB3, and MAP2 (Figure 2F).

During embryonic development, GDE2 promotes neurogenesis in non-cell-autonomous manner involving contact-dependent Notch signaling (Sabharwal et al., 2011). In neuroblastoma cells, however, GDE2-induced cell spreading and neurite formation was observed in both isolated and contacting cells (Figure S1B). Furthermore, conditioned medium from GDE2-overexpressing cells did not affect the morphology of parental cells (results not shown), arguing against the involvement of a diffusible factor produced by GDE2. It thus appears that GDE2 promotes neuroblastoma differentiation in a cell-autonomous manner, not requiring cell-cell contact.

Various human neuroblastoma cells, including SH-SY5Y, are responsive to retinoic acid (RA), a known inducer of neuronal differentiation that is used as a therapeutic in the clinic (Brodeur, 2003; Brodeur et al., 2014; Matthey, et al., 2009). RA upregulates GDE2 expression in the ventricular zone (Rao and Sockanathan, 2005) and in Neuro-2A cells (Yanaka et al., 2007), but it did not influence SH-SY5Y cells (Figure S1C). Upon forced overexpression in SH-SY5Y cells, GDE2 enhanced the neurite-inducing effect of RA (2 μM) (Figure S1D), strongly suggesting that GDE2 and RA act cooperatively to induce differentiation.

GDE2-overexpressing SH-SY5Y and IMR-32 cells adhered markedly more strongly to extracellular matrix molecules (laminin, fibronectin, collagen) than did the parental cells (Figure 2G). Cell-matrix adhesion is a major determinant of cell motility, and a motile phenotype is associated with increased incidence of metastases and poor prognosis in neuroblastoma (van Nes et al., 2013). By monitoring the random motility of SH-SY5Y and IMR-32 cells, we established that GDE2 overexpression renders the cells less motile than the wild-type cells (Figure 2H).

When expressed at relatively low levels, GDE2 was detected both at the plasma membrane and in intracellular compartments as revealed by confocal and super-resolution microscopy (Figures S1E and S1F). Plasma membrane GDE2 localized to distinct microdomains, possibly representing lipid rafts, and was particularly enriched at the tips of neurite-like extensions (Figure S1F).

Intracellularly, a relatively large subset of GDE2-containing vesicles was positive for established markers of recycling endosomes, notably Rab11 and the transferrin receptor (Grant and Donaldson, 2009; Welz et al., 2014) (Figures S1G and S1H). This suggests that internalized GDE2 follows a Rab11-driven recycling route as a way of regulation. We further note that, once internalized, GDE2 has its catalytic ectodomain exposed to the vesicle lumen and hence cannot act on cytosolic substrates.

**GDE2 Opposes RhoA-Mediated Neurite Retraction and Activates Rac**

Rho guanosine triphosphatases, particularly RhoA, Rac, and Cdc42, are key regulators of F-actin-driven processes, including neuronal differentiation, cell adhesion, motility, and invasion (Govek et al., 2005; Hall, 2012; Hall and Lalli, 2010; Ridley, 2015; Spuul et al., 2014). One hallmark of developing neurites is their susceptibility to acute retraction by RhoA-activating agonists such as lysophosphaticid acid (LPA), a lipid mediator acting on specific G-protein-coupled receptors. As such, LPA antagonizes the phenotypic differentiation of neuroblastoma and neural/stem progenitor cells (Frisca et al., 2013; Hirose et al., 1998; Jalink et al., 1994; Kranenburg et al., 1999; Moolenaar, 1995). Strikingly, GDE2-overexpressing N1E-115 cells were unable to retract their developing neurites and round up in response to LPA, whereas the parental cells responded vigorously (Figures...
Figure 3. GDE2 Opposes RhoA-Driven Neurite Retraction by LPA and Activates Rac

(A) Serum-starved N1E-115 cells expressing GDE2-mCh were stimulated with 1 μM LPA for the indicated times. GDE2-mCh-expressing cells are indicated by black arrowheads and non-transfected cells by white arrowheads. Scale bars, 10 μm.

(legend continued on next page)
and reduced cell-matrix adhesion (Figures 4D–4G). Moreover, by marked cytoskeletal reorganization, fewer focal adhesions, down (Figure 4B). When compared with shControl cells, GDE2 knockdown cells migrated faster and over longer distances than did shControl cells (Figure 4H). Shep2 cells were poorly responsive to LPA; upon GDE2 depletion, however, they showed more pronounced cytoskeletal contraction induced by LPA (results not shown). The knockdown phenotypes could be rescued by expression of RNAi-resistant GDE2, indicating that the observed effects are specific for GDE2 depletion (Figures 4D–4H). Thus, GDE2 knockdown evokes phenotypic changes opposite to those induced by GDE2 overexpression.

Having shown that GDE2 activates Rac1 and opposes RhoA, we next examined how GDE2 may dictate neuroblastoma phenotype at the gene expression level. We analyzed differential gene expression in GDE2 knockdown versus shControl Shep2 cells using genome-wide RNA sequencing (RNA-seq). In duplicate experiments, RNA-seq-based analysis revealed 121 differentially expressed genes (log2 fold change >1.5; p < 0.001) (Figure 4I and Table S1). The list of differentially expressed genes was markedly enriched (18%; 22/121) in those involved in neuronal differentiation, such as RELN, NTF, ROBO4, RNF112, PLPPLR43, KIR-RELS, and various genes (15%; 18/121) that regulate cell adhesion (LAMA4, COL6A3, COL13A1, FN1, ITGA10, ITGA11) and extracellular matrix organization (Table S1 and Figure 4I). It is also noteworthy that some 20% (26/121) of the GDE2-regulated genes encodes proteins involved in transmembrane receptor activity, including signaling by receptor tyrosine kinases (EPHB6, PDGFRg, TIE1, ERBB4), GPCRs, Wnt, Hedgehog, and transforming growth factor β (TGF-β) receptors, several of which play a role in neurodevelopment (Table S1). These data suggest a scenario in which a unique set of differentiation regulatory genes, along with Rac hyperactivation, dictates the observed GDE2 phenotypes, and may help explain the marked correlation between GDPD5 expression and patient survival.

**GDE2 Biochemical Activity: Release of GPI-Anchored Glypican-6**

What is the relevant catalytic activity of GDE2 and how does it relate to the observed differentiated phenotype? Immunoprecipitated GDE2 hydrolyzes glycerophosphocholine into glycerol 3-phosphate (Galazzini et al., 2008), but this reaction has no signaling relevance. We examined GDE2 enzymatic activity using cell-based assays and mutational analysis. Prompted by the discovery that chicken GDE2 induces the release of GPI-anchored RECK from HEK293 cells (Park et al., 2013), we measured GDE2 activity toward selected GPI-anchored proteins, including RECK and glypicans (GPCs). GPCs are a family of six heparan sulfate proteoglycans that function as co-receptors or ligands to Gprotein-coupled receptors (GPCRs) or transmembrane receptor-tyrosine kinase (RTK) receptors, several of which play a role in neurodevelopment (Table S1). These data suggest a scenario in which a unique set of differentiation regulatory genes, along with Rac hyperactivation, dictates the observed GDE2 phenotypes, and may help explain the marked correlation between GDPD5 expression and patient survival.

**GDE2 Depletion Reverses the GDE2 Overexpression Phenotype and Uncovers GDE2-Regulated Gene Expression**

Since low GDPD5 expression correlates with poor clinical outcome, we examined how GDE2 depletion affects neuroblastoma phenotype through lentiviral small hairpin RNA (shRNA) transduction. To this end, we used Shep2 cells, since these cells show relatively high GDPD5 expression among 24 human neuroblastoma cell lines analyzed (Figures 4A and S3). Using two (out of five) independent shRNAs, we achieved efficient GDE2 knockdown (Figure 4B). When compared with shControl cells, GDE2-depleted Shep2 cells were less well spread and smaller in size, and showed a reduction in basal Rac1 activity, which was now sensitive to LPA stimulation (Figure 3D). Upon GDE2 overexpression, however, the magnitude of LPA-induced Rac1 activity, which was now reduced by about 3-fold (Figure 3D). Decreased Rac activity was also observed in SH-SY5Y cells (Figure 3D). Furthermore, overexpressed GDE2 opposed constitutively active RhoA(V14A) in promoting cell contraction, again consistent with an activated Rac phenotype (Figure 3E). From these results, along with previous findings on Rac-RhoA antagonism, we conclude that high GDE2 expression prevents neurite retraction by activating Rac and opposes RhoA action both at the level of RhoA-guanosine triphosphate (GTP) accumulation and more downstream.

(B) Quantification of LPA-induced cell rounding over time in control and GDE2-overexpressing cells (n = 200 cells; mean ± SEM). *p < 0.05; **p < 0.001, unpaired t-test.

(C) Rac activity was measured in cells expressing empty vector (pcDNA3) or GDE2-GFP. Active GTP-bound Rac was assessed by pull-down assays and compared with total Rac levels in lysates of the same transfectants. (Left) Effect of LPA stimulation on Rac-GTP levels. (Middle) Densitometry analysis of Rac-GTP versus total Rac levels in the presence or absence of LPA (mean ± SEM of three independent experiments). *p < 0.05, unpaired t-test. (Right) Western blot showing Rac activity in SH-SY5Y cells as a function of GDE2 expression and LPA stimulation.

(D) (Left) Activation of RhoA in N1E-115 cells expressing either GDE2-mCherry (red trace) or mCherry-CAAX (black trace) was monitored by an FRET-based biosensor, as described by Kedziora et al. (2016). In parental N1E-115 cells, LPA evoked an immediate increase in RhoA activity that gradually leveled off to a sustained elevated level (Figure 3D). Upon GDE2 overexpression, however, the magnitude of LPA-induced RhoA activation was reduced by about 3-fold (Figure 3D). Decreased Rac activation was also observed in SH-SY5Y cells (Figure 3D). Furthermore, overexpressed GDE2 opposed constitutively active RhoA(V14A) in promoting cell contraction, again consistent with an activated Rac phenotype (Figure 3E). From these results, along with previous findings on Rac-RhoA antagonism, we conclude that high GDE2 expression prevents neurite retraction by activating Rac and opposes RhoA action both at the level of RhoA-guanosine triphosphate (GTP) accumulation and more downstream.

See also Figure S2; Movies S1 and S2.
regulate diverse signaling pathways, either positively or negatively, particularly those involved in morphogenesis and neurodevelopment (Allen et al., 2012; Filmus et al., 2008; Veugelers et al., 1999). We co-expressed GDE2 and its candidate substrates, confirmed their proper expression at the plasma membrane (Figures S4A–S4C and data not shown), and assayed cell lysates and the medium for substrate release by immunoblotting, essentially as described by Park et al. (2013).

We confirmed that chicken GDE2 promotes the release of RECK, as did exogenous PI-PLC (Figure 5A). Unexpectedly, however, our efforts to detect RECK release by human GDE2 were unsuccessful (Figure 5A), suggesting that GDE2 substrate preferences may vary among vertebrates. We then examined the GPI-anchored GPCs as potential substrates. Human GDE2 induced the release of GPC3 and GPC6, but not that of GPCs 1, 2, 4, and 5 (Figures S4D and 5C). This indicates that GDE2 shows selectivity toward GPC family members, likely due to structural differences in the respective heparan sulfate chains and/or the GPI-anchor core.

GPC6 is widely expressed among neuroblastoma cell lines (Figure S4E), while GPC3 expression is more variable (Figure S4F). SH-SY5Y and N1E-115 cells express GPC6 but not GPC3 (Figures S4E–S4G and 7A). We therefore focused our subsequent experiments on GPC6. As one would predict, when co-expressed in N1E-115 cells, GDE2 and GPC6 co-localized in part to the same membrane microdomains (Figures S4B and S4C).

**Mutating His233 Abolishes GDE2 Function**

Uncertainty still exists as to the active site of GDE2. Residue His275 was reported to be critical for GDE2-induced neurogenesis (Rao and Sockanathan, 2005). Another potential catalytic residue is His233, located in a conserved GHRG motif (Yanaka, 2007) (Figure 5B). As shown in Figure 5C, mutants GDE2(H233A) and GDE2(H233A/275A) failed to induce the release of GPC6, whereas the H275A mutation reduced GDE2 activity only partially. This result defines His233 as a key catalytic residue.

Previous in vivo studies suggested that GDE2 activity is enhanced after reduction of a putative disulfide bond (Cys25-Cys576) linking the N- and C-terminal tails (Yan et al., 2009) (Figure 5B), but the catalytic activity of relevant Cys mutants was not examined. In our GPC release assay, the supposedly hyperactive mutant GDE2(C25S), lacking the putative disulfide bond, showed the same catalytic activity as wild-type GDE2 (Figure 5D). This argues against a critical role for Cys25-Cys576 disulfide bonding/reduction in regulating GDE2 activity under the present assay conditions. Finally, to establish whether GDE2 acts in cis (same cell) or/and in trans (adjacent cell), we mixed GDE2-expressing cells (lacking GPC6) with GPC6-expressing cells (lacking GDE2) and measured GPC6 release. GDE2-expressing cells were incapable of promoting GPC6 release from adjacent cells (Figure 5E). It thus appears that GDE2 acts in cis, consistent with GDE2 promoting differentiation in a cell-autonomous manner, not requiring intercellular contacts.

When expressed in N1E-115 cells, catalytically dead GDE2(H233A) failed to promote cell spreading and neurite formation. GDE2(H275A) induced an intermediate phenotype, while the C25S mutation had no effect (Figures 6A–6C), consistent with the above biochemical data. In addition, GDE2(H233A)-overexpressing cells showed a normal contractile response to LPA (results not shown). Thus, GDE2 catalytic activity is necessary and sufficient to induce neuroblastoma cell differentiation and inhibit agonist-induced neurite retraction.

**GPC6 Underexpression Phenocopies GDE2 Overexpression and Correlates with Favorable Disease Outcome**

We next asked to what extent GPC6 release may account for the biological activity of GDE2. Using SH-SY5Y cells, which express GPC6 as the only relevant GPC (Figure 7A), we established that doxycyclin-induced GDE2 expression promotes the release of endogenous GPC6 from the cell surface as measured by flow cytometry (Figure 7B).

GPC6 release from the cell surface predicts loss of GPC6 function. We stably knocked down GPC6 in SH-SY5Y cells by lentiviral transduction of different shRNAs (Figure 7C). GPC6 knockdown cells showed enhanced basal Rac1 activity, which was sensitive to LPA stimulation (Figure 7D), and increased neurite outgrowth (Figure 7E). To validate the knockdown result, we...

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**Figure 4. GDE2 Depletion Reverses the GDE2 Overexpression Phenotype and Uncovers GDE2-Regulated Gene Expression**

(A) Immunoblot of GDE2 expression in the indicated cell lines, using anti-GDE2 antibody. See Figure S3 for GDPD5 mRNA expression analysis.

(B) Left) GDPD5 expression (relative to GAPDH) in control and GDE2-depleted Shep2 cells stably expressing shRNAs against GDE2. Maximal GDE2 knockdown was obtained with hairpins #4 and #5. Error bars denote mean ± SEM. **p < 0.01, ***p < 0.001, unpaired t test. (Right) Immunoblot analysis of GDE2 protein expression using five different hairpins, as indicated. Actin was used as loading control.

(C) Rac-GTP levels in control and GDE2-depleted Shep2 cells (using hairpin #4).

(D) Cell morphology and cytoskeletal organization (phalloidin and vinculin staining) in control and GDE2-depleted Shep2 cells. Expression of RNAi-resistant GDE2 rescues the phenotype, as indicated. Black scale bars, 100 μm; white scale bars, 10 μm.

(E) Immunoblot showing GDE2 expression in control, GDE2-depleted, and “GDE2-rescued” Shep2 cells (transfected with RNAi-resistant GDE2).

(F) Quantification of focal adhesion area in control, GDE2-depleted, and GDE2-rescued Shep2 cells using ImageJ software (n = 20 cells; mean ± SEM). **p < 0.0001, one-way ANOVA.

(G) Cell-matrix adhesion of control, GDE2-depleted, and rescued Shep2 cells. Cells were plated on fibronectin or collagen. At 30 min after plating, adherent cells were fixed and stained with crystal violet for quantification. Graph shows mean ± SEM of two independent experiments. **p < 0.01, ***p < 0.001, unpaired t test.

(H) Analysis of random cell motility. (Left) Traces from individual cells are shown (control, GDE2-depleted, and GDE2-rescued cells, as indicated). Tracks were generated from time-lapse movies. Images were captured every 10 min for 12 hr. (Right) Quantification of mean migration distances (n ≥ 60 cells, mean ± SEM). **p < 0.01, ***p < 0.0001, unpaired t test.

(I) (Left) Heatmap showing differentially expressed genes upon GDE2 silencing in Shep2 cells as determined by whole-genome RNA-seq (cutoff: log2 fold change >1.5; p < 0.001). Experiments were performed in duplicate, as indicated. For the complete list of genes with gene ontology (GO) annotations, see Table S1. (Right) Bar graph shows the GO terms that are significantly enriched among the differentially expressed genes. Transmembrane receptor signaling includes pathways mediated by G-protein-coupled receptors, receptor protein tyrosine kinases, TGF-β, Wnt, and Hedgehog.

See also Table S1.
knocked out GPC6 using CRISPR/Cas9-based genome editing (Figures 7F, S5A, and S5B). CRISPR/Cas9-mediated GPC6 knockout led to even more prominent neurite outgrowth than was observed with shRNA (Figure 7F). Neurites induced upon GPC6 silencing were fully resistant to LPA-induced contraction (results not shown). In addition, GPC6 depletion resulted in enhanced cell-matrix adhesion and reduced cell motility (Figures 7G and 7H). It thus appears that loss of GPC6 function is phenotypically equivalent to GDE2 overexpression. We also examined a possible contributing role of GPC3 in regulating neurite outgrowth using cells (IMR-32) that express both GPC3 and GPC6 (Figures S4E and S4F), but found no effect of GPC3 depletion on neurite formation (Figures S5C and S5D).

Finally, low GPC6 expression correlated significantly with favorable outcome in neuroblastoma patients (Figure 8A); no significant correlation was found for GPC3 (Figure S6). When GDPD5 and GPC6 expressions were combined, patients classified as GDPD5<sup>high</sup>/GPC6<sup>low</sup> showed the best disease outcome, whereas the GDPD5<sup>low</sup>/GPC6<sup>high</sup> group had the poorest outcome (Figure 8B). This result is consistent with the functional interaction between GDE2 and GPC6 observed in neuroblastoma cell culture.

DISCUSSION

In this study, we have identified and characterized transmembrane ecto-phosphodiesterase GDE2 as an inducer of neuroblastoma differentiation and as a powerful prognostic marker. We find that GDE2, previously shown to promote neurogenesis (Rao and Sockanathan, 2005), acts through an enzymatic mechanism involving cleavage of the GPI-anchor of GPC6. In the simplest model compatible with our findings, GDE2 releases GPC6 from the cell surface to modulate the activity of an as yet unknown transmembrane effector (Figure 8C), which leads to Rac activation, suppression of RhoA activity, and altered gene expression. Together, these events direct neuroblastoma differentiation as evidenced by neurite outgrowth, increased cell-matrix adhesion, reduced cell motility, and blockade of agonist-induced neurite retraction. The set of GDE2-regulated genes was markedly enriched in those involved in neuronal differentiation, cell-matrix adhesion, and transmembrane receptor signaling. Future studies should assess whether a subset of these genes contributes to the GDE2-induced phenotypes and/or may represent a gene signature predictive of clinical outcome.

Although GDE2 can also release GPC3, and GPC3 has been implicated in the progression of various types of cancer (Filmus and Capurro, 2013; Han et al., 2016; Peters et al., 2003), we find that (1) GPC3 is not expressed two of our model neuroblastoma cell lines (SH-SY5Y and N1E-115), (2) GPC3 knockdown does not affect neurite outgrowth in IMR-32 cells, and (3) GPC3...
expression levels, unlike those of GPC6, do not correlate with clinical outcome. Nevertheless, it will be interesting to explore whether and how GDE2-mediated release of GPC3 may regulate signaling pathways and cellular phenotypes in other types of cancer or during development.

The cell-autonomous GDE2-GPC6 signaling axis underlying neuroblastoma differentiation contrasts with the non-cell-autonomous GDE2-RECK-Notch pathway that promotes neurogenesis in the spinal cord (Park et al., 2013; Sabharwal et al., 2011). Our efforts to detect RECK release (Park et al., 2013) by human GDE2 were unsuccessful, suggestive of differential substrate preferences among vertebrate GDE2 enzymes. Moreover, GDE2 was unable to release GPCs 1, 2, 4, and 5, indicating that GDE2 is highly selective toward GPI-anchored substrates under our experimental conditions. Some caution is needed, however, since the GDE2 activity assays were conducted in HEK293 cells, and the availability of GPI-anchored substrates may depend on local membrane lipid organization and structure (Raghupathy et al., 2015). In addition, GDE2 substrate selectivity may be dictated by structural modifications in the oligosaccharide core of the respective GPI anchors and/or, in the case of GPCs, by the nature of the heparan sulfate chains located close to the cell surface (Veugelers et al., 1999), and hence may physically interact with the GDE2 ectodomain. We identified His233 as key catalytic residue of GDE2, but as yet remains unclear whether GDE2 acts as a PLC- or PLD-type phosphodiesterase.

Regardless of how GDE2 selects and hydrolyzes its GPI-anchored substrates, the evidence from the present and previous studies (Park et al., 2013) suggests that GDE2 may signal in both cell-autonomous and non-cell-autonomous modes to induce neuronal differentiation, depending on cell and tissue context.

Our findings raise a number of new questions. Foremost, how does GDE2-induced cleavage of GPC6 at the cell surface activate downstream signaling events? In other words, what is the GPC6-regulated transmembrane effector? Despite many advances in heparan sulfate proteoglycan research, our understanding of GPC outside-in signaling is still very limited, and that of GPC6 in particular. In fact, GPC6 is the least studied and arguably the most enigmatic GPC family member. Where studied, GPCs can bind specific ligands, including Wnt and Hedgehog proteins, and present them to cognate receptors. However, the emerging picture of GPC-regulated Wnt signaling is complex, involving both canonical and non-canonical signaling cascades, and to date has not led to a unifying model; moreover, findings are sometimes contradictory (Capurro et al., 2005, 2014; Yiu et al., 2011). Whatever the possible role of GPC6 in Wnt signaling, the finding that GDE2 acts cell autonomously, not requiring nearby cells or extrinsic factors, seems difficult to reconcile with a role for secreted Wnt ligands in GDE2-induced neuronal differentiation; however, we cannot formally rule out a possible cooperation with secreted ligands such as Wnt.

Interestingly, emerging evidence suggests that GPCs can also function as ligands themselves by binding to transmembrane receptors, namely type II receptor protein tyrosine phosphatases (RPTPs) (Coles et al., 2011, 2015). Type II RPTPs influence...
Figure 7. GPC6 Silencing Phenocopies GDE2 Overexpression and Correlates with Favorable Outcome

(A) Glypican mRNA expression (relative to GAPDH) in SH-SY5Y cells (qPCR analysis). Error bars denote mean ± SEM (triplicate measurements).

(B) (Left) Cell-surface expression of GDE2-mCherry before (black) and after (red) doxycyclin (Dox) treatment of SH-SY5Y cells, as detected by flow cytometry. (Right) Cell-surface expression of GPC6 in GDE2-negative (black) and GDE2-positive (red) SH-SY5Y cells, as detected by flow cytometry. Control or Dox-induced (legend continued on next page)
neuronal morphology and have multiple intracellular binding partners, including regulators of Rac/Rho activity (Chagnon et al., 2010; Coles et al., 2015). Heparan sulfate proteoglycans can directly interact (in cis) with the ectodomain of RPTPα to modulate RPTP oligomerization and transmembrane signaling (Coles et al., 2011, 2015). Loss of a GPC ligand might then lead to altered RPTP clustering at the plasma membrane with a consequent change in localized levels of tyrosine phosphorylated proteins involved in neuronal differentiation, cytoskeletal organization, and cell-matrix adhesion (Figure 4I and Table S1).

Figure 8. Prognostic Significance of GPC6 and Signaling Scheme of GDE2
(A) Kaplan-Meier analysis of overall survival in a set of 498 neuroblastoma patients (RPM cohort). Patients were classified using the same GPC6 cutoff value. See also Figure S6.
(B) Kaplan-Meier analysis based on the combined expression status of GDPD5 and GPC6 (AMC patient cohort).
(C) Proposed GDE2 signaling scheme. In this model, GDE2 acts through GPI-anchor cleavage of GPC6, leading to GPC6 release from the cell surface with consequent loss of interaction with an as yet unknown transmembrane effector or receptor. This leads to Rac1 activation, increased cell adhesion and spreading, neurite outgrowth, reduced cell motility, and inhibition of LPA-G12/13−RhoA-driven neurite retraction. Additionally GDE2 regulates a unique set of genes involved in neuronal differentiation, cytoskeletal organization, and cell-matrix adhesion (Figure 8C). GDE2 cell-surface expression may be regulated by a Rab11-driven endocytic recycling pathway (Figures S1G and S1H), as illustrated. See text for further details.

Rab11- and Trfr-positive recycling endosomes, supporting a model in which GDE2 activity is regulated at the level of cell-surface expression by membrane trafficking (Figure 8C). Interestingly, a recent study supports a scenario in which GDE2 is regulated by intracellular trafficking (Yan et al., 2015). Regulation of GDE2 activity by C-terminal tail phosphorylation is another plausible scenario that warrants further investigation (Topanurak et al., 2013).

GDE2 cells were labeled with secondary antibody alone (second Ab) or anti-GPC6 antibody. Bar graph shows quantification (mean ± SEM) from three independent experiments. *p < 0.05, paired t test.
(C) GPC6 knockdown in SH-SYSY cells as determined by qPCR. GPC6 mRNA levels were normalized to GAPDH. **p < 0.01, ****p < 0.001, unpaired t test.
(D) Western blot showing that GPC6 depletion increases basal and LPA-stimulated Rac activity (SH-SY5Y cells). Bar graph shows quantification of relative Rac-GTP levels in the presence or absence of LPA (mean ± SEM of three independent experiments). *p < 0.05, unpaired t test.
(E) GPC6 depletion leads to longer neurites (arrowheads) in SH-SY5Y cells. Scale bars, 100 μm. Bar graph shows quantification of neurite length in control versus GPC6 knockdown cells (n = 250, mean ± SEM). ****p < 0.0001, unpaired t test.
(F) CRISPR/Cas9-mediated GPC6 knockout promotes neurite outgrowth in SH-SY5Y cells (black arrows). Scale bars, 100 μm. Bar graph shows percentage of neurite-bearing cells in CRISPR-GPC6 knockout cells (n ≥ 500 cells, n = 11 colonies, mean ± SEM). ****p < 0.0001.
(G) shControl and shGPC6 were plated on fibronectin or laminin. At 1 hr after plating, adherent cells were fixed and stained with crystal violet for quantification. Error bars denote mean ± SEM of two independent experiments. *p < 0.05, unpaired t test.
(H) Quantification of average distances of neurite-bearing cells in CRISPR-GPC6 knockout cells (n ≥ 500 cells, n = 11 colonies, mean ± SEM). ****p < 0.0001, **p < 0.001, unpaired t test.

See also Figure S5.
The identification of GDE2 as an inducer of neuroblastoma cell differentiation and as a favorable prognostic marker is consistent with the finding that patient survival is largely dependent on the degree of neuronal differentiation (Louis and Shohet, 2015). New therapeutic approaches are urgently needed in neuroblastoma, but their development has been hampered by the paucity of tractable oncogenic drivers (Molenaar et al., 2012; Pugh et al., 2013) and an incomplete understanding of neuroblastoma differentiation. In this respect, the present results open up possibilities for exploring new therapeutic approaches. In particular, pharmacological stimulation of GDE2 activity could be a promising strategy, not least because exo-/ecto-phosphodiesterases are convenient drug targets. Therefore, the development of drug-like allosteric activators of GDE2 could be valuable for targeted differentiation therapy in neuroblastoma. Further structural and preclinical studies will be needed to confirm the feasibility and efficacy of this approach.

EXPERIMENTAL PROCEDURES

Human mRNA Datasets and Survival Analysis
All mRNA gene expression analyses were performed within R2: genomics analysis and visualization platform (http://r2.amc.nl). We have made use of the following publicly available datasets: Neuroblastoma Oberthuer (ArrayExpress: E-TABM-38), Neuroblastoma SEQC (GEO: GSE62564), and Neuroblastoma Versteeg (GEO: GSE16476 88/122). Kaplan scanning was performed within R2 (http://r2.amc.nl). In brief, for each gene or other numerical characteristic R2 calculates the optimal cutoff expression level, dividing the patients in a good and bad prognosis cohort. Samples within a dataset are sorted according to the expression of the investigated gene and divided into two groups on the basis of a cutoff expression value. All cutoff expression levels and their resulting groups are analyzed for survival, with the provision that minimal group number is eight (or any other user-defined value) samples. For each cutoff level and grouping, the log-rank significance of the projected survival is determined according to the expression of the investigated gene and divided into two groups on the basis of a cutoff expression value. All cutoff expression levels and their resulting groups are analyzed for survival, with the provision that minimal group number is eight (or any other user-defined value) samples. For each cutoff level and grouping, the log-rank significance of the projected survival is calculated as described by Bewick et al. (2004). The best p value and corresponding cutoff value is selected. This cutoff level is reported and used to generate Kaplan-Meier graphs.

Materials, Cell Culture, Antibodies, and Expression Vectors
Reagents, cell culture, expression vectors, and transfection protocols are described in Supplemental Experimental Procedures. A GDE2 polyclonal antibody was raised against the very C-terminal tail of human GDE2 (sequence MVRHQPLQYYEPQ) and affinity purified, as described in Supplemental Experimental Procedures. A GDE2 polyclonal antibody was raised against the very C-terminal tail of human GDE2 (sequence MVRHQPLQYYEPQ) and affinity purified, as described in Supplemental Experimental Procedures. A GDE2 polyclonal antibody was raised against the very C-terminal tail of human GDE2 (sequence MVRHQPLQYYEPQ) and affinity purified, as described in Supplemental Experimental Procedures.

Cell Adhesion and Motility Assays
Cell adhesion and random cell motility were measured as detailed in Supplemental Experimental Procedures.

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