GDE transmembrane ecto-enzymes : novel signaling functions through GPI-anchor cleavage

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Chapter 5

GDE2 and GDE3: differences in regulation and substrate recognition

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Abstract

The glycosylphosphatidylinositol (GPI) anchor is a C-terminal protein modification that tethers proteins to the plasma membrane. Because of their very nature, GPI-anchored proteins are unlikely to be internalized like classical transmembrane receptors to shut off their activities. Instead, they can be cleaved by GPI-specific phospholipases. Recent studies have identified a family of enzymes, the so called GDEs, that selectively cleave and release GPI-anchored proteins; yet it remains unknown how they are regulated and recognize their specific substrates. Here we report that GDE2 and GDE3, when co-expressed, co-localize at the plasma membrane but are differentially regulated, with GDE2, but not GDE3, being detected at Rab11-positive vesicles. We find that the GDE2 N- and C-terminus are both involved in vesicle localization, while the extracellular catalytic domain determines substrate recognition. Swapping the respective GPI-attachment signals had no effect on substrate specificity. Although preliminary, our findings suggest that GDE2 and GDE3 are differentially regulated and that substrate specificity is largely determined by the protein rather than the attached PI lipid moiety.

Introduction

In eukaryotic cells, proteins can span the membrane or be tethered to the outer membrane leaflet via a glycosylphosphatidylinositol (GPI) anchor. This unique and complex modification is attached to the protein in the endoplasmic reticulum (ER) before being transported to the plasma membrane [29]. The class of GPI-anchored proteins is very diverse, ranging from enzymes to receptors, ligands, complement factors and adhesion molecules [146]. Consequently, impairments in the GPI-anchor due to acquired mutations can cause diseases like paroxysmal nocturnal hemoglobinuria [261, 262]. Because GPI-anchored proteins do not share a common function, the anchor itself appears to attribute a general feature to the proteins; one of which is the cleavage by phospholipases [263]. A bacterial phospholipase C (PI-PLC) cleaves GPI-anchored proteins from the membrane, corroborating the function of GPI anchors as substrates for phospholipases [64]. However, mammalian GPI-specific phospholipases have long been elusive [4, 69, 76, 264]. Recent studies have identified two members of a family of glycerophosphodiester phosphodiesterases (GDEs), that cleave GPI-anchored proteins from the membrane; notably GDE2 and GDE3 [17, 110, 116]. GDE2, the best characterized family member, cleaves the GPI-anchored protein RECK [110]. By cleaving RECK in the developing spinal cord, GDE2 indirectly inactivates a Notch ligand (Delta-like1). As a consequence, Notch signaling is inactivated in adjacent progenitor cells to initiate differentiation. GDE2 also promotes differentiation of neuroblastoma cells in a cell-autonomous way, through cleavage and release of glypican-6 [17]. GDE2 activity is specific for glypican-6 and glypican-3, leaving other glypicans unaffected [17]. GDE2's closest family member, GDE3, was recently shown to cleave uPAR [116]. GDE3 cleaves uPAR in a phospholipase-C like manner. Homology modeling of GDE2 and GDE3 revealed striking differences in electrostatic surface properties [116]. Together, these results suggest that GDE2 and GDE3 have evolved to selectively cleave and release GPI-anchors proteins.

A possible clue as to how GDEs recognize different substrates is the structural heterogeneity of the GPI-anchor that may hinder and modulate substrate cleavage [29, 146, 147]. For example, palmitoylation of the inositol group confers resistance to a PI-PLC attack [265]. Moreover, GPI-anchoring may also affect protein localization to the apical or basolateral membrane, as well as modulating protein clustering [48, 266-268].

Here we examine the substrate specificity of GDE2 and GDE3 by analyzing their localization and how the GPI anchor may dictate substrate recognition. We show that GDE2 and GDE3 co-localize on the plasma membrane but are differentially regulated. Replacing the N-terminus of GDE2 with GDE3 affected endosome localization but not substrate recognition. By contrast, swapping the GDE2 and GDE3 ecto-domains did affect substrate specificity. Furthermore, replacing the GPI anchors of GDE substrates had no effect on their specific release. Together our results support the notion that GDE's cleave GPI-anchored proteins through specific protein-protein interactions.

Results

GDE2 and GDE3 co-localize at the plasma membrane but are differentially regulated

GDE2 and GDE3 have a different tissue distribution and have specific substrates [101, 116]. We have previously shown that GDE2 localizes to intracellular vesicles in N1E-115 cells but GDE3 localization remains to be determined [17]. When expressed at relatively low levels in N1E-115 cells – a well characterized neuroblastoma cell line that we have used in previous GDE2 studies – GDE2 and GDE3 co-localize to distinct microdomains at the plasma membrane (Figure 1A). The localization of substrates glypican 6 and uPAR was analyzed to assess if localization would affect substrate specificity. When expressed in N1E-115 cells, glypican 6 and uPAR co-localize to the same membrane domains (Figure S1), arguing against localization-dependent substrate specificity. We next assessed if GDE2 and GDE3 may also co-localize intracellularly. We co-transfected GDE2 (GFP- or HA-tagged) with GDE3 (mCh- or GFP-tagged) in HEK293 and N1E-115 cells. A relatively large pool of GDE2 was detected in intracellular vesicles. Remarkably, however, GDE3 was not detected in these vesicles, as shown by confocal microscopy (Figure 1B, C) and super-resolution microscopy (Figure S2A). Quantification of their relative localization, using an imageJ macro, is shown in Figure S2B. Consistent with this, intracellular GDE2 co-localized with Rab11 but GDE3 did not (Figure 1D), suggesting that surface expression of both enzymes is differentially regulated.

Differences in GDE localization do not determine enzymatic activity or substrate specificity

GDE2 and GDE3 contain intracellular N- and C-termini that might be involved in regulatory processes. To test whether these cytosolic tails determine localization, enzyme activity or/and substrate specificity; we made use of chimeric constructs. We swapped the N- and C-terminal tails of GDE2 with those of GDE3 and analyzed the localization of the chimeric proteins in N1E-115 cells. Wild-type GDE2 and GDE2 with an GDE3-C-terminal tail (GDE2(C-GDE3)) partially localized to intracellular vesicles (Figure 2A). Surprisingly, GDE2 containing the GDE3-N-terminal tail (GDE2(N-GDE3)) or GDE3-N and -C-terminal tail, was detected only at the plasma membrane. Because altered localization could be caused by the deletion of an GDE2-specific tail, we generated additional N- and C-terminal deletion mutants; these were found to co-localize with wild-type GDE2 (Figure S3A). It thus seems that the N-terminus of GDE3 prohibits endosome localization. To assess how this might affect enzymatic activity, glypican 6 or uPAR were co-transfected with the chimeric constructs, followed by immunoblotting of the conditioned medium (Figure 2B). No differences were observed, suggesting that GDE2 recycling is not a determinant of GDE2 activity.

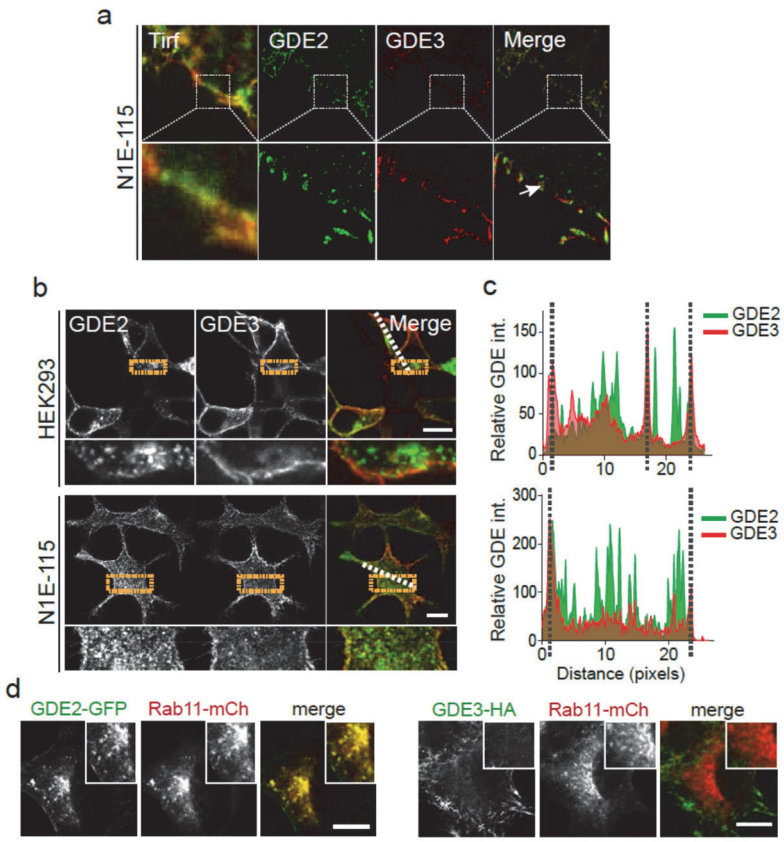


Figure 1. GDE2 and GDE3 colocalize at the plasma membrane but not in intracellular vesicles
(A) GDE2 and GDE3 co-localize to specific microdomains on the plasma membrane. Dual-color super-resolution images of N1E-115 cells, co-expressing GDE2-HA and GDE3-GFP. White arrow depicts distinct microdomain. **(B)** Confocal images of HEK293 and N1E-115 cells co-expressing GDE2-GFP and GDE3-mCh. Only GDE2 localizes to intracellular vesicles as shown by a line intensity profile **(C)**; quantification of the dashed white line. Bar,30 μm. **(D)** GDE and Rab11-mCh localization in N1E-115 cells. Bar, 10 μm.

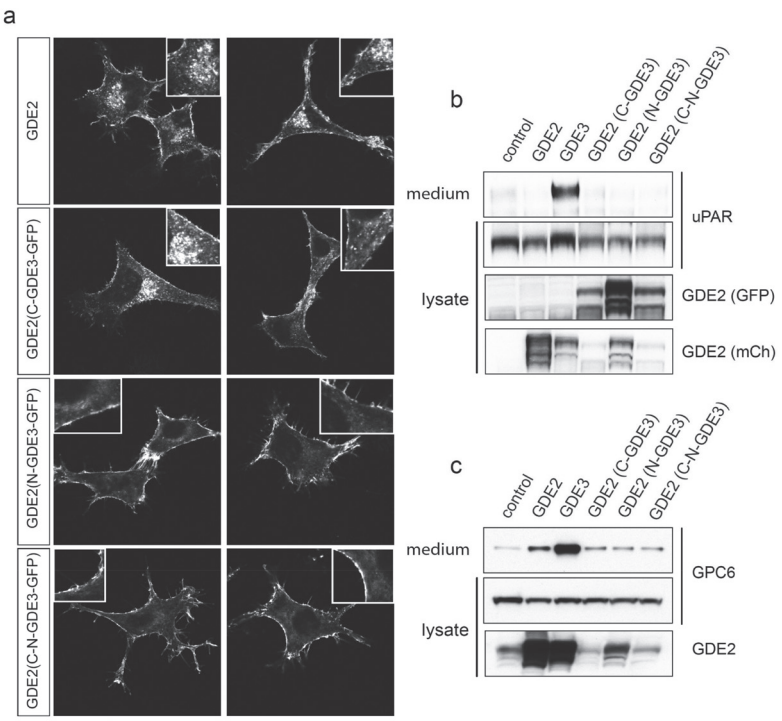


Figure 2. Swapping the N-termini of GDE2 with GDE3 prevents vesicle localization without affecting enzymatic activity

(A) Confocal images of N1E-115 cells transfected with wild-type GDE2-GFP, GDE2(C-GDE3-GFP), GDE2(N-GDE3-GFP) and GDE2(C-N-GDE3-GFP), as indicated. **(B,C)** HEK293 cells were co-transfected with either glypican 6-HA or uPAR-GFP together with wild-type GDE2, GDE3 or the indicated mutants. uPAR or glypican 6 release was analyzed by immunoblotting of the conditioned medium and corresponding lysates.

The GDPD ectodomain determines GDE substrate specificity

Swapping the GDPD domains of GDE2 and GDE3 did not affect subcellular localization (Figure 3A). However, it altered its substrate specificity into that of GDE3. Of note, GDE2 containing the GDE3 GDPD domain was less efficient in cleaving uPAR compared to wild-type GDE3 (Figure 3B). Thus, other regulatory domains must contribute to catalytic activity. Unfortunately, swapping the ecto-domain of GDE3 with that of GDE2 was unsuccessful since the chimeric construct was not expressed at the plasma membrane (Figure 3B). A possible explanation could be misfolding or protein instability.

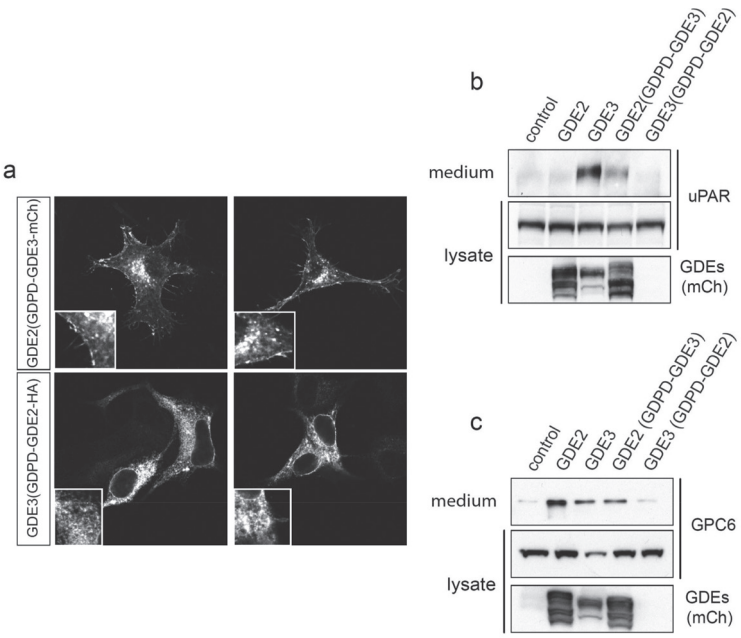


Figure 3. GDE2 substrate selectivity is dictated by the extracellular GDPD domain
(A) Confocal images of N1E-115 cells were transfected with GDE2(GDPD-GDE3-mCh), or GDE3(GDPD-GDE2-HA), as indicated. **(B,C)** HEK293 cells were co-transfected with either glypican 6-HA or uPAR-GFP together with wild-type GDE2, GDE3 or the indicated mutants. uPAR or glypican 6 release was analyzed by immunoblotting of the conditioned medium and corresponding lysates.

GDE substrate selectivity does not depend on the GPI-attachment signal

We next asked what determines substrate specificity. To address this, we focused on glypican-6 and uPAR as substrates. Previous studies have shown that the GPI anchor is a requisite for cleavage by GDE's [110, 116]. Because modifications in the GPI anchor core structure can affect protein clustering or cleavage by phospholipases, we swapped the GPI-anchor attachment signal of uPAR with that of glypican 6 and tested substrate release by GDE's (Figure 4) [48, 266-268]. The uPAR protein consists of three domains (I-III) with a small disordered region at the C-terminus. At the N-terminus, a signal peptide targets the premature protein to the endoplasmic reticulum were a C-terminal signal is responsible for GPI-anchor attachment at the ω-site (Figure 4A, B). Two chimeric constructs were generated: uPAR with the propeptide of glypican 6 and uPAR with the propeptide plus the disordered region replaced by glypican 6 (Figure 4C); which did not affect localization (data not shown). To test release by GDE2 and GDE3, HEK293 cells were co-transfected with uPAR or the chimeric constructs. GDE2 was unable to release any of the chimeric constructs (Figure 4D). It thus appears that GDE substrate recognition does not simply depend on the respective GPI anchors.

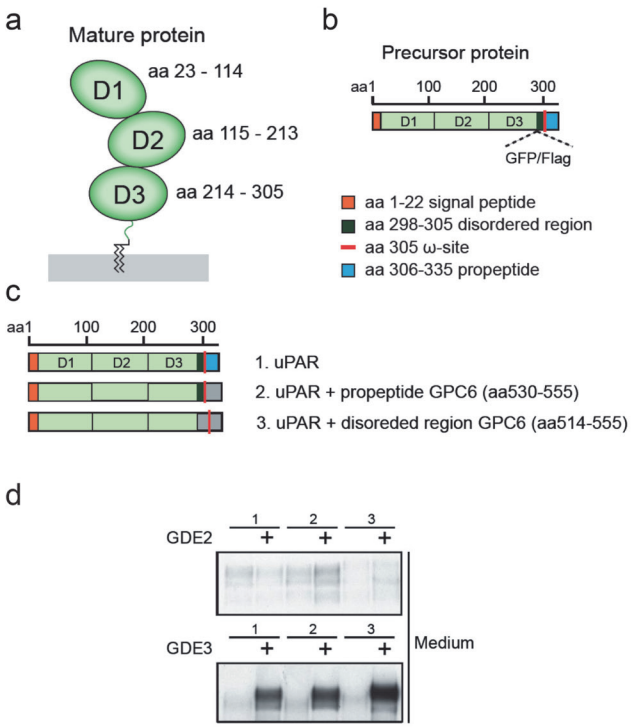


Figure 4. GDE substrate selectivity does not simply depend on GPI-anchor heterogeneity **(A)** Schematic representation of the mature uPAR protein as expressed on the plasma membrane. **(B)** Schematic representation of the immature uPAR protein with the N-terminal signal peptide and the C-terminal GPI-attachment signal. **(C)** Illustration of wild-type and glypican 6 chimeric uPAR constructs, as indicated. The GPI-attachment signal was replaced with that of glypican 6. **(D)** HEK293 cells were co-transfected with either uPAR-GFP or a chimeric construct (1,2 or 3) together with wild-type GDE3 (+) or an empty vector control. uPAR release was analyzed by immunoblotting of the conditioned medium.

Discussion

In this preliminary report, we show that GDE2 and GDE3 co-localize to specific domains at the plasma membrane but are differentially regulated. Remarkably, GDE2 cleaves only a subset of the GPI-anchored proteins released by GDE3. While exchange of the GDPD domain of GDE2 with that of GDE3 resulted in the cleavage of uPAR (a substrate for GDE3 but not GDE2), it was not equally efficient as GDE3, suggesting that other domains are involved in regulating GDE2 activity. Since vesicular localization is exclusive to GDE2, its activity might be regulated through recycling and membrane expression. Moreover, transmembrane receptors and adhesion molecules are known to be internalized to regulate their activity,

where the C-terminal tail is often used as a docking site for adapter proteins [269, 270]. Interestingly, while GDE2 and GDE3 share a high-level sequence similarity, their C-termini are not conserved [116]. To test whether the C-terminus is involved in GDE2 internalization or recycling, we made use of deletion mutants. Unfortunately deleting the N or C-terminus had no effect on GDE2 activity (data not shown) or vesicular localization. Interestingly, a previous study suggested that GDE2 activity is regulated through the cleavage of an intracellular disulfide bond between the N and C-terminus [118]. By contrast, the present deletion mutants together with an earlier study - where we mutated the respective cysteine residues - suggest that GDE2 activity is not regulated in this way [17]; but it should be noted that deletion of the complete GDE2 C-terminal tail resulted in a misfolded protein. Therefore, the remaining amino acids could still be essential for its recycling or internalization. However, this is unlikely since swapping the full-length C-terminal tail with GDE3 – which does not localize to intracellular vesicles - had no effect. Our data suggest that the N-terminus of GDE3 prevents GDE2 internalization (or recycling). If so, replacing the N-terminus of GDE3 with GDE2 should mimic GDE2 localization. Further studies should address if membrane expression levels alone, or other factors like disulfide bridges in the ectodomain [119], affect GDE2 enzymatic activity.

Selective cleavage by GDE2, in contrast to bacterial PI-PLC, suggests a higher order substrate recognition beyond the core GPI-anchor itself [17, 110, 116]. Specific GPI-anchor modifications could serve this function by dictating the sensitivity of GPI-anchored proteins to a GDE2 attack [146, 147]. This is an attractive hypothesis since it would allocate multiple regulatory steps to the specific cleavage by GDE's. To test this, we swapped the GPI-anchor attachment signal, and the 5'prime unstructured region of uPAR with glypican 6. We next assessed cleavage by GDE2, but without success. We therefore conclude that GDE2 cleaves GPI-anchored proteins through specific protein-protein interactions. A notion that is supported by homology modeling of GDE2 and GDE3, revealing striking differences in electrostatic surface properties [116]. Future structural studies should address the question how protein-protein interaction contribute to GDE2 substrate specificity.

Experimental Procedures

Cell Culture and Materials

HEK293 and N1E-115 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO2. Antibodies used: anti-HA from Sigma; anti-mCh and anti-GFP (home-made). Bacterial PI-PLC from Molecular Probes. Transient transfections were done using a calcium phosphate protocol or XtremeGene 9 agent (Roche).

Expression Vectors

Human cDNA GDE2 and GDE3 were subcloned into a pcDNA3 (-mCherry and –EGFP) plasmid as previously described [17, 116]. The pCAGGS-HA-glypican 6 was kindly provided by Dr. Sungjin Park (Johns Hopkins Medical School, Baltimore, USA) and uPAR-GFP is previously described [153]. GDE2 N and C-terminal truncation mutants were generated by pcr amplification. Swapping the GDE2 and GDE3 GDPD domain or C and N-terminus (as annotated by uniprot; Q8WTR4 and Q9HCC8) was done by pcr amplification. Thereafter, the GDPD ecto-domain was cloned into the GDE backbone using Gibson assembly (Gibson Assembly Cloning kit, New England Biolabs). For the uPAR-GFP chimeric constructs, uPAR; aa1-aa305 and aa1-aa298 was fused to glypican 6 aa530-aa555 and aa514-aa555 respectively.

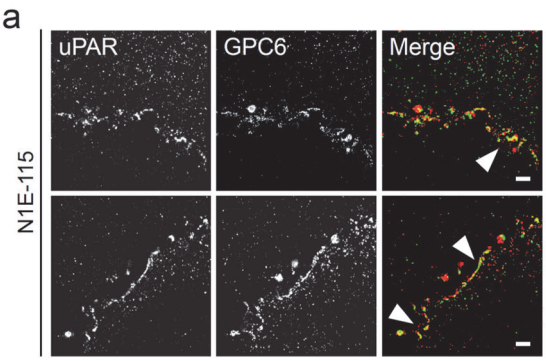
Western Blotting

For Western blotting, cells were washed with cold PBS, lysed in RIPA buffer supplemented with protease inhibitors followed by a centrifugation step. A BCA protein assay kit (Pierce) was used tom measure protein concentration and LDS sample buffer (NuPAGE, Invitrogen) was added to the lysate or medium. Samples were loaded on SDS-PAGE pre-cast gradient gels (4-12% Nu-Page Bis-Tris, Invitrogen) followed by transfer to nitrocellulose membrane. Membranes were blocked by 5% skimmed milk in TBST followed by overnight incubation with the primary antibodies at 4° C in TBST supplemented with 2% skimmed milk. Horseradish-peroxidase conjugated secondary antibodies (DAKO, Glostrup, Denmark) were incubated for 1 hr at room temperature; ECL Western blot reagent was used to detect proteins.

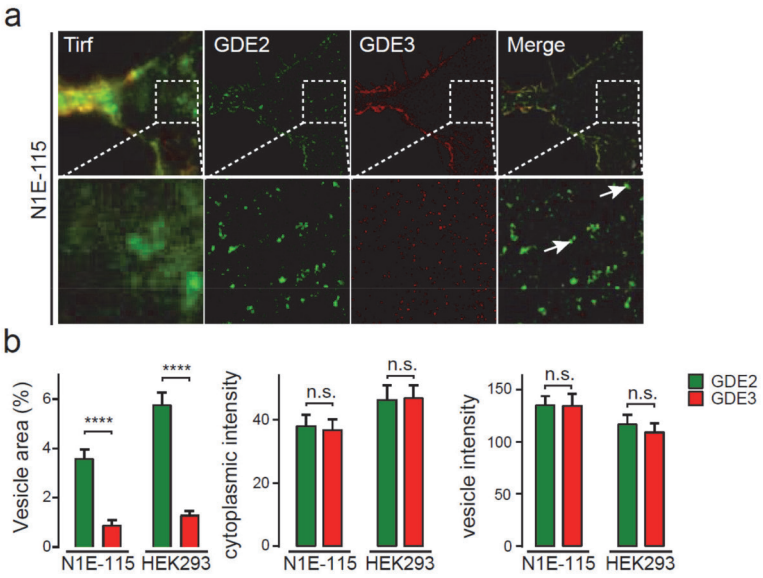
Microscopy

Cells were cultured on 24 mm, #1,5 coverslips, washed and fixed with 4% PFA, permeabilized with 0.1% Triton X-100 and blocked with 2% BSA for 1 hr. Incubation with primary antibodies was done for 1 hr followed by incubation with Alexa-conjugated antibodies. For confocal microscopy, cells were washed with PBS, mounted with Immnuno-MountTM (Thermo Scientific) and visualized on a LEICA TCS-SP5 confocal microscopy (63 x objective). The total vesicle area of GDE2 and GDE3 was measured using confocal pictures of co-transfected N1E-115 cells. An imageJ macro analyzed bright spots in a predefined region representing the intra-cellular area. Background intensity and vesicle intensity were measured as control. Super-resolution imaging was done using a SR-GSD Leica microscope equipped with an oxygen scavenging system, as previously described [137]. In short, 15000 frames were taken in TIRF mode, at 10 ms exposure time. After post image analysis, movies were analyzed and corrected using the ImageJ plugin Thunderstorm (http://imagej.nih.gov/ij/) followed by correction with an ImageJ macro using the plugin Image Stabilizer.

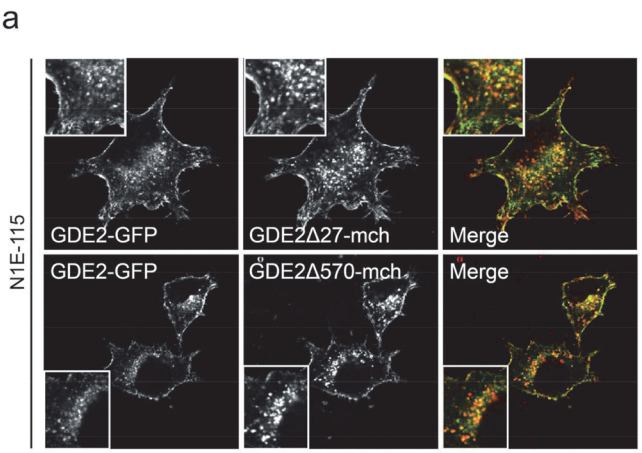
Supplementary figures



Supplementary Figure 1. Membrane localization of glypican 6 and uPAR
(A) Membrane localization of glypican 6-HA and uPAR-GFP in N1E-115 as detected by super-resolution microscopy. White arrows depict distinct microdomains at the plasma membrane. Bar, 1 µm.



Supplementary Figure 2. GDE2 and GDE3 subcellular localization
(A) Localization of GDE2-HA and GDE3-GFP in N1E-115 cells as detected by dual color super-resolution microscopy. White arrows point to intracellular vesicles **(B)** Quantification of the total vesicle area of GDE2 and GDE3 in HEK293 and N1E-115 cells. Vesicle area was measured using an ImageJ macro that detects bright vesicles in confocal microscopy images. Cytoplasmic intensity and vesicle intensity were measured as background control (mean ± SEM). **** p value < 0.0001 (unpaired t-test).



Supplementary Figure 3. Localization of N- and C-terminal truncated GDE2 constructs
(A) Confocal images of N1E-115 cells co-transfected with wild-type GDE2-GFP together with GDE2Δ27-mCh or GDE2Δ570-mCh, as indicated.