Altered Met receptor phosphorylation and LRP1-mediated uptake in cells lacking carbohydrate-dependent lysosomal targeting

Megan Aarnio-Peterson, Peng Zhao, Seok-Ho Yu, Courtney Christian, Heather Flanagan-Steeet, Lance Wells, and Richard Steet

From the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602

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Acid hydrolases utilize a carbohydrate-dependent mechanism for lysosomal targeting. These hydrolases acquire a mannose 6-phosphate tag by the action of the GlcNAc-1-phosphotransferase enzyme, allowing them to bind receptors and traffic to endosomes. Loss of GlcNAc-1-phosphotransferase results in hydrolase hypersecretion and profound lysosomal storage. Little, however, is known about how these cellular phenotypes affect the trafficking, activity, and localization of surface glycoproteins. To address this question, we profiled the abundance of surface glycoproteins in WT and CRISPR-mediated GNPTAB−/− HeLa cells and identified changes in numerous glycoproteins, including the uptake receptor LRP1 and multiple receptor tyrosine kinases. Decreased cell surface LRP1 in GNPTAB−/− cells corresponded with a reduction in its steady-state level and less amyloid-β-40 (Aβ40) peptide uptake. GNPTAB−/− cells displayed elevated activation of several kinases including Met receptor. We found increased Met phosphorylation within both the kinase and the docking domains and observed that lower concentrations of peroxanadate were needed to cause an increase in phospho-Met in GNPTAB−/− cells. Together, these data suggested a decrease in the activity of the receptor and non-receptor protein tyrosine phosphatases that down-regulate Met phosphorylation. GNPTAB−/− cells exhibited elevated levels of reactive oxygen species, known to inactivate cell surface and cytosolic phosphatases by oxidation of active site cysteine residues. Consistent with this mode of action, peroxide treatment of parental HeLa cells elevated phospho-Met levels whereas antioxidant treatment of GNPTAB−/− cells reduced phospho-Met levels. Collectively, these findings identify new mechanisms whereby impaired lysosomal targeting can impact the activity and recycling of receptors.

Soluble acid hydrolases are targeted to the lysosome via a carbohydrate-dependent mechanism. This mechanism involves the addition of mannose 6-phosphate (M6P) tags on the N-glycans of newly made hydrolases by the heterohexameric enzyme UDP-GlcNAc:lysosomal enzyme N-acetylglucosamin-1-phosphotransferase (GlcNAc-1-phosphotransferase; encoded by GNPTAB and GNPTG genes) (1, 2). The GNPTAB gene encodes the α/β subunits of the enzyme, involved in both hydrolase recognition and catalysis, whereas the GNPTG gene encodes the γ subunit, which plays a role in facilitating efficient mannose phosphorylation and enzyme stability (3–10). Defects in GNPTAB and GNPTG cause the lysosomal diseases mucolipidosis II (MLII), MLIII α/β, and MLIII γ, characterized by hypersecretion of hydrolases, profound lysosomal storage in many mesenchymal cell types, and a broad spectrum of clinical manifestations (3, 11, 12). The majority of lysosomal enzymes reach the lysosome via M6P-dependent targeting but additional tissue- and cell type–specific mechanisms of carbohydrate independent sorting have been shown to exist (13). Lysosomal hydrolases can also be targeted to lysosomes via secretion and recapture. Receptors including LDL receptor and LRP1 have been implicated in trafficking of nonphosphorylated cathepsin D and B, and can function in the absence of GlcNAc-1-phosphotransferase (14).

Although lysosomal storage remains the hallmark of MLII, relatively little is known about how the localization and function of cell surface glycoproteins are affected upon the loss of lysosomal targeting. Several studies have reported abnormal recycling and trafficking of cell surface glycoproteins in cells with lysosomal storage (15–19). These trafficking defects can arise from multiple potential mechanisms, including the secondary storage of glycolipids or other molecules that interfere with the vesicle trafficking machinery or alter processes such as endocytosis or autophagy. Endosomal accumulation of glycoproteins was directly linked to abnormal recycling caused by cholesterol storage (15). Cell surface glycoproteins are also susceptible to the action of extracellular glycosidases, which mediate cell surface glycoprotein turnover (20). Thus, higher levels of secreted glycosidases in GNPTAB−/− cells because of loss of M6P targeting may result in increased processing and altered cell surface residence of glycoproteins by affecting their interaction with galectins or other factors (21, 22). Defining these mechanisms is likely to provide important new clues to the molecular pathogenesis of lysosomal diseases.

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This article contains supplemental Tables S1–S3.

1 To whom correspondence should be addressed: 315 Riverbend Road, Athens, GA 30602. Tel.: 706-583-5550; Fax: 706-542-4412; E-mail: rstee@crrc.uga.edu.

2 The abbreviations used are: M6P, mannose 6-phosphate; ML, mucolipidosis; SEEL, selective exo-enzymatic labeling; p-Met, phospho-Met; ROS, reactive oxygen species; DPBS, Dulbecco’s phosphate-buffered saline; RTK, receptor tyrosine kinase; Aβ40, amyloid-β-40.
In this study, we took advantage of chemical glycobiology methods to profile the abundance of cell surface glycoproteins in parental and CRISPR-mediated GNPTAB\(^{-/-}\) HeLa cells in an effort to identify how loss of M6P-dependent lysosomal targeting impacts these glycoproteins. Our findings reveal changes in the abundance of multiple cell surface glycoproteins, including reduced levels of the uptake receptor LRP1 and receptor tyrosine kinase Met. Characterization of the functional consequences and molecular basis for these changes revealed new mechanisms of action whereby loss of lysosomal targeting and lysosomal storage alter receptor activity and receptor-mediated uptake.

Results

Selective exo-enzymatic labeling (SEEL)-based profiling of GNPTAB\(^{-/-}\) HeLa cells reveals altered cell surface residence of multiple glycoprotein receptors

To address how loss of lysosomal hydrolyase targeting affects cell surface sialoglycoproteins, their abundance was profiled in parental HeLa, GNPTG\(^{-/-}\), and GNPTAB\(^{-/-}\) cells using one-step SEEL with the sialyltransferase ST6Gal1 (7, 23, 24). Cells were labeled with recombinant rat ST6Gal1 and biotinylated CMP-sialic acid in the presence or absence of neuraminidase and lysates by Western blot analysis. Differences were noted in the profile of the major labeled glycoproteins, with the most striking changes noted between parental HeLa and GNPTAB\(^{-/-}\) cells (Fig. 1A). Higher reactivity was detected in GNPTAB\(^{-/-}\) cells treated without neuraminidase, likely reflecting an increase in free terminal galactose acceptors. GNPTAB\(^{-/-}\) cells also exhibited greater heterogeneity and increased mobility of the major labeled glycoproteins in the presence of neuraminidase.

Following neuraminidase-coupled SEEL, biotinylated glycoproteins were enriched by immunoprecipitation, resolved by SDS-PAGE, and subjected to proteomic analysis by LC-MS-MS (Fig. 1B). In light of the differences noted in the profile of labeled glycoproteins, we focused the proteomic analysis on the parental and GNPTAB\(^{-/-}\) lines (Fig. 1C; see supplemental Table S1). Consistent with the ability of SEEL to only modify glycoproteins at the cell surface, the vast majority of assigned proteins were consistent with the ability of SEEL to only modify glycoproteins at the cell surface, the vast majority of assigned proteins were
**Increased Met receptor phosphorylation in GNPTAB**

**cells**

Figure 2. Surface reduction of the reuptake receptor LRP1 results in decreased ligand uptake in GNPTAB−/− cells. A, representative β-LRP1 immunoblot following biotin immunoprecipitation of ST6Gal1 SEEL–labeled parental HeLa and GNPTAB−/− cells (n = 5). 20 μg of protein was loaded for input (I) and flowthrough (F) fractions, and 100 μg of protein was loaded for the elute (E) fraction. B, RT-PCR of LRP1 from parental HeLa, GNPTG−/−, and GNPTAB−/− cells with RPL4 as a control gene. C, AF647-Aβ40 cellular uptake represented in relative fluorescent units (RFU). Cells were treated with 2 μM Aβ40 for 16 h. Mean ± S.D. values obtained from four biological replicates with at least three technical replicates for each experiment. **, p < 0.01.

**Increased phosphorylation of receptor tyrosine kinases was observed in GNPTAB−/− HeLa cells**

SEEL-based proteomics identified increased cell surface abundance of multiple receptor tyrosine kinases in GNPTAB−/− cells (Fig. 1D). Although this method gives information regarding the abundance and localization of these receptors, it does not yield insight into receptor activity. To survey the phosphorylation status of receptor tyrosine kinases in the GNPTAB−/− cells, we next performed phospho-receptor tyrosine kinase (RTK) arrays that monitor the global phosphorylation status of these receptors. As shown in Fig. 3A, numerous RTKs exhibited increased phosphorylation in GNPTAB−/− cells, suggesting increased activation and/or sustained activity. Quantification by densitometry of three independent runs demonstrated a 2- to 3-fold increase in the phosphorylation of c-Met, EphA7, EphB2, and Axl in the GNPTAB−/− (Fig. 3B). Activation of EGF receptor (EGFR) was not significantly affected in GNPTAB−/− cells. FGFR1 and FGFR3 also displayed increased phosphorylation in GNPTAB−/− cells, despite the fact that neither receptor was detected at the cell surface using SEEL.

**GNPTAB−/− and GNPTG−/− exhibit increases in the steady-state level and phosphorylation of Met receptor**

The Met receptor is highly enriched in epithelial cells of several organs, including the liver and kidney, and plays a central

known cell surface glycoproteins, including receptors, cell adhesion proteins, and ion channels. For the most abundant glycoproteins, fold change between GNPTAB−/− and parental HeLa cells was plotted using a log2 scale (Fig. 1D). These data demonstrate lower cell surface abundance of multiple glycoproteins including the uptake receptor LRP1, the mucin MUC16, and the receptor tyrosine phosphatases (PTPRK and PTPRJ). Increased abundance of several receptor tyrosine kinases (AXL, MET, EPH2A) and adhesion proteins (ITGB4, L1CAM, MCAM) was also noted in GNPTAB−/− cells. This indicates that the reduced cell surface reduction, labeled lysates were immunoprecipitated with anti-biotin and LRP1 subsequently analyzed by immunoblot (Fig. 2A). These results show that the majority of LRP1 is present at the cell surface in the parental HeLa cells but less LRP1 is detected in both the input and the eluted fractions of the GNPTAB−/− cells. This indicates that the reduced cell surface abundance of LRP1 in these cells likely stems from lower overall levels of this protein. The electrophoretic mobility of LRP1 was also increased in GNPTAB−/− cells, possibly reflecting abnormal glycan or protein processing. RT-PCR was performed next to ask whether this decrease corresponded with reduced transcript abundance but no differences were noted (Fig. 2B). LRP1 not only mediates uptake and clearance of amyloid peptides in the brain (25–28), it also plays a role in M6P-independent recapture of lysosomal hydrolases. Therefore, decreased abundance of LRP1 may further exacerbate the cellular storage phenotype. To assess whether reduced LRP1 affects ligand uptake in GNPTAB−/− cells, we performed uptake assays using a fluorescently labeled amyloid-β peptide (AF647-Aβ40). Parental HeLa and GNPTAB−/− cells were incubated with AF647-Aβ40 overnight and peptide uptake measured in cell lysates. Consistent with LRP1 lower surface abundance, relative fluorescent units were 35% lower in GNPTAB−/− lysates (Fig. 2C).

**GNPTAB−/− cells exhibit reduced LRP1-dependent uptake**

SEEL analysis identified a significant decrease in the LRP1 receptor on the cell surface of GNPTAB−/− cells (Fig. 1D). This was of interest because of its proposed role in M6P-independent uptake and acid hydrolase sorting (14). To confirm LRP1 cell surface reduction, labeled lysates were immunoprecipitated with anti-biotin and LRP1 subsequently analyzed by immunoblot (Fig. 2A). These results show that the majority of LRP1 is present at the cell surface in the parental HeLa cells but less LRP1 is detected in both the input and the eluted fractions of the GNPTAB−/− cells. This indicates that the reduced cell surface abundance of LRP1 in these cells likely stems from lower overall levels of this protein. The electrophoretic mobility of LRP1 was also increased in GNPTAB−/− cells, possibly reflecting abnormal glycan or protein processing. RT-PCR was performed next to ask whether this decrease corresponded with reduced transcript abundance but no differences were noted (Fig. 2B). LRP1 not only mediates uptake and clearance of amyloid peptides in the brain (25–28), it also plays a role in M6P-independent recapture of lysosomal hydrolases. Therefore, decreased abundance of LRP1 may further exacerbate the cellular storage phenotype. To assess whether reduced LRP1 affects ligand uptake in GNPTAB−/− cells, we performed uptake assays using a fluorescently labeled amyloid-β peptide (AF647-Aβ40). Parental HeLa and GNPTAB−/− cells were incubated with AF647-Aβ40 overnight and peptide uptake measured in cell lysates. Consistent with LRP1 lower surface abundance, relative fluorescent units were 35% lower in GNPTAB−/− lysates (Fig. 2C).

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**GNPTAB−/− and GNPTG−/− exhibit increases in the steady-state level and phosphorylation of Met receptor**

The Met receptor is highly enriched in epithelial cells of several organs, including the liver and kidney, and plays a central
Increased Met receptor phosphorylation in GNPTAB\textsuperscript{−/−} cells

role in epithelial to mesenchymal transitions (29–32). Met showed both the largest difference in signal and the largest difference in cell surface abundance between HeLa and GNPTAB\textsuperscript{−/−}, making this receptor a logical choice for further study. We first sought to validate the findings from both the SEEL and the RTK array analyses. Western blots of whole cell lysates using an antibody to the phospho-tyrosines within the kinase domain (Tyr-1234 and Tyr-1235) demonstrated very low levels of Met receptor phosphorylation in parental HeLa cells, whereas this was increased in both GNP\textsuperscript{TG\textsuperscript{−/−}} and GNPTAB\textsuperscript{−/−} cells (Fig. 4A). Quantitative analysis of Met and phospho-Met levels showed that Met levels were increased by nearly 3-fold in GNPTAB\textsuperscript{−/−} cells compared with the parental HeLa line, but phospho-Met levels were increased less than 125-fold, indicating a high level of activation and/or sustained activity of this receptor at these sites (Fig. 4B). An intermediate increase in Met receptor and phosphorylated Met receptor were noted in GNP\textsuperscript{TG\textsuperscript{−/−}}, suggesting a relationship between Met receptor activation and the degree to which lysosomal targeting is impaired. To determine whether other Met phosphorylation sites were increased in GNPTAB\textsuperscript{−/−}, we repeated these experiments using an antibody specific for the phospho-tyrosine within the docking domain (Tyr-1349). We found that the levels of phosphorylation at these sites were greatly increased in the GNPTAB\textsuperscript{−/−} cells but only marginally elevated in the GNP\textsuperscript{TG\textsuperscript{−/−}} cells (Fig. 4, C and D). These data indicate that Met phosphorylation is increased at two different domains within the receptor.

Increased abundance of the Met receptor at the cell surface in GNPTAB\textsuperscript{−/−} cells was confirmed by SEEL labeling followed by biotin immunoprecipitation and immunoblotting (Fig. 4E). To examine the subcellular localization of phospho-Met, parental and GNPTAB\textsuperscript{−/−} cells were immunohistochemically stained for LAMP1 and phospho-Met and analyzed by confocal microscopy. Phospho-Met was readily detected in both LAMP1-positive and -negative vesicular structures in GNPTAB\textsuperscript{−/−} but not parental HeLa cells (Fig. 4F). We quantified the level of p-Met in both the cytosolic (LAMP1-negative) and lysosomal (LAMP1-positive) pools in the two cell lines (Fig. 4G). Cytosolic p-Met levels were significantly higher in GNPTAB\textsuperscript{−/−} cells. The substantial overlap between phospho-Met and the lysosomal marker LAMP1 in the GNPTAB\textsuperscript{−/−} cells suggested that a portion of phospho-Met localizes to this compartment. Because a detectable portion of phospho-Met was present in LAMP1-negative vesicles, we asked whether the increase in Met receptor phosphorylation correlated with an increase in the expression of Met-responsive genes (Fig. 4H). The transcript abundance of ITGB1, a known Met-responsive gene (33), was increased 31% in GNPTAB\textsuperscript{−/−}. Further, the transcript abundance of EPHX2 (whose expression is known to be negatively regulated by Met activity (34)) was decreased by 60%.

GNPTAB\textsuperscript{−/−} cells have decreased phosphatase activity toward Met and increased reactive oxygen species (ROS)

Elevated Met receptor phosphorylation suggests its activity is sustained in the GNPTAB\textsuperscript{−/−} cells, whereas it is effectively deactivated in the parental HeLa cells. Met activity is regulated by the dephosphorylation of specific tyrosine residues by both cell surface protein-tyrosine phosphatases, such as PTPRJ/Dep-1, and non-receptor phosphatases such as PTP1B and TCPTP (35–38). We asked whether a reduction of phosphatase activity differentially impacts Met phosphorylation by treating cells with increasing concentrations of the general phosphatase inhibitor pervanadate and immunoblotting for phospho-Met (Tyr-1234 and Tyr-1235) and Met receptor. As shown in Fig. 5A, inhibition of global phosphatase activity resulted in increased phospho-Met levels in all cell types but lower amounts of pervanadate were needed to raise phospho-Met levels in GNPTAB\textsuperscript{−/−}, suggesting less protein-tyrosine phosphatase activity. The ratio of phospho-Met to Met receptor was quantified in Fig. 5B. Roughly the same ratio increase was noted at 1 \(\mu M\) in GNPTAB\textsuperscript{−/−} as was seen at 10 \(\mu M\) in the parental HeLa cells, supporting the idea that less active phosphatases are present to deactivate Met in GNPTAB\textsuperscript{−/−}.

The activity of both receptor and non-receptor tyrosine phosphatases is sensitive to increases in ROS. High levels of ROS are known to inactivate protein-tyrosine phosphatases that act on Met including PTPRJ and PTP1B (36, 37, 39–42). Our data on Met phosphorylation showed that phosphorylation of tyrosines within the kinase and docking domains was elevated, pointing to decreased activity of more than one phosphatase. Many lysosomal storage diseases are known to exhibit increased cellular ROS because of lysosomal storage and improper breakdown of mitochondria (43–47). Thus, elevated ROS in the GNPTAB\textsuperscript{−/−} cells may account in part for the increase in phospho-Met levels within multiple domains of the receptor. Using a fluorescent indicator of intracellular ROS, we examined ROS levels and found that GNPTAB\textsuperscript{−/−} cells have a 50% increase in ROS compared with parental HeLa (Fig. 5C). We next asked whether short-term treatment of parental HeLa

**Figure 3.** GNPTAB\textsuperscript{−/−} cells have increased levels of multiple phosphorylated receptor tyrosine kinases. **A,** representative image of a human phospho-RTK array analysis of HeLa parental control and GNPTAB\textsuperscript{−/−} cells (n = 3). Receptors with high signal or large change in signal between HeLa parental control and GNPTAB\textsuperscript{−/−} are labeled. **B,** fold change of the signal intensity of individual spots comparing GNPTAB\textsuperscript{−/−} to HeLa parental control obtained from three biological replicates. Error bars denote mean \(\pm S.D.\)
cells with hydrogen peroxide (at concentrations previously shown to inactivate phosphatases and increase kinase phosphorylation (40)) was sufficient to elevate Met receptor phosphorylation. Hydrogen peroxide treatment was capable of increasing Met receptor phosphorylation within the kinase domain in a dose-dependent manner with no appreciable change in Met receptor levels (Fig. 5D), suggesting ROS-mediated inactivation of phosphatases is sufficient to raise phospho-Met levels in the parental HeLa line. To assess whether increases in ROS are linked to increases in phospho-Met levels in GNPTAB−/− cells, these cells were treated with the antioxidant Trolox for 96 h followed by immunoblotting for phospho-Met (Asp-26) and Met (Fig. 5E). Trolox treatment consistently reduced phospho-Met levels in GNPTAB−/− cells without substantially altering Met receptor levels.

Lastly, we attempted to rescue GNPTAB−/− cells with WT GNPTAB cDNA to ask whether the biochemical alterations, including increased Met receptor activation, in the CRISPR-mediated knock-out cells was specific to loss of lysosomal targeting. The glycosidases β-galactosidase and β-hexosaminidase are mannose 6-phosphorylated and trafficked to the lysosome in parental HeLa cells but not in GNPTAB−/−, as evidenced by the reduction in intracellular activity of both glycosidases (Fig. 6A). Following 120 h of transient transfection, the intracellular glycosidase activities were measured to ensure that WT GlcNAc-1-phosphotransferase was functionally
expressed. As shown in Fig. 6A, transfection with WT GNPTAB increased intracellular glycosidase to near normal levels despite only an estimated 30–35% transfection efficiency. It is likely that the overexpression of WT GNPTAB results in hydrolase targeting to a greatly increased number of lysosomes in the GNPTAB−/− cells, thus exaggerating the effects of transient GNPTAB expression. We next asked whether the partial restoration of lysosomal targeting was sufficient to reduce Met receptor phosphorylation (Fig. 6B). GNPTAB expression slightly lowered Met receptor phosphorylation in the GNPTAB−/− cells but this difference was not statistically significant in replicate experiments. Of note, levels of LAMP1 were also slightly reduced, which indicates that transient GNPTAB expression may not be capable of fully reducing lysosomal storage in the transfected cell population.

**Discussion**

In this study, we expand the molecular phenotypes in GNPTAB-deficient cells to include altered receptor activity and uptake. Taking advantage of selective cell surface labeling to profile differences in the residence of sialoglycoproteins, we identified altered abundance of multiple receptors and cell adhesion molecules, including the sorting receptor LRP1 and the receptor tyrosine kinase Met, on the surface of GNPTAB−/− cells. Our subsequent analysis of Met revealed an unexpected increase in its phosphorylation status. The mechanisms that underlie this phenotype were investigated, revealing a loss in the ability to deactivate Met, most likely because of ROS-mediated inactivation of protein-tyrosine phosphatases.

Prior work in MLII fibroblasts identified numerous alterations at the cellular levels when lysosomal targeting is impaired (16, 45). For example, Otomo et al. (16, 45) demonstrated impaired endocytosis and receptor recycling in MLII fibroblasts that could be partially rescued by a total enzyme replacement strategy. This suggests that some of the differences in the cell surface abundance of receptors in the GNPTAB−/− HeLa cells may arise because of storage-dependent effects on receptor internalization and/or trafficking. Moreover, autophagic
Increased Met receptor phosphorylation in GNPTAB<sup>−/−</sup> cells

flux and mitochondrial defects were also detected in the MLII fibroblasts, which likely explains the increase in ROS within the GNPTAB<sup>−/−</sup> HeLa cells. Impaired autophagy allows damaged mitochondria to persist in these cells, providing a source of damaging ROS. One of the proteins exhibiting the greatest decrease in abundance at the cell surface of GNPTAB<sup>−/−</sup> cells was the sorting receptor LRP1. This decrease corresponded with a functional reduction in uptake and internalization of its ligand Aβ40. Although the mechanistic basis for the decreased residence of this receptor is unclear, lower levels of LRP1 may reflect a recycling problem in these cells, consistent with effects on receptor recycling noted in other storage disease cells (15, 17, 19).

LRP1 has been shown to play a role in the reuptake of non-phosphorylated lysosomal hydrolases, although the steady-state levels of this receptor were not affected in mouse embryonic fibroblasts. It is plausible that reduction in its surface levels in some MLII cell types could potentiate lysosomal storage by preventing efficient recapture of secreted hydrolases. Further indication of reduced LRP1 as a disease modifier is evidenced in neurodegeneration and Alzheimer's disease pathology (48–50). LRP1 serves an essential role in brain lipid metabolism, and loss of LRP1 leads to altered brain lipid homeostasis, causing global changes in brain function. Amyloid-β, which is the main pathogenic molecule that accumulates in Alzheimer's disease brains, is typically cleared by LRP1. Work by several groups suggests that reduction or inactivation of LRP1 leads to a decrease in amyloid-β efflux across the blood-brain barrier (26, 28, 48). Interestingly, amyloid-β–induced oxidative modification of LRP1 can mediate LRP1 dysfunction (51). Because GNPTAB<sup>−/−</sup> cells exhibited increases in ROS, it is possible that the functional loss of LRP1 may be related to oxidative stress. We did not however detect any consistent elevation in LRP1 protein following treatment of GNPTAB<sup>−/−</sup> cells with the antioxidant Trolox.

Oxidative stress is a consequence of lysosomal storage, which can be caused by improper degradation of defective cellular components or organelles such as mitochondria and can lead to many negative effects (44). Cellular increases in ROS affect the activity of protein-tyrosine phosphatases by inactivation of the catalytic cysteine (40). The protein-tyrosine phosphatases PTPRJ and PTP1B have been shown to be susceptible to this kind of oxidative inactivation (36, 37, 39–42). SEEL followed by proteomics further identified decreased surface abundance of PTP1B in the GNPTAB<sup>−/−</sup> cells. Met receptor tyrosine kinase and its adaptor protein Gab1 are known substrates of PTPRJ (35). Thus it is possible that under the oxidative conditions in GNPTAB<sup>−/−</sup> cells, PTPRJ, or other phosphatases such as PTP1B are partially inactivated and unable to dephosphorylate Met, leading to the sustained Met phosphorylation we detected within two different domains.

The involvement of phosphatases may explain changes in cell surface abundance of other glycoproteins including cell adhesion molecules. For example, PTPRJ/Dep-1 and PTPRF have been shown to regulate integrin activation and recycling, respectively (52, 53). Thus, a decrease in phosphatase activity could lead to altered surface residence of these proteins. The detection of phosphorylated Met in the lysosome of the GNPTAB<sup>−/−</sup> cells suggests that this form of the receptor may traffic to this organelle and then fail to turn over due to a lack of necessary hydrolases and/or phosphatases. Nonetheless, the effects of sustained Met phosphorylation could be observed upon the expression of at least two Met-responsive genes, so it is likely that at least some of the phospho-Met (in particular the cytosolic pool) is functionally active. The increase in Met phosphorylation may ultimately derive from a combination of decreased phosphatase-dependent inactivation and terminal storage of activated receptor in lysosomes. Based on the degree of storage and autophagic impairment in different patient tissues, it is likely that the increased Met phosphorylation we observed in the HeLa cells may be operational in vivo. This response will also vary with how well certain cell types manage fluctuations in storage-induced ROS levels.

Another intriguing possibility to consider is the fact that Met receptor levels are increased as a protective effect against oxidative stress. Work by several groups has shown that loss of c-Met in cells, particularly hepatocytes, results in loss of redox homeostasis and an acceleration of ROS-mediated damage (34, 54–57). Thus, increased Met receptor expression or activity may reflect a compensatory response by the GNPTAB<sup>−/−</sup> cells to mitigate the impact of higher ROS levels.

Despite restoring glycosidase levels back to near normal levels, we were unable to achieve substantial rescue of the Met phosphorylation following introduction of WT GNPTAB. This is mostly likely attributed to the inability of transient GNPTAB transfection to correct the storage phenotype in these cells, as evidenced by only a slight decrease in LAMP1 levels following transfection, and to the low transfection efficiency of the large GNPTAB cDNA. An initial concern of ours was the possibility that elevated Met receptor activity was a result of clonal selection following CRISPR/Cas editing in the HeLa cells. The observation of an intermediate increase in phospho-Met levels in the GNPTG<sup>−/−</sup> cells makes this unlikely in our view and instead suggests that the effect on Met phosphorylation is tied directly (through trapping in this organelle) or indirectly (through ROS production) to the degree of lysosomal storage.

This study highlights another example whereby impaired lysosomal targeting leads to increased signaling of cell surface receptors. Studies in model organisms including zebrafish have identified increased TGFβ signaling as a function of the direct action of secreted hydrolases on latent growth factors deposited within the extracellular matrix (ECM) of tissues like cartilage (58). The present evidence for increased Met receptor activation appears to point instead to an intracellular mechanism of action. It is intriguing to speculate that ROS generation in lysosomes not only inactivates protein-tyrosine phosphatases in the cytosol but also may reduce phosphatases in this compartment. This might explain why Met remains phosphorylated even after reaching the lysosome. The present study also highlights the ability of SEEL to identify changes in the abundance of cell surface glycoproteins and generate leads for further analysis. There are several candidates of interest for investigation including the wide range of cell adhesion molecules with altered abundance and/or expression in the GNPTAB<sup>−/−</sup> cells. Future studies will be focused on understanding whether
altered glycan processing of cell surface glycoproteins by secreted glycosidases contributes to altered surface residence.

**Experimental procedures**

**Reagents**

Recombinant rat α-(2,6)-sialyltransferase (ST6Gal1) was prepared as published previously (59). CMP-sialic acid biotin was synthesized as reported previously (23). *Vibrio cholerae* neuraminidase (type II) (N6514) and Protein G Sepharose Fast Flow beads (P3296) were purchased from Sigma-Aldrich. FastAP Thermosensitive Alkaline Phosphatase (EF0651), Protease Inhibitor Mini Tablets (88666), and compatible Silver Stain for Mass Spectrometry (24600) were purchased from Thermo Scientific. IgG fraction monoclonal mouse anti-biotin with and without HRP conjugation were purchased from Jackson ImmunoResearch Laboratories (200–032-211 and 200–002-211). Rabbit monoclonal anti-LRP1 was purchased from Abcam (ab92544). Monoclonal rabbit anti-Met (D1C2), rabbit anti–phospho-Met (Tyr-1234/1235) (Asp-26) and rabbit anti–phospho-Met (Tyr-1349) were purchased from Cell Signaling Technology (8198 and 3077). For cellular uptake assays, human amyloid-β peptide (1–40), HiLyte Fluor 647-labeled was purchased from AnaSpec (AS-60493) and was reconstituted in 100% DMSO at 500 μM. The cell-permeable vitamin E derivative Trolox was purchased from Enzo Life Sciences (AXL-270–267-M100).

**Cell lines and culture**

GNPTG−/− and GNPTAB−/− HeLa cells were generously provided by Stuart Kornfeld (Washington University School of Medicine, St. Louis, MO) and were generated by CRISPR/Cas9 genome editing (7). Cells were cultured in DMEM with 4.5 g/liter glucose and 1-glutamine (Lonza, 12–604F) and supplemented with 10% fetal bovine serum (VWR Life Science Sera-digm, 1500–500), and penicillin (100 IU/ml)–streptomycin (100 μg/ml) (Corning, 30–001–CI). Cells were cultured in a 5% CO₂ atmosphere, 37 °C humid incubator. Following 16 h of incubation, media were altered glycan processing of cell surface glycoproteins by secreted glycosidases contributes to altered surface residence.

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GNPTG−/− and GNPTAB−/− HeLa cells were generously provided by Stuart Kornfeld (Washington University School of Medicine, St. Louis, MO) and were generated by CRISPR/Cas9 genome editing (7). Cells were cultured in DMEM with 4.5 g/liter glucose and 1-glutamine (Lonza, 12–604F) and supplemented with 10% fetal bovine serum (VWR Life Science Sera-digm, 1500–500), and penicillin (100 IU/ml)–streptomycin (100 μg/ml) (Corning, 30–001–CI). Cells were cultured in a 5% CO₂ atmosphere, 37 °C humid incubator. Following 16 h of incubation, media were

**Western blotting, immunoprecipitation, and phospho-receptor tyrosine kinase assay**

Cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) and then lysed on ice with lysis buffer 17 (phospho-RTK kit) supplemented with protease inhibitor mixture. Lysed cells were centrifuged at 14,000 rpm for 8 min and the supernatant was removed and saved. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific, 23235). Immunoprecipitation with anti-biotin antibody–coated protein G beads was performed as reported previously (24). Proteins were separated by SDS-polyacrylamide gels (Bio-Rad, 161–0148), and transferred to nitrocellulose membranes (Bio-Rad, 162–0115). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline plus 0.1% Tween 20 or with 5% bovine serum albumin in Tris-buffered saline plus 0.1% Tween 20 in the case of phospho-Met immunoblotting. Immunoreactive bands were identified with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080) and imaged and quantified using a Bio-Rad ChemiDoc MP Imaging System. Human Phospho-RTK Arrays were performed according to manufacturer’s instructions (R&D Systems, ARY001B).

**RT-PCR and quantitative PCR (qPCR)**

For RT-PCR, RNA was extracted using TRizol (Invitrogen, 15596026) from trypsinized cell pellets. For qPCR, RNA was extracted from cell pellets using an RNeasy Plus Kit (Qiagen, 74134). CDNA was prepared using Quanta qScript XLT cDNA SuperMix (95161). Thermo Scientific PCR Master Mix (2X) (K0171) was used for RT-PCR reactions and Quantabio’s PerfeCTa SYBR Green FastMix (95072-250) was used for qPCR. The primers used for RT-PCR or qPCR are shown in supplemental Table S3.

**Amyloid-β-40 uptake assay**

1 × 10⁶ cells were plated per well in a 96-well tissue culture dish and allowed to adhere overnight. The following day, cells were treated with 2 μM human amyloid-β peptide (1–40), HiLyte Fluor 647-labeled (AnaSpec), or DMSO in complete DMEM growth media for 16 h in 5% CO₂ atmosphere, 37 °C humid incubator. Following 16 h of incubation, media were
removal and cells were rinsed twice with DPBS. Cells were then lysed in RIPA buffer and fluorescence was read using a Synergy plate reader.

Confocal immunofluorescence microscopy

6 × 10⁴ cells were plated on gelatin-coated coverslips in 12-well plates 1 day prior to staining. Immunofluorescence staining was performed according to manufacturer’s instructions (Cell Signaling Technology), but 0.15% Triton X-100 was used instead of 0.3%. Images were acquired on an Olympus FV1000 laser-scanning microscope outfitted with a 60X oil-immersion objective (NA 1.4). Images were taken using 0.45 μM Z-steps. Fluorescent data were quantified using Slidebook Software (Intelligent Imagining Innovations) in a blinded manner, such that the person quantitating the data did not perform the experiment or know the identity of the samples being quantified. The mean intensities of p-Met were determined for 40 regions of interest (20 LAMP1-positive and 20 LAMP1-negative regions) from three independent experiments. Co-localization of LAMP1 and p-Met was assessed by determining the Pearson’s coefficients for these same regions.

Pervanadate and Trolox treatment

6 × 10⁴ cells were plated in 12-well plates 1 day prior to treatment. Cells were incubated for 20 min at 37 °C with 1 μM, 5 μM, or 10 μM pervanadate. Following treatment, cells were rinsed, and SDS-PAGE and Western blotting were performed as described. For Trolox experiments, cells were plated at 50% confluence and treated with 5 μM Trolox dissolved in DMSO. Trolox incubations were performed for 96 h, with medium change and Trolox replacement after 48 h, followed by Western blot analysis.

Analysis of cellular ROS levels

Reactive oxygen species (ROS) levels were determined using the ROS indicator dye carboxy-H₂DCFDA. Cells were seeded at 75% confluence 1 day prior to ROS measurement. 2 mM carboxy-H₂DCFDA in DMSO was prepared fresh before each experiment. Cells were trypsinized and resuspended in DMEM containing 0.1% BSA and incubated with 20 μM carboxy-H₂DCFDA for 45 min in a rocking 37 °C chamber. Cells were quickly pelleted and resuspended in DPBS and ROS levels were read using a Synergy plate reader.

Rescue experiments with WT GlcNAc-1-phosphotransferase

GNPTAB−/− HeLa cells were transfected with 1 μg of WT GlcNAc-1-phosphotransferase using Lipofectamine 3000 (Invitrogen) several hours after plating at 75% confluence in 6-well plates. Following overnight incubation, transfection medium was removed and replaced with fresh growth medium. Enzyme assays or SDS-PAGE and Western blotting were performed 120 h after transfection.

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Author contributions—M. A.-P. conceived the study and designed, performed, and analyzed the experiments. P. Z. and L. W. designed, performed, and analyzed all the proteomics experiments shown in Figure 1. S.-H. Y. provided technical assistance and contributed to the analysis of the proteomics data. C. C. performed experiments and analyzed data. H. F.-S. contributed to the study design and performed all confocal microscopy. R. S. conceived the study and coordinated the research. R. S. and M. A. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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