AUTOPHAGY
2023, VOL. 19, NO. 7, 2143–2145
https://doi.org/10.1080/15548627.2023.2167376

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COMMENTARY AND VIEWS

LYSET/TMEM251- a novel key component of the mannose 6-phosphate pathway

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ABSTRACT

Degradation of macromolecules delivered to lysosomes by processes such as autophagy or endocytosis is crucial for cellular function. Lysosomes require more than 60 soluble hydrolases in order to catabolize such macromolecules. These soluble hydrolases are tagged with mannose6-phosphate (M6P) moieties in sequential reactions by the Golgi-resident GlcNAc-1-phosphotransferase complex and NAGPA/UCE/uncovering enzyme (N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase), which allows their delivery to endosomal/lysosomal compartments through trafficking mediated by cation-dependent and -independent mannose 6-phosphate receptors (MPRs). We and others recently identified TMEM251 as a novel regulator of the M6P pathway via independent genome-wide genetic screening strategies. We renamed TMEM251 to LYSET (lysosomal enzyme trafficking factor) to establish nomenclature reflective to this gene's function. LYSET is a Golgi-localized transmembrane protein important for the retention of the GlcNAc-1-phosphotransferase complex in the Golgi-apparatus. The current understanding of LYSET's importance regarding human biology is 3-fold: 1) highly pathogenic viruses that depend on lysosomal hydrolase activity require LYSET for infection. 2) The presence of LYSET is critical for cancer cell proliferation in nutrient-deprived environments in which extracellular proteins must be catabolized. 3) Inherited pathogenic alleles of LYSET can cause a severe inherited disease which resembles GlcNAc-1-phosphotransferase deficiency (i.e., mucolipidosis type II).

Abbreviations: GlcNAc-1-PT: GlcNAc-1-phosphotransferase; KO: knockout; LSD: lysosomal storage disorder; LYSET: lysosomal enzyme trafficking factor; M6P: mannose 6-phosphate; MPRs: mannose-6-phosphate receptors, cation-dependent or -independent; MBTPS1/site-1 protease: membrane bound transcription factor peptidase, site 1; MLII: mucolipidosis type II; WT: wild-type

ARTICLE HISTORY
Received 5 December 2022
Revised 6 January 2023
Accepted 6 January 2023

KEYWORDS
GlcNAc-1-phosphotransferase; Golgi-apparatus; mannose 6-phosphate; mucolipidosis type II; lysosomal enzyme trafficking; lysosome

Lysosomes are key degradative organelles that contain a variety of hydrolytic enzymes that are used for the intracellular digestion of biomolecules. Impairment of lysosomal homeostasis leads to over 70 rare inherited metabolic diseases known as lysosomal storage diseases (LSDs) [1]. Targeting of soluble lysosomal enzymes requires mannose 6-phosphate (M6P) tagging, which is initiated by the GlcNAc-1-phosphotransferase complex (GNPTAB-GNPTG) in the Golgi apparatus. Defective GlcNAc-1-phosphotransferase function is etiological for the severe LSD mucolipidosis type II (MLII) [2]. The M6P lysosomal sorting pathway is well-studied and was thought to be completely understood. However, three recent independent studies performing genome-wide CRISPR screens found TMEM251, (renamed LYSET [lysosomal enzyme trafficking factor]) to be a novel regulator and indispensable component of the M6P sorting pathway [3–5] (Figure 1).

Lysosomal cathepsin-mediated proteolytic function is critical for reovirus infection [6]. We performed genome-scale CRISPR screens using susceptibility to reovirus infection as a phenotypic selection scheme to identify genes important for lysosomal biogenesis [4]. The top genes identified using this screening strategy encode a highly expressed cathepsin (CTSL [cathepsin L]) as well as key components of the M6P-pathway including GNPTAB, GNPTG, MBTPS1/site1-protease, a Golgi resident protease required for cleavage and activation of the GlcNAc-1-phosphotransferase complex, and LYSET/TMEM251. We hypothesized and confirmed that LYSET is required for infection by other viruses that depend on cathepsin proteolytic activity (e.g., Ebola virus and severe acute respiratory syndrome coronavirus, SARS-CoV-2). In the second study by Pechincha et al., LYSET was found to be necessary for nutrient-deprived cancer cells to feed on extracellular proteins [3], and Zhang et al. found LYSET to be important for lysosomal protein degradation using a reporter-based assay [5].

To investigate whether LYSET affects M6P-mediated lysosomal enzyme transport, we determined the endogenous expression of relevant lysosomal enzymes in extracellular medium, intracellular lysates, and subcellular organelle fractions using immunoblotting, lysosomal enzyme activity assays and unbiased proteomic approaches. Aberrant secretion of lumenal lysosomal proteins and a global loss of cathepsin protease activity are observed in *LYSET* KO cells, indicating that LYSET deficiency causes a severe defect in M6P-specific lysosomal trafficking. Transmission electron microscopy revealed that the missorting of lysosomal enzymes is accompanied by accumulation of non-degraded material and lysosomes are increased in size and number. Using specific dyes, we found increased numbers of autophagosomes. Furthermore, we used glycoproteomics to detect M6P modifications directly on glycoproteins isolated from wild-type (WT), *LYSET*-KO, and *GNPTAB*-KO cells. In contrast to WT cells, M6P-modified glycopeptides are absent in both *GNPTAB* and *LYSET* KO cells,

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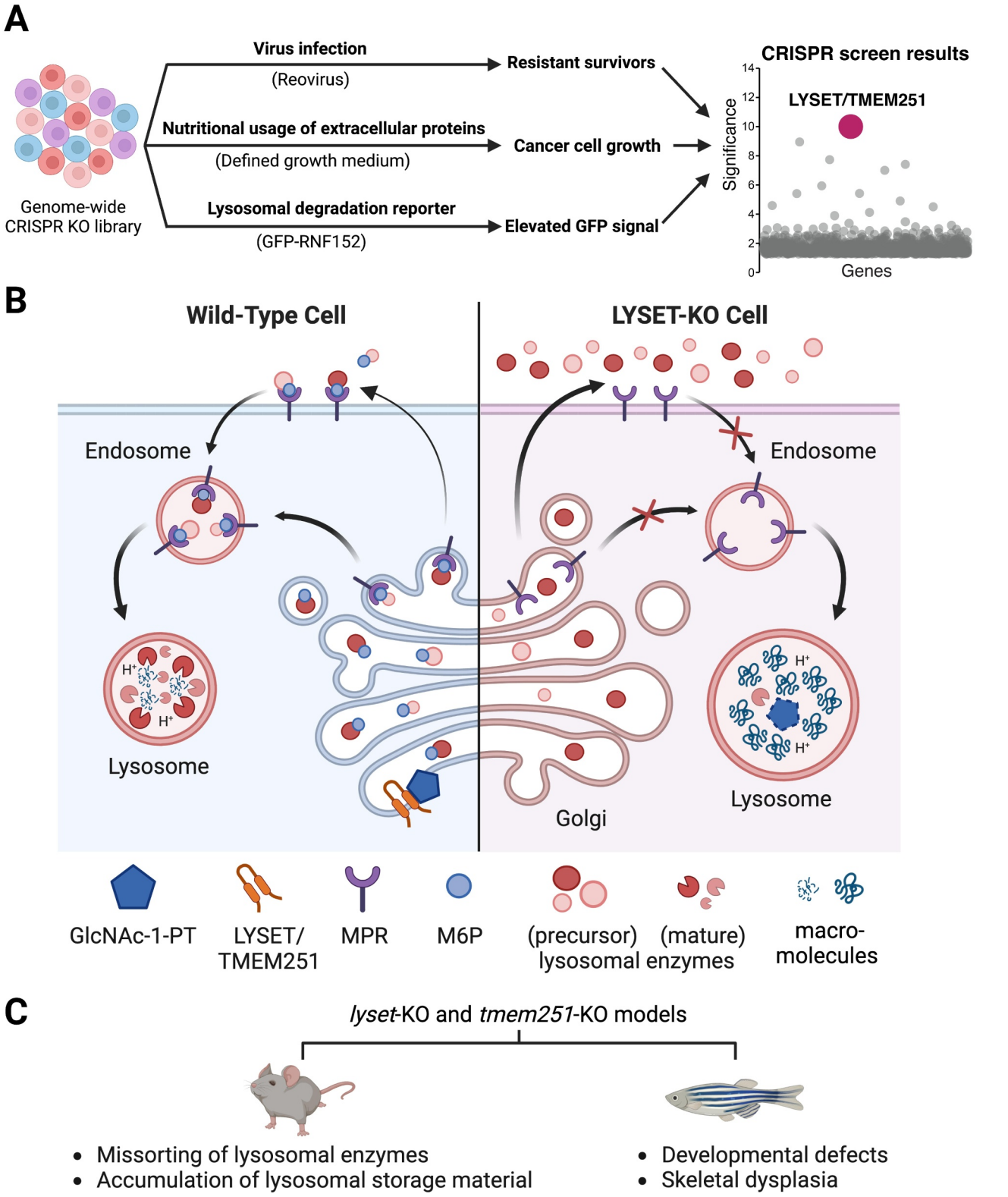

Figure 1. LYSET identification, function, and phenotypes. (A) Independent genome-wide CRISPR screens identified LYSET as essential for i) viral infection, ii) cell proliferation in nutrient-poor conditions where cancer cells depend on extracellular proteins as nutrient source, and iii) lysosomal degradation of a reporter protein (GFP-RNF152). All three screening approaches converge on LYSET/TMEM251 as one of the most significant candidate genes (exemplified representation of a CRISPR screen result). (B) GlcNAc-1-phosphotransferase (GlcNAc-1-PT) catalyzes the major step in the attachment of mannose 6-phosphate (M6P) residues to lysosomal soluble proteins. LYSET controls GlcNAc-1-PT function by binding to and retaining it in the Golgi apparatus. M6P-tagged lysosomal enzymes are recognized by MPRs mediating their transport to lysosomes. In *LYSET*-KO cells, GlcNAc-1-PT is transported to lysosomes where it is degraded. The subsequent disruption of M6P-tagging results in aberrant secretion of enzymes normally present in the lysosome, increased lysosome size and number and accumulation of macromolecules that are degraded in a wild-type cell (degradation represented as dotted lines). (C) Mouse and zebrafish *lyset*- and *tmem251*-KO models display phenotypes resembling those found in mucolipidosis type II patients. *Created with BioRender.com.*

indicating that LYSET plays a critical role in generating M6P modifications.

We then explored how LYSET affects the M6P modification by the GlcNAc-1-phosphotransferase complex. Immunoprecipitation analysis revealed that LYSET binds to GNPTAB. Strikingly, immunofluoresence experiments revealed that GNPTAB is relocalized to lysosomes in the absence of LYSET. To investigate this further, we characterized the expression of the endogenous GNPTAB in subcellular fractions. A near complete loss of endogenous GNPTAB protein levels is observed in the 100 K ER/Golgi-enriched fraction from *LYSET* KO cells. Moreover, preventing lysosomal degradation by blocking organellar acidification with bafilomycin A_1 or by protease inhibition with a combined treatment of E64d+leupeptin +pepstatin A results in elevated GNPTAB protein levels specifically in 20 K lysosome-enriched fractions as compared to untreated cells. Collectively, our data support a model that LYSET interacts with GNPTAB and plays an essential role in proper localization of GNPTAB in Golgi stacks. In the absence of LYSET, GNPTAB is mislocalized to the lysosomes where it is degraded, which consequently results in M6P tagging failure (Figure 1).

In an independent study Pechincha et al., found LYSET to be selectively required for cell growth and survival when cells depend on extracellular proteins as nutrient source. Consequently, LYSET-deficient cancer cells are severely impaired in their ability to form tumors in mice [3]. In a third independent study, Zhang et al. directly addressed proteins essential for lysosomal degradation by screening for genes that block degradation of RNF152, a lysosomal membrane protein that is normally degraded quickly as part of a pathway that controls lysosomal turnover [5]. All three studies demonstrate that LYSET binds to GNPTAB and that *LYSET* KO results in loss of mature GNPTAB. Based on the reduced presence of mature GNPTAB subunits and the reappearance of mature forms using protease inhibitors in *LYSET* KO cells, we and Pechincha et al. proposed that LYSET regulates proper localization and stability of mature GNPTAB subunits. Zhang et al. do not present experiments with protease inhibitors and interpret the loss of mature GNPTAB subunits as a cleavage defect based on immunoprecipitations showing association between LYSET, GNPTAB and MBTPS1/S1P. They proposed to rename TMEM251 to GNPTAB cleavage and activity factor/GCAF. Future experiments are needed to differentiate between the current mechanistic models of LYSET as (1) a Golgi retention factor for GNPTAB preventing its anterograde transport for lysosomal degradation or (2) as a factor regulating MBTPS1/S1P activity on GNPTAB.

Mucolipidosis type II is a hereditary lysosomal storage disease caused by GNPTAB mutations. Recently, an MLII-like genetic disorder in patients carrying biallelic LYSET mutations has been described [7]. The critical role of LYSET in regulating M6P-mediated lysosomal enzyme transport might underlie this genetic dysfunction. To better link LYSET with the disease, we generated *lyset/tmem251*-KO mice by gene editing in which typical diagnostic features of MLII, including elevated levels of lysosomal enzymes in blood serum and enlarged lysosomes with accumulated storage material, are observed. *tmem251*-KO in zebrafish by Zhang et al. leads to heart edema, severe developmental defects, and skeletal dysplasia, the latter being typical features of MLII. Consistent with these animal models of LYSET/TMEM251 deficiency, we observed that WT LYSET and nonpathogenic LYSET variants restore the cathepsin protein missorting and maturation in *LYSET* KO cells in complementation assays. However, human pathogenic LYSET mutations Y72Ter and R45W fail to do so. Moreover, the pathogenic allele R45W is incapable of rescuing the loss of endogenous GNPTAB. These results suggest that the interaction of LYSET with GNPTAB is critical for GNPTAB function. If this interaction is defective, M6P-dependent lysosomal protein sorting becomes severely compromised and likely manifests at the *in vivo* level as a lysosomal storage disorder akin to MLII.

Taken together, these works identify LYSET as a Golgi-resident protein essential for M6P-mediated lysosomal protein trafficking. Mechanistically, LYSET binds to GNPTAB and retains it in the Golgi apparatus for proper biological function. LYSET is required for infection by highly pathogenic viruses that rely on endo-lysosomal activation by cathepsins. LYSET plays an additional role in growth of tumor cells in nutrient-poor conditions. Furthermore, the key role of LYSET in lysosome biogenesis provides a mechanistic explanation for an MLII-like genetic LSD related to disease-associated LYSET variants, which may help to identify disease-causing mutations and provide a reference for clinical diagnosis. Collectively, these findings emphasize the relevance of LYSET and the M6P pathway in diverse disease conditions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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