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# Mannose 6-phosphate-dependent targeting of lysosomal enzymes is required for normal craniofacial and dental development

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ABSTRACT

Mucolipidosis II (MLII) is a severe systemic genetic disorder caused by defects in mannose 6-phosphate-dependent targeting of multiple lysosomal hydrolases and subsequent lysosomal accumulation of non-degraded material. MLII patients exhibit marked facial coarseness and gingival overgrowth soon after birth, accompanied with delayed tooth eruption and dental infections. To examine the pathomechanisms of early craniofacial and dental abnormalities, we analyzed mice with an MLII patient mutation that mimic the clinical and biochemical symptoms of MLII patients. The mouse data were compared with clinical and histological data of gingiva and teeth from MLII patients. Here, we report that progressive thickening and porosity of calvarial and mandibular bones, accompanied by elevated bone loss due to 2-fold higher number of osteoclasts cause the characteristic craniofacial phenotype in MLII. The analysis of postnatal tooth development by microcomputed tomography imaging and histology revealed normal dentin and enamel formation, and increased cementum thickness accompanied with accumulation of storage material in cementoblasts of MLII mice. Massive accumulation of storage material in subepithelial cells as well as disorganization of collagen fibrils led to gingival hypertrophy. Electron and immunofluorescence microscopy, together with $^{35}$S-sulfate incorporation experiments revealed the accumulation of non-degraded material, non-esterified cholesterol and glycosaminoglycans in gingival fibroblasts, which was accompanied by missorting of various lysosomal proteins (α-fucosidase 1, cathepsin L and Z, Npc2, α-L-iduronidase). Our study shows that MLII mice closely mimic the craniofacial and dental phenotype of MLII patients and reveals the critical role of mannose 6-phosphate-dependent targeting of lysosomal proteins for alveolar bone, cementum and gingiva homeostasis.


## 1. Introduction

Targeting of newly synthesized soluble lysosomal enzymes requires the generation of mannose 6-phosphate (M6P) residues by two Golgi-resident enzymes, *N*-acetylglucosamine (GlcNAc)-1-phosphotransferase and GlcNAc-1-phosphodiester α-*N*-acetylglucosaminidase [1,2]. The GlcNAc-1-phosphotransferase consists of two α-, two β- and two γ-subunits [3]. The α- and β-subunits are encoded by the *GNPTAB* gene as a single highly *N*-glycosylated 190-kDa type III membrane precursor protein and the *GNPTG* gene codes for the γ-subunit [4–6]. Upon arrival in the *cis*-Golgi compartment, the α/β-subunit precursor protein is cleaved by the site-1 protease to form the catalytically active α- and β-subunits [7]. The function of the γ-subunit is still unknown but recent data suggest that the interaction between γ- and α-subunits enhance the phosphorylation of subsets of lysosomal enzymes and facilitate the folding and transport of α/β-subunit precursor proteins to the Golgi apparatus [8–10]. Mutations in the *GNPTAB* gene cause the rare lysosomal storage disorder mucolipidosis type II (MLII), also known as I-cell disease [11]. Biochemically, MLII is characterized by the failure to form M6P residues which subsequently results in missorting and hypersecretion of

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lysosomal enzymes. The cellular deficiencies of multiple lysosomal enzymes in lysosomes lead to the accumulation of non-degraded macromolecules, such as proteins, lipids, or glycosaminoglycans (GAGs), and to lysosomal dysfunction. Clinically, MLII is characterized by impaired skeletal growth, progressive osteodystrophy, destructive bone lesions, psychomotor retardation and early death between 5 and 8 years of age due to cardiorespiratory failure [11–13]. Marked facial coarseness and gingival overgrowth are apparent soon after birth with gradual progression [12,14,15]. Delayed tooth eruption, impacted teeth and enamel defects have also been reported in a few case reports of MLII patients [15–17].

Tooth formation is a complex three-dimensional morphogenetic process that requires differentiation, function and communication between various secretory cells [18]. The bulk of the tooth is formed by ameloblasts and odontoblasts that produce enamel and dentin, respectively. The tooth supporting tissues comprise alveolar bone, gingiva and cementum that are formed by osteoblasts, gingival fibroblasts and cementoblasts, respectively. Bone and gingiva are constantly remodeled by bone-resorbing osteoclasts and gingival macrophages as a response to mechanical stimuli and periodontal pathogens [19,20]. Defects in a number of single lysosomal enzymes or membrane proteins have been reported to severely affect enamel formation [21], gingival homeostasis [22], root development [23–25], and tooth eruption [26,27]. Previously, we have generated a proper mouse model for MLII disease by single base insertion into the *Gnptab* gene corresponding to a mutation detected in an MLII patient [28]. In addition to cell type- and tissue-specific missorting of multiple lysosomal enzymes and subsequent accumulation of non-degraded material, the mice exhibit progressive brain atrophy, osteoporosis, primarily caused by a numerical increase of functionally active osteoclasts, and defective humoral immunity leading to premature death [28–31].

To systematically examine pathomechanisms of early craniofacial/dental abnormalities and gingival enlargement in MLII, we analyzed MLII mice in comparison with clinical and histological data of gingiva and teeth from MLII patients. Here, we demonstrate that MLII mice recapitulate craniofacial phenotypes of MLII patients and report on specific oral features affecting the tooth-supporting tissues, i.e. bone and cementum. A detailed examination of gingival fibroblasts revealed enzyme-specific missorting of lysosomal hydrolases and a massive accumulation of GAGs, fucose-containing glycostructures and non-esterified cholesterol contributing to gingival hypertrophy in MLII disease.

## 2. Materials and methods

### 2.1. Animals

MLII mice were generated by cytosine insertion into the murine *Gnptab* gene (c.3082insC) corresponding to an MLII patient mutation [4,28]. Mice were fed with standard rodent diet and maintained on a mixed C57Bl/6 129/SvJ genetic background in a pathogen-free animal facility at the University Medical Center Hamburg-Eppendorf. All mice were euthanized by $CO_2$ inhalation. Three to six female animals were analyzed per genotype at 1, 3 and 6 months of age. Animal procedures were performed in accordance with EU Directive 2010/63/EU for animal experiments and approved by the Amt für Gesundheit und Verbraucherschutz.

### 2.2. Micro-CT and opto-digital analysis

Heads of MLII mice and wild-type littermates were scanned with a high-resolution micro-CT (μCT 40, Scanco medical) with a voxel resolution of 15 μm. Quantification of calvarial and alveolar bone parameters were performed on micro-CT 3D-reconstruction using ImageJ (Image J 1.42, National Institute of Health). Alveolar bone loss was calculated as the area between the cementoenamel junction and the alveolar crest. Enamel volume and pulp volume were measured using the evaluation software provided by the manufacturer (Scanco medical). Teeth were colored using Photoshop (Photoshop Cs4, Adobe Systems Inc.). For soft tissue analysis, mandibular molars were imaged with a high-resolution inverted microscope (Olympus DSX500i) and a 10× objective lens (DSX-10×, Leica Microsystems). Optical microscopic images were analyzed using ImageJ (Image J 1.42, National Institute of Health).

### 2.3. Histology and ultrastructural analysis

Heads of MLII mice and wild-type littermates were fixed in 4% formaldehyde overnight and then transferred into 80% ethanol. In mice, bone and tooth mineralization increases with age, which makes it difficult to section undecalcified mice older than 3 months. Therefore, heads of 6 months old mice were decalcified in 20% EDTA solution, wax-embedded and serial sectioned at 4 μm. Sections were stained with hematoxylin and eosin according to standard protocols. For undecalcified histology, heads of 3 months old mice were dehydrated, embedded in methylmethacrylate and serial sections prepared at 4 μm. Cellular histomorphometry was carried out on standardized sagittal sections stained with touluidine-blue using an OsteoMeasure system (Osteometrics Inc., USA) as described previously [32]. Osteoclasts and osteoblasts were quantified in alveolar bone sections between the 1st and 2nd molar. Predentin to dentin ratio and the odontoblast number were quantified in the crown area of the 1st mandibular molar. Cementum thickness and the cementoblast number were quantified at the mesial root surface of the 2nd mandibular molar. The number of ameloblasts was determined in the mandibular incisor area. Quantitative backscattered electron imaging and transmission electron microscopy were carried out as previously described [32,33].

### 2.4. Immunohistochemistry

Twenty μm thick cryosections of gingiva were prepared from 6 months old MLII and wild-type mice, double-labeled with antibodies against Ki-67 (Abcam, 1:500), Lamp1 (CD107a from BD Pharmingen, 1:100), cathepsin K (Abcam, 1:1000) and osteocalcin (Alexis, 1:1000). Secondary Alexa 488- and Alexa 546-coupled antibodies were diluted 1:1000. Sections were coversliped in ProLong Gold with 4′,6-diamidin-2-phenylindol (DAPI, Molecular Probes/Invitrogen). In-situ cell death detection kit from Roche was applied according to the instructions of the company.

### 2.5. Patient analyses

The mutations found in the four MLII patients, sex, nationality and age at analysis are given in S1 Table or have been described previously [30,34,35]. All patient data were obtained during standard clinical care according to the Declaration of Helsinki. Written informed consent was obtained from all parents. Patients #1, #3 and #4 have been clinically examined by the authors (TK, NM). MRI scans and panorex radiographs were performed according to standard clinical procedures. The gingival biopsy from patient #4 was taken during gingivectomy under general anesthesia and analyzed in the Institute of Neuropathology of the University Medical Center Hamburg-Eppendorf according to standard protocols. Parents of patients #2 and #4 donated exfoliated deciduous teeth for ultrastructural analysis.

### 2.6. Generation of gingival fibroblasts

Gingival fibroblasts were isolated from wild-type and MLII mice at an age of 3–4 months. Palatal mucosal tissue was removed, minced and incubated with 12 U/5 ml dispase I (Roche) and 850 U/5 ml collagenase III (Biochrom) in phosphate-buffered saline (PBS) for