ubiquitylation (Fig. 2A and fig. S7D). Together, our data show that FANCI-FANC D2 acts upstream of HR in the context of replication-coupled ICL repair, but that it is not required for RAD51 recruitment to chromatin. Instead, the requirement for FANCI-FANC D2 in promoting HR can be explained by its role in promoting the incisions that underlie DSB formation (2). The FA pathway may also enhance HR via more direct mechanisms, because FA proteins also stimulate HR in the context of preformed DSbs (6, 7, 9, 16, 17).

Here, we report that in the context of replication-coupled ICL repair, the DSB generated in one sister chromatid through the action of FANC I-FANC D2 is fixed via strand invasion into the intact sister (fig. S8). We find that RAD51 binds efficiently to ICLs before a DSB has been generated (fig. S8, ii). Although lesion bypass likely displaces RAD51 from one sister chromatid, incisions and resection of the other sister creates a new docking site for RAD51, such that both ends of the DSB are coated with the recombination (fig. S8, iv). The interaction of RAD51 with ICL-stalled forks before DSB formation may function to prevent fork breakage/degradation (5, 15, 18) in favor of regulated incisions and/or to initiate strand invasion as soon as possible once the DSB has been formed. A major obstacle impeding our understanding of DSB repair has been the absence of cell-free systems. Combined with ChIP and the ability to inactivate or remove essential proteins, the system described here represents a powerful tool to elucidate the complex mechanism underlying DSB repair.

References and Notes
13. Supplementary methods are available as supporting material on Science Online.

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A Key Enzyme in the Biogenesis of Lysosomes Is a Protease That Regulates Cholesterol Metabolism
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Mucolipidosis II is a severe lysosomal storage disorder caused by defects in the α and β subunits of the hexameric N-acetylgalactosamine-1-phosophotransferase complex essential for the formation of the mannose 6-phosphate targeting signal on lysosomal enzymes. Cleavage of the membrane-bound α/β-subunit precursor by an unknown protease is required for catalytic activity. Here we found that the α/β-subunit precursor is cleaved by the site-1 protease (S1P) that activates sterol regulatory element–binding proteins in response to cholesterol deprivation. S1P-deficient cells failed to activate the α/β-subunit precursor and exhibited a mucolipidosis II–like phenotype. Thus, S1P functions in the biogenesis of lysosomes, and lipid-independent phenotypes of S1P deficiency may be caused by lysosomal dysfunction.

More than 50 soluble enzymes are targeted to lysosomes in a mannos 6-phosphate (M6P)–dependent manner. The formation of M6P residues on newly synthesized lysosomal enzymes is catalyzed by two multimeric enzyme complexes, N-acetylgalactosamine (GlcNAc)-1-phosphotransferase and GlcNAc-1-phosphodiester–α-N-acetylgalactosaminidase, allowing binding of the enzymes to M6P-specific receptors (1). The receptor–enzyme complexes

are then transported to the endosomal compartment, followed by low pH–induced dissociation and delivery of lysosomal proteins to lysosomes and recycling of receptors to the Golgi apparatus. The GlcNAc-1-phosphotransferase comprises a hexameric complex of three subunits (α2β2γ) with a molecular mass of 540 kD (2). The α and β subunits are encoded by a single gene, GNPTAB, and synthesized as a 190-kD precursor protein and processed by the site-1 protease (S1P) that activates sterol regulatory element–binding proteins (3). The loss of GlcNAc-1-phosphotransferase activity leads to the synthesis of lysosomal enzymes lacking M6P residues, resulting in missorting and intracellular deficiencies of multiple lysosomal hydrolases, and lysosomal storage of nondegraded material, which are used as diagnostic markers in MLII patients (6). Clinically, these patients are characterized by skeletal abnormalities, chondrodysplasia, cardiomyopathy, and motor and mental retardation, leading to early death (6). Cleavage of the α/β-subunit precursor between Lys928 and Asp929 by an unknown protease is a prerequisite for the enzymatic activity of the GlcNAc-1-phosphotransferase complex (7). Treatment with brefeldin A, a drug that disrupts Golgi trafficking, prevents the cleavage of the α/β-subunit precursor, suggesting that this reaction takes place in the Golgi apparatus (8).

To identify the protease responsible for the cleavage, we generated an α/β-subunit precursor miniconstruct that allows its efficient expression, spans the membrane twice, and lacks amino acids 431 to 819 (Fig. 1A). Antibodies to the human β subunit (9) allowed the detection of both the α/β-subunit precursor constructs and the cleaved 45-kD β subunit in baby hamster kidney (BHK) cells (Fig. 1B). Analysis of additional miniconstructs with stepwise deletions showed that 20 amino acids proximal to the cleavage site were required for proper proteolysis (Fig. 1B). Construct 3 was the best substrate and was used for all further experiments. To define structural requirements for efficient cleavage of the α/β-subunit precursor in more detail, we substituted residues Thr923 to Ser934 (according to the numbering of the full-length precursor) individually with alanines, Arg925, Leu927, and Lys928, which were most critical for cleavage of the phosphotransferase...
precursor (Fig. 1C and fig. S1). The expression of cleavage-resistant α/β-subunit precursors resulted in a 120/110-kD doublet. Endo H treatment showed that the 120-kD polypeptide contained complex sugar chains, which are also detectable in mature β subunits. Thus, the α/β-subunit precursor reaches the mid-Golgi apparatus where these sugar modifications occur (fig. S2).

The residues most critical for the cleavage of the α/β-subunit precursor were found to be homologous to the consensus recognition motif of the Golgi-resident site-1 protease, (R/K)X(hydrophobic)Z↓, where X represents any amino acid and Z preferentially Leu or Thr, but excluding Val, Pro, Glu, Asp, or Cys (Fig. 1D) (10). Site-1 protease (S1P; also known as subtilisin kexin isozyme-1, SKI-1), is encoded by the MBTPS1 gene and is a membrane-bound serine protease (11, 12). The prototypical membrane-bound S1P substrates are the sterol regulatory element–binding proteins SREBP1 and 2, which play a major role in lipid metabolism and cholesterol homeostasis (13). S1P is also responsible for the processing of numerous precursor proteins such as pro-BDNF (brain-derived neurotrophic factor), capsule glycoproteins of arenaviruses, transcription factors (activating transcription factor 6 (ATF6) and members of the cAMP response element–binding protein (CREB) family), and the autocalytic activation of proS1P (11, 14, 15). To examine whether S1P is responsible for the cleavage of the α/β-subunit precursor, we performed small interfering RNA (siRNA)-mediated down-regulation of MBTPS1 in HeLa cells, reducing its mRNA level to <10% of that in control cells (Fig. 2A). The subsequent transfection of MBTPS1 siRNA-treated cells with α/β-subunit precursor construct 3 revealed an almost complete inhibition of its cleavage (Fig. 2B). Next, we overexpressed the α/β-subunit precursor construct 3 and its mutant form, R925A, in Chinese hamster ovary cells (CHO-7) and in S1P-deficient CHO-7 cells, termed SRD-12B (16). The SRD-12B cell line was derived by mutagenesis followed by selection for cholesterol auxotrophy that is resistant to amphotericin. The mutant cells were then rescued by growth in the presence of cholesterol, oleate, and mevalonate and used for cloning of the S1P cDNA by complementation (17). Almost no MBTPS1 mRNA

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**Fig. 1.** Structural requirements for cleavage of the α/β-subunit precursor of the GlcNAc-1-phosphotransferase. (A) Schematic presentation of α/β-subunit constructs used in this study. Construct 1 shows the full-length α/β-subunit precursor and its domain structure (3). The proposed cleavage site (R925RLK928↓) is indicated in red. Constructs 2 to 6 are truncated α/β-subunit precursors missing amino acids 431 to 819/848/888/908/918, respectively. (B) BHK cells were transfected with constructs 1 to 6, followed by anti-β-subunit Western blotting, demonstrating that at least 20 amino acids proximal to the proposed cleavage site are required for cleavage. (C) BHK cells were transfected with construct 3 (wild-type; wt) or construct 3 with single mutations of the proposed cleavage site and analyzed by anti-β-subunit Western blotting. Constructs with mutations R925A, L927A, and K928A show no or reduced amounts of β subunits, indicating reduced cleavage. (D) Sequence alignment of the GlcNAc-1-phosphotransferase α/β-subunit precursor and known S1P substrates. Shading indicates the conserved cleavage consensus motif (R/K)(X)(hydrophobic)Z↓. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

**Fig. 2.** S1P-dependent cleavage of the α/β-subunit precursor. (A) MBTPS1 mRNA expression in down-regulated HeLa cells and (B) subsequent transfection with α/β-subunit precursor construct 3, followed by anti-β-subunit Western blotting. In MBTPS1 siRNA-treated cells, no β-subunit products were detected (mean ± SD; n = 3; ***P < 0.005). (C) MBTPS1 mRNA expression in CHO-7 and S1P-deficient cells (SRD-12B; mean ± SD; n = 3; ***P < 0.005). (D) SRD-12B cells fail to cleave α/β-subunit precursor. CHO-7 and SRD-12B cells were transfected with construct 3 or the R925A mutant, followed by anti-β-subunit and anti-myc Western blotting. Cotransfection with S1P-myc cDNA rescued the cleavage of the α/β-subunit precursor in SRD-12B cells.
was detected in SRD-12B cells (Fig. 2C). In CHO-7 cells the α/β-subunit precursor construct was cleaved, but its mutant form, R925A, was not, whereas no cleavage of the α/β-subunit precursor construct was observed in SRD-12B cells. Reexpression of myc-tagged S1P in SRD-12B cells completely rescued the cleavage of the α/β-subunit precursor and the formation of β subunits (Fig. 2D), supporting the role of S1P in the formation of mature GlcNAc-1-phosphotransferase subunits.

To examine the biological importance of S1P for lysosomal targeting, we determined the activities of four lysosomal hydrolases, which were found to be significantly reduced in the SRD-12B cells, with the exception of α-mannosidase. In the culture medium of SRD-12B cells, however, 6- to 10-fold increases of all enzyme activities tested were determined relative to media of CHO-7 cells, indicating missorting of newly synthesized enzymes. The hypersecretion of CHO-7, cells was set to 1 (mean ± SD; n = 5; ***P < 0.005). The marginal decrease in α-mannosidase activity in SRD-12B cells might be explained by the slow lysosomal turnover of the enzyme. The specific activities in CHO-7 cells were measured in cell extracts and conditioned media of CHO-7 and SRD-12B cells. The specific activities of the lysosomal enzymes β-hexosaminidase (β-hex), β-galactosidase (β-gal), α-mannosidase (α-mann), and α-fucosidase (α-fuc) were measured in cell extracts and conditioned media of CHO-7 and SRD-12B cells. The specific activities in CHO-7 cells were set to 1 (mean ± SD; n = 5; ***P < 0.005). The marginal decrease in α-mannosidase activity in SRD-12B cells might be explained by the slow lysosomal turnover of the enzyme present in different proteolytically processed polypeptides in α-mannosidase A and B isomeric complexes. The hypersecretion of α-mannosidase into the medium indicated mis-sorting of the newly synthesized enzyme. CHO-7 and SRD-12B cells were labeled with [35S]methionine for 1 hour and either harvested (−) or chased (+) for 4 hours, followed by immunoprecipitation of the lysosomal protease cathepsin Z from cell extracts and media (p, precursor; m, mature form). In SRD-12B cells, most newly synthesized cathepsin Z was secreted and exhibited decreased electrophoretic mobility due to complex-type oligosaccharides. (C) Cell extracts and media of CHO-7 and SRD-12B cells were analyzed by M6P Western blotting with the scFv M6P antibody fragment (18). Embryonic fibroblasts (MEF) of wild-type (wt) and MLII mice (Gnptabc<sup>c.3082insC</sup>) lacking GlcNAc-1-phosphotransferase activity were used as a control (5).

**Fig. 3.** Lysosomal enzymes are missorted in S1P-deficient cells. (A) The specific activities of the lysosomal enzymes β-hexosaminidase (β-hex), β-galactosidase (β-gal), α-mannosidase (α-mann), and α-fucosidase (α-fuc) were measured in cell extracts and conditioned media of CHO-7 and SRD-12B cells. The specific activities in CHO-7 cells were set to 1 (mean ± SD; n = 5; ***P < 0.005). The marginal decrease in α-mannosidase activity in SRD-12B cells might be explained by the slow lysosomal turnover of the enzyme present in different proteolytically processed polypeptides in α-mannosidase A and B isomeric complexes. The hypersecretion of α-mannosidase into the medium indicated mis-sorting of the newly synthesized enzyme. (B) CHO-7 and SRD-12B cells were labeled with [35S]methionine for 1 hour and either harvested (−) or chased (+) for 4 hours, followed by immunoprecipitation of the lysosomal protease cathepsin Z from cell extracts and media (p, precursor; m, mature form). In SRD-12B cells, most newly synthesized cathepsin Z was secreted and exhibited decreased electrophoretic mobility due to complex-type oligosaccharides. (C) Cell extracts and media of CHO-7 and SRD-12B cells were analyzed by M6P Western blotting with the scFv M6P antibody fragment (18). Embryonic fibroblasts (MEF) of wild-type (wt) and MLII mice (Gnptabc<sup>c.3082insC</sup>) lacking GlcNAc-1-phosphotransferase activity were used as a control (5).

**Fig. 4.** Impaired enzyme activation and accumulation of storage material in lysosomes of SRD-12B cells. (A) The M6P-dependent endocytosis of [125I]arylsulfatase B precursor (p) was not affected in SRD-12B cells whereas its subsequent proteolytic activation into mature forms (arrows) was impaired. (B) (Top) Electron micrographs revealed intracellular vacuoles resembling lysosomal structures and electron-dense storage material in SRD-12B cells. Scale bar, 1 μm. (Bottom) Higher magnification of CHO-7 and SRD-12B cells. N, nucleus; M, mitochondria. Scale bar, 0.5 μm. (C) Immunofluorescence microscopy showed accumulation of the lysosomal anionic lipid bis(monoacylglycerol) phosphate (BMP) and unesterified cholesterol stained by filipin (blue) in SRD-12B cells, which is partially colocalized with the lysosomal marker Lamp2 (green). Scale bar, 15 μm.
prominent accumulation of storage material of high electron density (Fig. 4B and fig. S4). In cells of patients or mice with various lysosomal storage disorders, secondary accumulation of lipids was observed (27). In SRD-12B cells, a variable number showed an accumulation of unesterified cholesterol and a moderate increase in staining intensity of the unusual lysophospholipid bis(monoacylglycerol)phosphate (BMP). Both lipids colocalized partially with the lysosomal marker protein Lamp2 (Fig. 4C and fig. S5). These data indicate that partial deficiencies of lysosomal enzymes in mutagenized and selected SRD-12B cells are sufficient to alter lysosomal functions.

Here we have provided evidence that S1P-mediated cleavage of the α/β-subunit precursor is associated with the activation of GlcNAc1-phosphotransferase, which is required for proper transport of lysosomal enzymes. The requirement of S1P for activation of the GlcNAc1-phosphotransferase activity, combined with its established role in lipid metabolism, indicates the importance of S1P for lysosome biogenesis and function. This may have implications for diagnosis of individuals with genetically undefined mucolipidoses II-like phenotypes such as Pacman dysplasia (22). Moreover, these findings raise the question of beneficial use of S1P inhibitors to reduce the synthesis of cholesterol, low-density lipoprotein (LDL), and fatty acids in treating cardiovascular disorders or as an antiviral therapy (23–25) owing to their unanticipated deleterious effects on lysosomal function.

### References and Notes


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**Long Unfolded Linkers Facilitate Membrane Protein Import Through the Nuclear Pore Complex**

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Active nuclear import of soluble cargo involves transport factors that shuttle cargo through the nuclear pore complex (NPC) by binding to phenylalanine-glycine (FG) domains. How nuclear membrane proteins cross through the NPC to reach the inner membrane is presently unclear. We found that at least a 120-residue-long intrinsically disordered linker was required for the import of membrane proteins carrying a nuclear localization signal for the transport factor karyopherin-α. We propose an import mechanism for membrane proteins in which an unfolded linker slices through the NPC scaffold to enable binding between the transport factor and the FG domains in the center of the NPC.

The nuclear envelope (NE) consists of an inner (INM) and outer nuclear membrane (ONM) connected by the pore membrane at sites where the nuclear pore complexes (NPCs) are embedded. The ONM is continuous with the endoplasmic reticulum (ER). NPCs are composed of a membrane-anchored scaffold that stabilizes a cylindrical central channel, in which nucleoporin (Nups) with disordered phenylalanine-glycine (FG)-rich regions provide the selectivity barrier (1). For a membrane protein to move through the NPC, its transmembrane (TM) domains must pass through the pore membrane, while its extraluminal soluble domain(s) must pass through the NPC by a mechanism yet to be clarified (2–4).

Some proteins reach the INM by diffusing through the pore membrane and adjacent lateral channels (5–8) and accumulate by binding nuclear structures (9, 10). Other membrane proteins have a nuclear localization signal (NLS), and binding to transport factors karyopherin-α and karyopherin-β1 is required to pass the NPC and reach the INM (11, 12). We sought to investigate the mechanism and path of nuclear transport of these integral INM proteins.

We first generated reporters using the Saccharomyces cerevisiae homolog of the human LEM domain–containing integral INM protein, Huh2. Huh2 is composed of a LEM domain, a bipartite NLS (hereafter h2NLS), a linker region (L), two TM segments flanking a luminal domain (LD), and a domain with homology to the C terminus of MAN1 (Fig. 1A) (12). The h2NLS is recognized by Kap60 (also known as Srp1 or Karyopherin-α), the yeast homolog of human Importin-α (12). Similar to Huh2, the reporter protein h2NLS-L–TM, consisting of green fluorescent protein (GFP) fused to amino acids 93 to 378 of Huh2, accumulated specifically at the NE (Fig. 1B). A control lacking the h2NLS, named L-TM, distributed over the NE and cortical ER. Although we could not resolve the INM from the ONM, we used the average pixel intensities at the NE and ER (NE-ER ratio) as a measure of INM accumulation (fig. S2, A and B). We validated this approach by confirming the localization of h2NLS-L–TM to the INM using immuno–electron microscopy (Fig. 1C and fig. S2C). h2NLS-L–TM accumulated 33-fold at the NE (Fig. 1B), whereas L-TM accumulated only 2-fold.

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