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Running Head: ER and lysosomal quality control of mutant IDS

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Keywords: endoplasmic reticulum, formylation, glycosaminoglycans, Hunter's disease, iduronate-2 sulfatase, nonsense mutations, lysosomal storage diseases, lysosome, molecular dynamics, mucopolysaccharidosis type II

ABSTRACT

Hunter's syndrome (mucopolysaccharidosis type II) is a rare X-linked lysosomal storage disorder caused by mutations in the iduronate 2-sulfatase (IDS) gene. Motivated by the case of a child affected by this syndrome, we compared the intracellular fate of wild type IDS (IDS_{WT}) and of four nonsense mutations of IDS (IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X}) generating progressively shorter forms of IDS associated with mild to severe forms of the disease. Our analyses revealed formylation of all forms of IDS at cysteine 84, which is a pre-requisite for enzymatic activity. After formylation, IDS_{WT} was transported within lysosomes, where it was processed in the mature form of the enzyme. The length of disease-causing deletions correlated with gravity of the folding and transport phenotype, which was anticipated by molecular dynamics analyses. The shortest form of IDS, IDS_{W337X}, was retained in the endoplasmic reticulum (ER) and degraded by the ubiquitin-proteasome system. IDS_{R443X}, IDS_{Y452X}, IDS_{L482X} passed ER quality control, were transported to the lysosomes, but failed lysosomal quality control resulting in their rapid clearance and in loss-of-function phenotype. Failure of ER quality control inspection is an established cause of loss-of-function observed in protein misfolding diseases. Our data reveal that fulfillment of ER requirements might not be sufficient, highlight lysosomal quality control as the distal station to control lysosomal enzymes fitness and pave the way for alternative therapeutic interventions.

INTRODUCTION

Lysosomal storage disorders (LSDs) are rare, inherited, progressive diseases characterized by impaired lysosomal activity with abnormal accumulation of macromolecules in the lumen of lysosomes (Marques and Saftig, 2019). Mucopolysaccharidosis type II (MPS II, Hunter's syndrome) (Hunter, 1917) is a rare X-linked recessive LSD characterized by mutations of the gene encoding for iduronate 2-sulfatase (IDS) (Bach et al., 1973; Stenson et al., 2014; Wraith et al., 2008). IDS deficiency leads to accumulation of glycosaminoglycans within the lysosomes of cells and tissues throughout the body (Muenzer et al., 2009). MPS II patients exhibit multi-organ symptoms, including progressive deafness, skeletal deformities, carpal tunnel syndrome, hepatosplenomegaly, frequent respiratory infections, cognitive impairment and hydrocephalus (Muenzer et al., 2009; Whiteman and Kimura, 2017). The *IDS* gene encodes a pre-pro-polypeptide of 550 residues. During ER translocation, side chains of 7 asparagine residues are N-glycosylated, the cysteine 84 is modified in formylglycine (oxoalanine) as key catalytic residue in the IDS active site (Schmidt et al., 1995) and 2 intramolecular disulfide bonds are formed (**Fig. 1A**) (Demydchuk et al., 2017). The native polypeptide is transported to the lysosomes, where it is proteolytically activated on removal of part of the N-terminus and cleavage of the SD2 regulatory peptide that remains non-covalently associated with the catalytic SD1 domain (**Fig. 1B**) (Demydchuk et al., 2017; Froissart et al., 1995). More than 500 IDS mutations have been reported, but little is known about consequences thereof on IDS biogenesis and lysosomal transport (Demydchuk et al., 2017). Analysis of few MPS II-causing IDS mutants recently led to ascribe the associated loss-of-function disorder to mutant's clearance via ER-associated degradation (ERAD) and to propose ERAD modulators as targets for therapeutic intervention (Osaki et al., 2019; Osaki et al., 2018). Motivated by the request of a family to study the IDS_{W337X} nonsense mutant, we compared its intracellular fate and the intracellular fate of three other MPS II-causing nonsense mutants (i.e., IDS_{R443X}, IDS_{Y452X}, IDS_{L482X}) with that of the IDS_{WT} protein. The clinical correlation between patients with the same mutation is imperfect. However, according to the literature, the truncation at residue 337 invariably correlates with severe MPS II (Keeratichamroen et al., 2008; Kosuga et al., 2016; Sukegawa et al., 1995), whereas truncations at residues 443, 452 and 482 are characterized by intermediate to mild forms of the loss-of-function disease (Brusius-Facchin et al., 2014; Filocamo et al., 2001; Froissart et al., 1998; Keeratichamroen et al., 2008; Kosuga et al., 2016; Sohn et al., 2012; Sukegawa et al., 1995; Vafiadaki et al., 1998). Here we performed molecular dynamic simulations that revealed structural instability only for IDS_{W337X}. These predictions were eventually supported by *in cellulo* analyses showing that IDS_{W337X} does not pass ER quality control and is cleared by ER-associated degradation (ERAD). IDS_{R443X}, IDS_{Y452X}, IDS_{L482X} and IDS_{wt} pass ER quality control and are transported within lysosomes. However, in

contrast to IDS_{wt}, which is processed into the enzymatically active form, IDS_{R443X}, IDS_{Y452X}, IDS_{L482X} are rapidly degraded. All in all, in addition to available literature indicating the failure to pass ER quality control and the subsequent intervention of the ubiquitin proteasome system as causative of loss-of-function phenotypes ((Ulloa-Aguirre et al., 2004) and (Osaki et al., 2019; Osaki et al., 2018) for the specific case of MPS II), our data involve the lysosomal compartment as a major site of clearance of structurally aberrant gene products linked to LSDs.

Material and Methods

IDS plasmids, primers, antibodies and inhibitors

IDS_{WT} and IDS_{W337X} constructs were ordered by GenScript (Piscataway, USA). IDS_{R443X}, IDS_{Y452X}, IDS_{L482X} were generated in our lab amplifying pcDNA3.1 containing the IDS_{WT} sequence by PCR using 5'-GATCACACCGGTACCCCACCATGCCGCCA-3' as forward primer and, as reverse primers, 5'-ACCTTCCTGAAGCATTTTGCGGCCGCCCCACACTAG-3' for IDS_{R443X}, 5'-ACTTGGAAGAGGATCCGGCGGCCGCCCCACACTAG-3' for IDS_{Y452X} and 5'-ATTCTGACAAGCCGAGTGCGGCCGCCCCACACTAG-3' for IDS_{L482X}. HA-tag antibodies are from Sigma-Aldrich (Buchs, Switzerland), anti-IDS from R&D Systems (Minneapolis, USA). PS341 (Millenium Pharmaceuticals) and BafA1 (Calbiochem and Sigma Aldrich, Buchs, Switzerland) were used at final concentration of 10 µM and 50-100 nM, respectively.

Cell lines and transient transfection

HEK and MEF were cultured in 4.5 g/L D glucose D-MEM (Thermo Fisher Scientific, Waltham, USA) containing 10% bovine fetal serum (FBS). Cells were seeded in 6 cm culture dishes/12-well plates. Transfection was performed using JetPrime reagent (Polyplus transfection, Illkirch, France) and 1-1.5 µg of total IDS plasmid. Experiments were performed 17 h post-transfections.

Cell lysis and western blots

Transfected cells were washed with phosphate-buffered saline (PBS) with 20 mM *N*-ethylmaleimide (NEM) and were subsequently lysed on ice for 20 min in 2% CHAPS (Anatrace, Maumee, USA) in HEPES-buffered saline at pH 6.8 containing 20 mM NEM and protease inhibitor cocktails. After centrifugation at 10'000 x g for 10 min, postnuclear supernatants were collected and aliquoted into Eppendorf tubes. Lysates were reduced and denatured using sample buffer supplemented with dithiothreitol (DTT) for 5 min at 95°C. Samples were then separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were subsequently transferred to PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad, Cressier, Switzerland). Rabbit anti-HA and goat anti-IDS were used as primary antibodies, whereas anti-proteinA horseradish peroxidase (HRP) and anti-goat HRP as secondary antibodies. Proteins were revealed with the Luminata Forte Western HRP substrate (Millipore, Schaffhausen, Switzerland). Chemiluminescence was detected by ImageQuant LAS 4000 system in both standard and high acquisition (GE Healthcare Life Sciences, Glattbrugg, Switzerland).

Metabolic labeling, immunoprecipitations and radiolabeled protein detection

Proteins produced by HEK cells were radiolabeled for 10 min using D-MEM containing 0.1 mCi [^{35}S]-methionine/cysteine (pulse solution). Chase was performed in D-MEM supplemented with non-radiolabeled methionine and cysteine. Cells were lysed as described above. Where needed, PS341 or BafA1 were added to the chase solution at concentrations described above. PNSs were immunoprecipitated using protein A/protein G beads and anti-HA/anti-IDS at 4°C for 2.5 h. Subsequently, beads were washed twice with 0.5% CHAPS and proteins were separated in reducing SDS-PAGE. Gels were dried and exposed to autoradiography films (GE Healthcare, Fuji) to reveal radiolabeled bands using Typhoon FLA 9500 software, version 1.0. Protein quantification was performed using ImageQuant software (Molecular Dynamics, GE Healthcare).

Immunofluorescence analyses

1.5 x 10^5 MEFs were seeded in 12-well plates on Alcian blue coverslips, transfected as previously described and incubated with/without 50 nM BafA1. After 12 h, cells were washed twice with PBS and fixed in 3.7% formaldehyde for 20 min at room temperature (RT). Then, cells were washed three times with PBS and permeabilized using a solution composed of 10% goat serum, 15 mM glycine, 0.05% saponin and 10 mM HEPES in PBS (PS) for 15 min at RT. After permeabilisation, cells were incubated with the anti-IDS diluted 1:100 and anti-LAMP1 diluted 1:50 in PS for 90 min at RT. Subsequently, cells were washed three times (5 min each) in PS, supplemented with Alexa Fluor-conjugated secondary antibody diluted 1:300 in PS for 40 min, washed with PS and water, mounted with Vectashield (Vector Laboratories, Burlingame, USA) containing 4',6-diamidino-2-phenylindole (DAPI). Pictures were collected by Leica TCS SP5 microscope with a 63x/1.4 N.A. objective (Leica HCX PL APO lambda blue 63.0 x 1.40 OIL UV, Wetzlar, Germany).

Formylation assessment by mass spectrometry

After immunoprecipitation, proteins were eluted from the beads by heating beads at 50 °C for 10 min in sample buffer containing 2% SDS (nonreducing conditions). Proteins were then separated by SDS-PAGE on a 12% acrylamide gel over a distance of 4.0 cm. After rapid Coomassie Blue staining, bands were excised and in-gel reduced and alkylated with chloroacetamide and digested with chymotrypsin (Promega) as described (Wilm et al., 1996). Data-dependent LC-MS/MS analyses of extracted peptide mixtures after digestion was carried out on a Fusion tri-hybrid orbitrap mass spectrometer (Thermo Fisher Scientific) interfaced to a Dionex RSLC 3000 nano-HPLC. Peptides were separated on a 60 min gradient from 5% to 50% acetonitrile in 0.1% formic acid at 0.3 µl/min on a PepMap column (75 µm ID x 25 cm, 2.0 µm, 100Åµ, Dionex). Full MS survey scans were performed at

120'000 resolution. In data-dependent acquisition controlled by Xcalibur 2.1 software (Thermo Fisher), the twenty most intense multiply charged precursor ions detected in the full MS survey scan were selected for collision-induced dissociation and analysis in the linear trap with an isolation window of 1.6 m/z and then dynamically excluded from further selection during 60s. Collections of tandem mass spectra from all five fractions for each sample were pooled for database searching using Mascot (Matrix Science, London, UK; version 2.5.0) against the release 2014_08 of the SWISSPROT database restricted to human taxonomy. Mass tolerances used were 10 ppm for the precursors and 0.5 Da for CID fragments. The software Scaffold 4.4.1.1 (Proteome Software Inc.) was used to validate MS/MS based peptide identifications (minimum 95% probability (Keller et al., 2002) and protein (min 99 % probability (Nesvizhskii et al., 2003)), perform dataset alignment and subtraction as well as parsimony analysis to discriminate homologous hits.

IDS enzymatic activity assay

HEK293 cells were washed twice on ice with PBS without calcium and magnesium. Afterwards, 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS was used to gently detach the cells. HEKs were collected in an Eppendorf tube and centrifuged at 1'500 x g for 5 min. EDTA solution was removed by washing the pellet twice with PBS. After washing, cells were centrifuged at 1'500 x g for 5 min. Enzymatic activity assay was performed by fluorimetric determination of IDS-mediated hydrolyzation of 4-methylumbelliferyl-α-L-iduronide-2-sulphate to 4-methyl umbelliferone (Voznyi et al., 2001) (Moscerdam, Oegstgeest, Netherlands).

Molecular dynamics (MD) simulations

MD simulations were performed using the X-ray structure of the IDS (PDB ID code 5FQL) (Demydchuk et al., 2017) as a starting point. The five systems used in MD simulations (IDS_{WT}, IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X}) were obtained deleting the residue between the truncation site and the C-terminal end of the protein. All systems, consisting of the protein and the covalently bound glycans included in the x-ray structure, were then prepared for MD with 'protein preparation wizard module' available in the Schrodinger suite for molecular modelling. This procedure automatically adds missing atoms to side chains, models short unresolved protein loops and assigns the correct atom names according to the chosen force field. The short loop connecting SD1 to SD2, not solved in the x-ray structure and susceptible to proteolytic cleavage was modelled in this preparation step. The protonation state of protein residues was assigned using Propka considering a reference pH of 7.4 (Olsson et al., 2011). The protein structures were then solvated in box of water with minimal distance from the protein surface of 10 Å. The neutrality of the system

was ensured adding the proper number of counter ions. All the non-solvent molecules were described by the OPLS3 (Harder et al., 2016) force field, while TIP3P model (Jorgensen et al., 1983) was used for water molecules. The systems were equilibrated following the equilibration protocol recommended for Desmond MD simulations: (1) Brownian Dynamics (100 ps) in a NVT enable (T=10 K) applying harmonic restraints on solute heavy atoms (force constant 50 kcal/mol/ Å); (2) NVT (T=10K) MD simulation of 12 ps in NVT ensemble conserving the same restraints applied in (1); (3) NPT (T=300K and P=1atm) MD simulation (12 ps) conserving the same restraints applied in (1); (4) NPT (T=300K and P=1atm) MD simulation (24 ps) without restraints. Finally, all the systems were simulated for 500 ns. The pressure and the temperature were fixed at 300 K and 1 atm by the Martyna-Tobias-Klein NPT thermostat scheme. RMSD value were calculated considering only the backbone atoms using the tool implemented in the VMD program (Humphrey et al., 1996). RMSF has been calculated by the g_rmsf tool implemented in GROMACS 2016.5. considering backbone atoms (Abraham et al., 2015).

RESULTS

Molecular dynamics (MD) simulations

MD simulations allow prediction of protein stability (Adcock and McCammon, 2006; Amir et al., 2019). In our analyses, we compared the structural stability of the 550-residues, full-length IDS protein (IDS_{WT}, **Fig. 1A**) with the structural stability of four forms of IDS linked to MPS II and truncated at position 482 (IDS_{L482X}), 452 (IDS_{Y452X}), 443 (IDS_{R443X}) or 337 (IDS_{W337X}), respectively (**Fig. 1C**). To this end, we monitored structural quantities such as Root Mean Square Fluctuations (RMSF) and Root Mean Square Deviation (RMSD) to assess protein stability and deviation from the reference IDS_{WT} crystal structure (Demydchuk et al., 2017; Dong et al., 2018; Khan et al., 2016). The larger RMSF and RMSD values for IDS_{W337X} (**Fig. 1D**) hint at higher instability of IDS_{W337X}, compared to IDS_{WT}, IDS_{L482X}, IDS_{Y452X} and IDS_{R443X}, all characterized by similar RMSF and RMSD values (**Fig. 1D**). Newly synthesized polypeptides are subjected to a stringent quality control in the ER. Structural instability of IDS_{W337X} led us to anticipate the retention of this mutant polypeptide in the ER and its rapid degradation, thus explaining the loss-of-function disease with severe outcome linked to this mutation. What about the other missense mutations for which MD analyses failed to reveal significant stability impairments, but are linked to a loss-of-function disease?

Expression of WT and nonsense IDS mutants

To verify the reliability of the predictions made by MD simulations and to establish the consequences of loss-of-function truncations on IDS maturation and fulfillment of ER quality control requirements for lysosomal transport, we compared the intracellular fate of IDS_{WT} with that of IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X}. The five HA-tagged proteins were ectopically expressed in human embryonic kidney cells (HEK293) or in mouse embryonic fibroblasts (MEF). After detergent solubilization, cellular proteins were separated in SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (western blot, WB). IDS_{wt} and the four IDS variants were revealed with a polyclonal, IDS-specific antibody (**Fig. 2A**), or with an anti-HA tag antibody that recognizes the C-terminus of the IDS proteins (**Fig. 2B**). Consistent with the expected processing pattern (**Fig. 1A, B**) (Froissart et al., 1995), the WT protein was separated in four distinct bands labeled as FL (full-length, unprocessed precursor), 50kD, SD1 and SD2. These polypeptides are revealed with the IDS-specific antibody (**Fig. 2A**, lane 1). Only FL was visible in the WB revealed with the anti-HA antibody (**Fig 2B**, lane 7). This was expected since the HA-tag displayed at the polypeptide C-terminus is lost when IDS_{wt} is processed into mature fragments within the lysosome.

IDS_{L482X} ends 39 residues after the expected processing site, which is at position 443 of the IDS polypeptide. However, the polypeptide runs as a single polypeptide band when separated by gel

electrophoresis (**Fig. 2A**, lane 2). This reveals that the truncation of most of the SD2 domain impairs proteolytic maturation of IDS_{L482X} that would generate polypeptides running at the same level as the 50kD and the SD1 generated on processing of IDS_{wt} (**Fig. 2A**, lane 1). The electrophoretic mobility of the IDS_{L482X} with an apparent MW of 66 kD indicates defective processing at the C-terminus (consistently, the polypeptide retains the HA-epitope, **Fig. 2B**, lane 8) and a possible defect in removal of the pro-peptide (**Fig. 1C**). The other variants examined in this study are truncated immediately after (IDS_{Y452X}), or at the physiologic site of IDS cleavage (IDS_{R443X}), respectively (**Fig. 1C**). They have an apparent MW of 60 and 55 kD (**Fig. 2A**, lanes 3 and 4, respectively), which is consistent with the presence of the HA-tag (confirmed in **Fig. 2B**, lanes 9 and 10, respectively) and a possible defect in removal of the pro-peptide (**Fig. 1C**). IDS_{W337X} conserves all N-glycans of the SD1 domain but lacks 25% of the amino acid stretch and one of the two intramolecular disulfide bonds (**Fig. 1C**). It runs with an apparent MW of 47 kD, it is poorly recognized by the anti-IDS polyclonal antibody (**Fig. 2B**, lane 5), but is very efficiently detected by the anti-HA antibody (**Fig. 2B**, lane 11). All in all, analyses of total cell lysates revealed high level expression of all IDS variants examined in this study. Based on the WB analyses we can speculate that only IDS_{WT} is appropriately processed within lysosomes into the mature form.

Activity measurement and formylation of IDS variants

Oxidation of the thiol group of Cys84 in the Cys-Ala-Pro-Ser-Arg- sequon to 2-formylglycine by formylglycine-generating enzyme (FGE) (Dierks et al., 1999) is required for activity of eukaryotic sulfatases and occurs in the ER either co-, or post-translationally (Schmidt et al., 1995). Mass spectrometry of chymotryptic fragments of IDS_{WT}, IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X} (see methods section) revealed that all proteins were appropriately modified at cysteine 84. However, when the enzymatic activity was monitored by fluorimetric determination of the IDS-mediated hydrolyzation of 4-methylumbelliferyl-α-L-iduronide-2-sulphate to 4-methylumbelliferone according to a well-establish diagnostic assay (Voznyi et al., 2001), only cells expressing IDS_{WT} were considered competent (**Fig. 2C**). Lack of enzymatic activity for cells expressing IDS_{W337X} was not surprising. IDS_{W337X} is linked to severe MPS II, it lacks 25% of the catalytic domain including one catalytic residue (Lys347) and the Cys_{422}-S-S-Cys_{432} disulfide bond, which is crucial for enzymatic activity. Moreover, our MD analyses predict extensive instability of this polypeptide (**Fig. 1D**). However, enzymatic activity was also undetectable in cells expressing the IDS_{L482X}, IDS_{Y452X} and IDS_{R443X} (**Fig. 2C**) for which MD analyses failed to reveal structural instability and which contain the entire catalytic domain with the ten active site residues that are highly conserved amongst sulfatases (D45, D46, C84, R88, K135, H138, H229, D334, H335, and K347) (Demydchuk et al.,

2017). Lack of enzymatic activity This is consistent with our speculation based on **Fig. 2A-B** that only IDS_{WT} is appropriately processed within lysosomes into the mature form.

Time-resolved analyses of IDS fate

To determine the fate of newly synthesized IDS variants, cells expressing the model polypeptides were pulse labeled with ^{35}S-methionine and cysteine for 10 min. Radiolabeled IDS was immunoisolated from cell lysates at increasing times after the pulse (10-360 min, **Fig. 3A-E**). Immunoisolation of radiolabeled IDS variants with the anti-HA that recognizes the unprocessed forms of the proteins revealed that in all cases, these forms disappeared during the chase (**Fig. 3A-E**, lanes 1-6). The immunoisolation of the radiolabeled IDS variants with an anti-IDS antibody revealed that disappearance of the HA epitope correlated with disappearance of the IDS signal for IDS_{L482X} (**Fig. 3B**, lanes 7-12), IDS_{Y452X} (**Fig. 3C**, lanes 7-12), IDS_{R443X} (**Fig. 3D**, lanes 7-12) and IDS_{W337X} (**Fig. 3E**, lanes 7-12) variants, showing that these proteins were degraded during the chase. In contrast, for the WT protein, disappearance of the HA epitope (**Fig. 3A**, lanes 1-6) resulted from processing of the polypeptide C-terminus as indicated by the appearance of proteolytic fragments recognized by the anti-IDS antibody (**Fig. 3A**, arrows in lanes 10-12) corresponding to the 50 kD and the SD1 proteolytic fragments shown by WB (**Fig. 2A**, lane 1). These results show that the truncated variants of IDS were degraded during the chase, whereas the WT form was proteolytically processed within the lysosomes in the enzymatically active form of IDS and are consistent with the finding reported above that IDS activity is only measured in cells expressing the WT form of the protein.

Lysosomal and proteasomal degradation of truncated IDS variants

Protein folding is error-prone and the efficiency of the folding process is reduced by mutations in the polypeptide chains. Most folding-defective polypeptides produced in the ER are eventually dislocated into the cytosol for proteasomal degradation in processes defined as ER-associated degradation (ERAD). In the particular case of Hunter's disease, ERAD has been proposed as the catabolic pathway that clears from cells mutant forms of IDS thus causing the loss-of-function phenotype characterizing the disorder (Osaki et al., 2019; Osaki et al., 2018). To verify the involvement of ERAD in disposal of the truncated versions of IDS analyzed in our work, cells expressing IDS_{WT}, IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X} were metabolically labeled for 10 min and chased for 10 min to visualize the initial amount of IDS expressed in the cells (**Fig. 3F-J**, lane 13 for HA-tagged IDS and lane 17 for total IDS) or for 240 min to appreciate the disappearance of HA-tagged IDS (**Fig. 3F-J**, lane 14), or of the total IDS signal (lane 18) during the chase. The 240 min chase time was also performed for cells grown in the presence of an inhibitor of lysosomal (BafA1, **Fig. 3F-J**, lanes 15

and 19) or proteasomal activity (PS341, lanes 16 and 20). For IDS_{WT}, as shown in **Fig. 3A**, lanes 1-6), the HA-epitope disappears during the chase (**Fig. 3F**, lane 13-14). IDS_{WT} is in fact processed on delivery within lysosomes to the enzymatically active form (**Fig. 3A**, lanes 7-12 and **3F**, lanes 17-18) (Froissart et al., 1995). Proteasomal inactivation does not affect disappearance of the HA-epitope (**Fig. 3F**, lane 16) nor the processing of IDS_{WT} (lane 20). In contrast, lysosomal inactivation inhibits both disappearance of the HA epitope (**Fig. 3F**, lane 15) and the formation of the processed forms of IDS_{WT} (lane 19). This is consistent with the fact that IDS_{WT} is delivered within lysosomes, where it is proteolytically cleaved in the active form of the protein (**Fig. 1B**) (Froissart et al., 1995). For the truncated variants IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} that disappear during the chase without forming distinguishable proteolytic fragments (**Fig. 3B-D**), proteasomal inactivation does not significantly affect clearance (**Fig. 3G-I**, lane 16 and 20), whereas lysosomal inactivation stabilizes the proteins (lanes 15, 19). In sharp contrast, clearance from cells of the shortest variant of IDS analyzed in this study (IDS_{W337X}) is inhibited on proteasomal (**Fig. 3J**, lanes 16, 20), but not on lysosomal inactivation (lanes 15, 19). All in all, IDS_{WT} is delivered within lysosomes, where it is activated on proteolytic processing. IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} pass ER quality control and are eventually delivered within lysosomes, where they undergo proteolytic degradation. Consistent with MD analyses that highlighted the IDS_{W337X} as the most perturbed form of the protein, this aberrant polypeptide remains trapped by the ER quality control machinery, which selects it for ERAD.

Lysosomal delivery of wt and truncated IDS variants

We reasoned that if our conclusions were correct, confocal laser scanning microscopy (CLSM) should reveal lysosomal localization of IDS_{WT} at steady state. In contrast, IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} that are degraded by lysosomal enzymes, should be visible within lysosomes only upon lysosomal inactivation. Lysosomal inactivation should not result in accumulation of IDS_{W337X} within lysosomes, as this IDS variant is an ERAD substrate. To verify this, cells expressing IDS_{WT}, IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} and IDS_{W337X} were incubated for 4 hr with BafA1 before processing for CLSM. As predicted, in these cells, all IDS variants (**Fig. 4A-D**), but IDS_{W337X} (**Fig. 4E**), did accumulate in lysosomes displaying LAMP1 at their limiting membrane. On BafA1 wash-out, only IDS_{WT} was stable in the lysosomes (**Fig. 4F**), where it is proteolytically processed to the mature form (**Fig. 2A**, lane 1, **3A**, lanes 7-12, **3F**, lanes 17-19) and acquire enzymatic activity (**Fig. 2C**). IDS_{L482X}, IDS_{Y452X}, IDS_{R443X} disappeared from the lysosomal lumen consistent with their intracellular degradation (**Fig. 3B-D**), which is executed in the lysosomes (**Fig. 3G-I**, lanes 15 and 19). IDS_{W337X}, which is linked to the most severe form of MPS II, is dramatically misfolded (**Fig. 1D**), does not pass ER quality control and is translocated in the cytosol for proteasomal degradation (**Fig. 3J**, lanes 16 and 20).

DISCUSSION

Our analyses of the intracellular fate of IDS_{WT} and of 4 disease-linked truncated versions of IDS, reveal a correlation between structural instability as predicted by MD calculations and failure to pass ER quality control checkpoints for the shortest IDS variant under investigation. IDS_{W337X}, which is linked to severe forms of MPS II, is retained in the ER and is dislocated across the ER membrane for proteasomal degradation. As such, IDS_{W337X} is a classical substrate of the ERAD machinery, whose failure to attain a native structure prevents transport at the organelle of destination, in this case the lysosome, and results in a loss-of-function LSD. The other truncated versions of IDS analyzed in our study are not distinguished from the wild type form of the protein by MD analyses. They all pass ER quality control to some extent and are appropriately transported within lysosomes. In sharp contrast to IDS_{WT}, however, truncated forms of IDS are rapidly degraded in the lysosomal lumen and their activity remains below detection, thereby explaining the loss-of-function disease state of the carriers of these nonsense mutations. The ER is site of production of all secretory and membrane-bound proteins and of proteins destined to the cellular organelles, including the lysosome. It is considered the site, where the gene products are subjected to a stringent quality control that selects structurally imperfect gene products for degradation thus resulting in loss-of-function diseases. As such, modulation of processes occurring within the ER such as post-translational modifications, chaperone-assisted protein folding, selection for secretion or for degradation are strong candidates for therapeutic intervention and chemical and pharmacologic chaperones that enhance folding of mutant gene products are entering the clinics (Noack et al., 2014; Pilla et al., 2017). Our data reveal that passing ER quality control and arrival at the intracellular site of destination, might not be sufficient to warrant healthy status. They highlight lysosomal quality control as the distal station to control lysosomal enzymes fitness and pave the way for alternative therapeutic interventions.

Acknowledgments

We thank the members of Molinari's lab for discussions. M. Quadroni (Protein Analysis Facility of the University of Lausanne). M.M. is supported by Signora Alessandra, AlphaONE Foundation, Foundation for Research on Neurodegenerative Diseases, Swiss National Science Foundation, Comel and Gelu Foundations.

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