A Golgi-localized Mannosidase (MAN1B1) Plays a Non-enzymatic Gatekeeper Role in Protein Biosynthetic Quality Control*

Received for publication, January 21, 2014, and in revised form, March 11, 2014 Published, JBC Papers in Press, March 13, 2014, DOI 10.1074/jbc.M114.552091

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Background: The capacity of Golgi-localized MAN1B1 to promote ERAD was investigated.

Results: The MAN1B1 catalytic domain is dispensable for ERAD, which helped identify an important conserved, non-enzymatic decapeptide sequence within the luminal stem.

Conclusion: The tempo-spatial expansion of quality control in the secretory pathway includes evolutionary gain-of-function for MAN1B1.

Significance: Evolutionary distinctions can exist in how quality control systems operate.

Conformation-based disorders are manifested at the level of protein structure, necessitating an accurate understanding of how misfolded proteins are processed by the cellular proteostasis network. Asparagine-linked glycosylation plays important roles for protein quality control within the secretory pathway. The suspected role for the MAN1B1 gene product MAN1B1, also known as ER mannosidase I, is to function within the ER similar to the yeast ortholog Mns1p, which removes a terminal mannose unit to initiate a glycan-based ER-associated degradation (ERAD) signal. However, we recently discovered that MAN1B1 localizes to the Golgi complex in human cells and uncovered its participation in ERAD substrate retention, retrieval to the ER, and subsequent degradation from this organelle. The objective of the current study was to further characterize the contribution of MAN1B1 as part of a Golgi-based quality control network. Multiple lines of experimental evidence support a model in which neither the mannosidase activity nor catalytic domain is essential for the retention or degradation of the misfolded ERAD substrate Null Hong Kong. Instead, a highly conserved, vertebrate-specific non-enzymatic decapeptide sequence in the luminal stem domain plays a significant role in controlling the fate of overexpressed Null Hong Kong. Together, these findings define a new functional paradigm in which Golgi-localized MAN1B1 can play a mannosidase-independent gatekeeper role in the proteostasis network of higher eukaryotes.

Although DNA is the natural carrier of inherited genetic information, the functional implementation of a gene lies within the structural maturation of the encoded protein (1–3).

Quality control during protein synthesis and folding is, therefore, essential for cellular health (4). During these processes it is critical to remove misfolded proteins and unassembled complexes, not only to prevent the deployment of dysfunctional molecules but to deter their persistent interaction with cellular protein folding machinery that may disrupt protein homeostasis (i.e. proteostasis) (5, 6). In recent years, intense investigation has begun to elucidate how the proteostasis network operates to manage the cellular proteome. The generated knowledge and emerging insights are expected to identify novel sites for the therapeutic intervention of numerous genetic diseases manifested at the level of aberrant protein structure (4).

Two of the central responsibilities for the proteostasis network are to first promote the conformational maturation of newly synthesized polypeptides and then orchestrate the selective retention and elimination of those molecules unable to attain native structure (7). These operations are perhaps best illustrated in the secretory pathway, a collection of organelles and vesicles through which newly synthesized secretory and membrane proteins are deployed (8, 9). Although proteostasis network components are distributed along these various compartments, conformational maturation is initiated in the endoplasmic reticulum (ER), which functions as the site for nascent protein translocation.

The majority of secretory proteins are co-translationally labeled with asparagine-linked oligosaccharides (N-glycans) (10). N-Glycans and the enzymes that covalently modify these appendages, are considered important players in promoting the successful folding of newly synthesized N-glycoproteins (2, 10–13). Distinct modifications, including the removal of terminal mannose units from N-glycans, are utilized to somehow

* The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; MAN1B1, mannosidase, α, class 1B, member 1; N1K, Null Hong Kong; COP, coat protein complex; KIF, kifunensine; EV, empty vector; WCL, whole cell lysate; DPS, decapeptide sequence; EDEM, ER degradation-enhancing mannose-like protein; SEL1L, sel-1 suppressor of lin-12-like (C. elegans) (human); ANOVA, analysis of variance.

** This work was supported, in whole or in part, by National Institutes of Health Grant RO1 DK064232. This work has also been supported by a grant from the Alpha1-Foundation (to R. N. S.) and Huffington Center on Aging Biology Training Grant T32AG000183-19 (to M. J. I.).

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target misfolded N-glycoproteins into a multistep disposal system designated ER-associated degradation (ERAD), culminating in their dislocation into the cytosol for elimination by 26 S proteasomes (6, 14, 15). The type II membrane protein MAN1B1, also designated ER mannosidase I (ERManI), is suspect to function in the mammalian ERAD system similar to its ortholog in budding yeast (Mns1p), which catalyzes the hydrolysis of a specific terminal α1,2-linked mannose unit (16–20). This initial step induces additional mannose trimming to promote recognition of the misfolded N-glycoprotein by downstream ERAD machinery before removal from the ER and subsequent degradation (6, 21, 22). Despite this traditional view, recent evidence from multiple investigators has begun to re-evaluate how N-linked glycan processing contributes to mammalian ERAD (11, 23–28). In support of this growing debate, we recently reported that MAN1B1 resides in the Golgi complex in numerous human cell lines rather than in the ER (29). Additional studies have begun to implicate its function as a possible cargo receptor based on its location, capacity to co-immuno-precipitate with the N-glycoprotein ERAD substrate Null Hong Kong (NHK), and the presence of γ-COP binding motifs in its N-terminal cytoplasmic tail (30). In this context, MAN1B1 was proposed to participate within a putative retention complex to promote the capture of escaped ERAD substrates in the Golgi complex and their loading into COPI vesicles for recycling back to the ER.

Understanding the manner in which biosynthetic protein maturation and disposal systems operate in any given organism is essential to ensure the efficacy of mechanism-based disease interventions. To this end, we recently discovered that a single nucleotide polymorphism in the MAN1B1 gene capable of suppressing the translation of the encoded MAN1B1 polypeptide is tightly linked to the early onset of end-stage liver disease in α1-antitrypsin deficiency (31). Considering that the human homolog resides in the Golgi complex and its reduced expression diminishes both enzymatic and potential non-enzymatic functions of the protein, in the present study we examined the contribution of both properties to the Golgi-based quality control system. Based on several lines of experimental evidence, we have substantiated that the capacity for Golgi-localized MAN1B1 to influence the fate of overexpressed NHK occurs in a non-enzymatic manner. In support of this conclusion and the evolutionary repurposing of MAN1B1, we identified a highly conserved, vertebrate-specific non-enzymatic luminal decapptide sequence that contributes to both ERAD substrate retention and elimination.

**EXPERIMENTAL PROCEDURES**

*Materials and Reagents—* Common chemicals and buffers were from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) was from Mediatech, Inc. Opti-MEM transfection media and fetal bovine serum (FBS) were from Invitrogen. Sodium fluoride (NaF), sodium orthovanadate (Na3VO4), PMSF, and CHAPS were from Sigma. Nonidet P-40 and Protein-G-agarose beads were from Calbiochem. Kifunensine (KIF) and MG-132 were from Cayman Chemical. Xpert protease inhibitor mixture was from GenDEPOT. Peptide N-glycosidase F was from New England Biolabs. The QuikChange site-directed mutagenesis kit was from Agilent Technologies. Expre<sup>35</sup>S<sup>35</sup>S Protein Labeling Mix was from PerkinElmer. Hoechst 33342, Prolong Gold, Lipofectamine 2000, and TaqDNA polymerase were from Invitrogen. SuperSignal West Pico ECL substrate was from Thermo Scientific.

*Antibodies—* Anti-human MAN1B1 clone 1D6 was previously generated by our group (29). Anti-human α1-antitrypsin (#A0409), anti-FLAG (#F1804), and anti-β-actin (#A1978) were from Sigma. HRP-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were from GE Healthcare. Alexa Fluor 488-conjugated donkey anti-mouse and 568-conjugated donkey anti-rabbit secondary antibodies were from Invitrogen.

*Plasmids, Constructs, and siRNA—* Generation of FLAG-tagged WT MAN1B1 cDNA (FL-WT) was previously described (29). FL-1–240 was generated through amplification from the FL-WT expression construct using forward primer 5′-AAGA-GAAACCTTATGGCTGCTGCGAGG-GA-3′ containing a HindIII site and reverse primer 5′-AATGCGGCCGCCTACT-GTGTCCCTGCG-GGTTGGCAC-3′ containing a NotI site, then cloned back into the pFLAG-CMV vector. FL-D463N, FL-E330Q, FL-1–240/ΔDPS, and QQQ mutants were generated using the QuikChange site-directed mutagenesis kit per manufacturer’s protocols, and primers for these constructs were designed using the QuikChange primer design program, available upon request. All mutations and truncations were verified by DNA sequencing at the Baylor College of Medicine sequencing core. The NHK cDNA construct was generated as previously described (20). Human MAN1B1 siRNA (#44435) was purchased from Ambion.

*Cell Culture, Transient Transfection, and Steady-state Experiments—* HeLa cells were cultured in DMEM supplemented with 10% FBS. For steady-state experiments, 1 day before transfection cells were passaged, counted, and plated in 24-well plates (unless indicated otherwise) to reach ~80% confluency. The following day cells were transfected with the indicated cDNA construct using Lipofectamine 2000 per the manufacturer’s protocol in Opti-MEM media. Approximately 24 h post-transfection, cells were washed 3× in PBS and incubated for 3 h in serum-free media. For KIF treatment groups, cells were pretreated with 100 μM KIF in complete medium then chased with KIF throughout the incubation. Plates were placed on ice, and media were collected, gently centrifuged to remove floating cells, and transferred to new tubes on ice. Media fractions were prepared by adding 4× sample buffer and heating at 95 °C for 5 min. Cells were washed 3× in ice-cold PBS then lysed on ice in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 1× Xpert protease inhibitor mixture) for 30 min. Lysates were collected, and whole cell lysates prepared by adding 2× sample buffer, vortexing, and heating at 95 °C for 5 min. Samples were resolved by either 10% or 4–20% SDS-PAGE as indicated, transferred onto nitrocellulose membranes, and immunoblotted. Immunoreactive proteins were detected by ECL and quantified by densitometry using NIH ImageJ software.

*Co-immunoprecipitation—* For endogenous MAN1B1 and NHK immunoprecipitation, HeLa cells were plated in 100-mm dishes and transfected with the indicated cDNA construct. 48 h
MAN1B1 Gain-of-function in Protein Quality Control

Post-transfection, KIF treatment groups were treated for 4–6 h with 100 \( \mu M \) KIF. Cells were washed 3× in ice-cold PBS then lysed on ice in CHAPS lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% CHAPS, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM PMSF, 1× Xpert protease inhibitor mixture) for 30 min and centrifuged at 13,000 rpm at 4 °C for 30 min. Supernatants were collected and quantified using the BCA assay. Equal amounts of protein were mixed with primary antibodies and Protein-G-agarose beads, then incubated overnight at 4 °C. The next day, beads were stringently washed 4× in lysis buffer by rotating tubes 8–10×. Immunocomplexes were eluted from the beads in 2× sample buffer and heated at 70 °C for 10 min. After resolution by 10% SDS-PAGE, gels were transferred onto nitrocellulose membranes and immunoblotted. Immunoreactive proteins were detected by ECL. For FLAG immunoprecipitation, the same protocol was followed except HeLa cells were plated in 35-mm plates, 0.5% Nonidet P-40 was substituted for CHAPS, and immunocomplexes were resolved by 4–20% SDS-PAGE.

Metabolic Pulse-chase Radiolabeling and Immunoprecipitation—For experiments involving MAN1B1 knockdown, HeLa cells were plated in 60-mm dishes and transfected with either non-coding control siRNA or MAN1B1-specific siRNA, then transfected 24 h later with NHK. The next day, cells were passaged into 6-well plates for each time point and a 24-well plate for parallel steady-state analysis. Before serum starvation, the KIF treatment group was pretreated for 1 h with 100 \( \mu M \) KIF and treatment was continued throughout the experiment. Cells were serum-starved in methionine-free media for 1 h then pulse-labeled with a mixture of \(^{[35]S}\)Met and \(^{[35]S}\)Cys for 30 min and chased until collection at the indicated time points. At collection, media samples were gently centrifuged to remove floating cells and transferred to new tubes. Cells were lysed on ice for 30 min in Nonidet P-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM PMSF, 1× Xpert protease inhibitor mixture) then centrifuged at 13,000 rpm at 4 °C for 30 min. Supernatants were collected and with media fractions were mixed with primary antibodies and rotated overnight at 4 °C. The next day, Protein-G-agarose beads were added to each sample and rotated at 4 °C for 3 h. Beads were stringently washed 4× with lysis buffer and gentle vortexing. Beads incubated with media were treated with peptide N-glycosidase F for 1 h at 37 °C per manufacturer’s protocols. Immunocomplexes were eluted from beads in 2× sample buffer and heated at 70 °C for 10 min. After resolution by 4–20% SDS-PAGE, gels were fixed for 30 min in a 2% sallycic acid, 30% methanol solution and dried before detection by fluorography. For the parallel steady-state assays, cells were pretreated with KIF for 1 h and then treated in serum-free media for 3 h before media collection and lysis. Cells were lysed in the same Nonidet P-40 buffer, and after centrifugation, pellets were washed in lysis buffer, spun, and dissolved in 2× sample buffer before SDS-PAGE. The same procedure was used for pulse-chase experiments involving co-transfection of MAN1B1 constructs with NHK or QQQ, but pulse-labeling was reduced to 15 min, and parallel steady-state assays were prepared using whole cell lysates as described in the earlier section. Results were quantified using NIH ImageJ software.

Immunofluorescence Microscopy—HeLa cells were cultured on 22-mm glass coverslips in 6-well plates before transfection with indicated cDNA constructs using Lipofectamine 2000 per manufacturer’s protocol in Opti-MEM media. The following was performed at room temperature unless noted. 24 h post-transfection, cells were washed 3× in PBS and fixed in 4% paraformaldehyde for 20 min, washed 4× in PBS, and permeabilized on ice in extraction buffer (20 mM HEPS pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl\(_2\), 300 mM sucrose) for 20 min. Slides were washed 2× with PBS, 0.1% Nonidet P-40 and blocked in 10% horse serum, PBS, 0.1% Nonidet P-40 for 1 h. Cells were immunostained for 2 h with primary antibodies, washed 4× with PBS, 0.1% Nonidet P-40, labeled with fluorescent secondary antibodies for 1 h, then washed 4× with PBS, 0.1% Nonidet P-40. Counterstaining with Hoechst 33342 was performed for 10 min, washed 3× in PBS, then mounted with Prolong Gold. Images were acquired on a Zeiss LSM710 confocal microscope. The objective was a 63× 1.4 NA oil-immersion lens. For image acquisition, Zen Imaging software was used, the pinhole was 1 airy unit, the pixel dwell was 3.15 μs, and zoom was set to 1.8×. Post-acquisition processing was performed using NIH ImageJ software.

Multiple Sequence Alignment—Multiple amino acid sequence alignments were conducted using Clustal Omega software (32). Sequence conservation was viewed and scored using Jalview 2 software (33). The sequences examined were Homo sapiens (gi: 37182054), Pans troglodytes (gi: 410308108), Rattus norvegicus (gi: 183985961), Mus musculus (gi: 187952927), Canis familiaris (gi: 73967467), Xenopus laevis (gi: 148232068), and Danio rerio (gi: 153792498).

Statistical Analyses—Data are expressed as the mean ± one S.E. One-way ANOVA with Tukey’s HSD post hoc test was used for sample comparison. Significance was calculated using SPSS Statistics software, with \( p \leq 0.05 \) considered statistically significant.

RESULTS

The Co-immunoprecipitation of MAN1B1 and NHK Is Not Dependent on N-Linked Glycans—Only the soluble luminal enzymatic domain of human MAN1B1 has been crystallized (34–37), underscoring the need to identify additional domains that could participate in the Golgi-based ERAD substrate retrieval system. In our most recent study, a direct interaction between the cytoplasmic tail of human MAN1B1 and γ-COP was shown to contribute to a Golgi-based cargo retrieval system that promotes the capturing of NHK for recycling back to the ER before ERAD (30). Co-immunoprecipitation between MAN1B1 and NHK that increased in response to γ-COP siRNA knockdown was also identified. Because numerous cargo receptors function as lectins that directly interact with the carbohydrate moiety of their N-glycosylated substrates (38–40), we therefore asked whether a similar interaction exists between the N-glycans of NHK and the active site of MAN1B1. Our initial approach was to block substrate access to the MAN1B1 active site by incubating cells with KIF, a general α1,2-mannosidase inhibitor (36, 41). HeLa cells were transfected with either empty vector (EV) or a construct that over-expresses recombinant NHK to ensure that a surplus of the
translated molecules would escape to the Golgi complex as previously reported (29, 30). Endogenous MAN1B1 was immunoprecipitated from cell lysates, fractionated by SDS-PAGE, and probed for the presence of NHK. NHK is not endogenously expressed in HeLa cells, and was therefore absent from EV-transfected lysates (Fig. 1A–C, lanes 1). In NHK-transfected HeLa cells, NHK was detected in the endogenous MAN1B1 immunoprecipitates from both KIF-treated and untreated cells, and KIF treatment substantially enhanced the association (Fig. 1A, lanes 5 and 6) likely because of its hindered degradation. The association was further confirmed in a reciprocal experiment from the same cell lysates, where endogenous MAN1B1 was detected in NHK immunoprecipitates (Fig. 1B, lanes 5 and 6). Because the endogenous, mature O-glycosylated 90-kDa MAN1B1 protein specifically localizes to the Golgi complex rather than the ER (28, 29), its detection in both immunoprecipitates validated that NHK was somehow associated with Golgi-localized MAN1B1.

In another approach, a glycan-deficient form of NHK (designated QQQ) was generated by mutating each of the three N-glycosylated residues at Asn-43, Asn-83, and Asn-247 to glutamine. HeLa cells were transfected with the QQQ construct and incubated in the presence or absence of KIF, after which endogenous MAN1B1 was immunoprecipitated from cell lysates. Consistent with results from the prior experiment, QQQ was detected in endogenous MAN1B1 immunoprecipitates regardless of KIF treatment (Fig. 1C, lanes 5 and 6). Taken together, the combined results demonstrate that the association between NHK and endogenous MAN1B1 does not require an interaction with N-linked oligosaccharides, implying that indirect interactions with other members of a proposed cargo retrieval complex might exist (30).

**The Quantitative Expression of MAN1B1, Apart from Its Catalytic Activity, Influences the Golgi-based Quality Control of NHK**—In a previous study we reported that the siRNA-mediated knockdown of endogenous MAN1B1 significantly increases the secretion of NHK from HeLa cells (30). Because KIF treatment had failed to interfere with the co-immunoprecipitation of endogenous MAN1B1 and NHK from HeLa cells (Fig. 1, A and B), experiments were designed to question whether the aforementioned secretion phenotype after MAN1B1 knockdown results from the absence of its enzymatic activity or a deficiency in other unknown, non-enzymatic properties. HeLa cells were transfected with non-coding control siRNA or a previously characterized siRNA targeting human MAN1B1 (29), then transfected the next day with the recombinant NHK expression construct. After 24 h, the fate of newly synthesized NHK was monitored by 35S metabolic pulse-chase radiolabeling. The overexpression of recombinant NHK in HeLa cells was used as a reporter for misfolded protein capturing and ERAD because these processes are saturable in this model system, as compared with the more robust proteostasis network of hepatoma cells (20). The ability of transfected NHK to be secreted represents a breach of the ER retention system.
and ensures that NHK has reached the Golgi, where MAN1B1 is proposed to execute its role in protein quality control. Therefore, monitoring intracellular and extracellular levels of NHK allowed for the detection of changes to the proteostasis network after co-transfection with recombinant MAN1B1.

The intracellular and secreted levels of NHK after treatment with control siRNA represent the basal capacity of Golgi-based capturing and ERAD systems in HeLa cells. Under these conditions, NHK was gradually degraded over the 5-h chase period whereas a fraction of molecules escaped and was secreted (Fig. 2, A (lanes 1–4), B, and C). The secreted protein was de-glycosylated with peptide N-glycosidase F to sharpen the bands for optimal quantification. Simultaneous treatment with KIF inhibited the increased electrophoretic mobility in NHK over time, indicative of suppressed mannose trimming from the N-glycans of NHK (20, 29) that substantially decreased the rate of intracellular degradation (Fig. 2, A (lanes 5–8), B, and C). Moreover, under these conditions NHK secretion was slightly hindered in response to KIF treatment (Fig. 2, A (lanes 5–8), B, and C). In contrast, the decrease in intracellular NHK in cells transfected with the MAN1B1 siRNA coincided with a significant increase in extracellular NHK over time (Fig. 2, A (lanes 9–12), B, and C), representing a breach of the endogenous retention system.

A parallel steady-state representation of the pulse-chase experiment using passages from the same treated cells (Fig. 2, D (lanes 9–12)), compare lanes 2–4) and extracellular NHK (Fig. 2, C (lanes 5–8)), compare lanes 2–4) after co-transfection with recombinant MAN1B1. Under these conditions, NHK was gradually degraded over the 5-h chase period whereas a fraction of molecules escaped and was secreted (Fig. 2, A (lanes 1–4), B, and C). Moreover, under these conditions NHK secretion was slightly hindered in response to KIF treatment (Fig. 2, A (lanes 5–8), B, and C). In contrast, the increase in intracellular NHK in cells transfected with the MAN1B1 siRNA coincided with a significant increase in extracellular NHK over time (Fig. 2, A (lanes 9–12), B, and C), representing a breach of the endogenous retention system.

A parallel steady-state representation of the pulse-chase experiment using passages from the same treated cells displayed the efficiency of MAN1B1 knockdown (Fig. 2, D and F) but also revealed that MAN1B1 knockdown led to a substantial, repeatable increase in the presence of an Non-ident P-40-insoluble fraction of NHK (Fig. 2, D (lane 8), and E). This population was undetectable during pulse-chase experiments because this assay can only measure soluble NHK. Steady-state analyses uncovered that diminishing the endogenous MAN1B1 concentration significantly increased the total intracellular NHK pool (both soluble and insoluble) as compared with KIF-treated cells, indicating that knockdown of MAN1B1 not only hinders NHK retention, but also reduces its solubility and degradation. NHK insolubility likely stems from its accumulation and subsequent aggregation within the secretory pathway without MAN1B1 because insoluble ERAD substrates accumulate in response to lowered expression levels of UGTT1, a fundamental, ER-based gatekeeper for N-glycoprotein proteostasis (42).

Another critical observation was that, despite the nearly 90% reduction in endogenous MAN1B1 expression in response to the siRNA treatment, mannose trimming from NHK N-glycans persisted, as indicated by the gradual increase in NHK electrophoretic mobility over time (Fig. 2A, compare lanes 9–12 with KIF-treated and -untreated groups) and under steady-state conditions (Fig. 2D, compare lanes 2–4). Taken together, these results provided the first implication that the MAN1B1 concentration, and not necessarily its mannosidase activity, contributes to the Golgi-based quality control of NHK. In addition, the intracellular removal of mannose from NHK's N-glycans does not depend solely on MAN1B1.

Neither the Catalytic Activity nor the Luminal Catalytic Domain of MAN1B1 Is Required to Accelerate NHK ERAD—Earlier studies have demonstrated that experimentally elevated
levels of recombinant human MAN1B1 are sufficient to increase NHK degradation (20, 29, 30, 43–45). However, results from the aforementioned experiments brought into question the contribution of mannosidase activity as the exclusive component by which MAN1B1 quantitatively participates in the Golgi-based quality control of NHK. To further address this issue, mammalian expression constructs were generated that encode N-terminal FLAG-tagged versions of recombinant human MAN1B1 with distinct mutations in its catalytic core (Fig. 3A). FL-D463N, reported to cause a 99.9% reduction in enzyme efficiency (Km/Km) with no detectable binding affinity (Kd) for a Man9GlcNAc2 glycopeptide ligand (35, 46, 47) was generated, as was FL-E330Q, which is reported to cause a 96.5% decrease in enzymatic efficiency but a 100-fold increase in binding affinity (35, 46, 47). To completely rule out any contribution from the enzymatic domain, we also generated a construct that encodes the human MAN1B1 mutant designated FL-1–240, which encodes only the first 240 amino acids corresponding to the N-terminal cytoplasmic tail, transmembrane domain, and luminal stem, thus eliminating the entire luminal catalytic domain (Fig. 3A).

The influence of each FLAG-tagged human MAN1B1 mutant on the fate of recombinant NHK was monitored in HeLa cells under steady-state conditions and compared with EV and FLAG-tagged WT MAN1B1 (designated FL-WT). Co-transfection with EV led to the appearance of recombinant NHK in the culture media (Fig. 3C, lane 1). Co-transfection with FL-WT led to a significant decrease in both the intracellular and secreted populations of NHK as compared with EV control (Fig. 3, B (lane 2), C, and D) and previously reported (20, 29, 30). Remarkably, co-transfection with the catalytically deficient FL-D463N or FL-E330Q mutants both significantly diminished the intracellular and extracellular NHK levels comparable with that of FL-WT (Fig. 3, B (lanes 3 and 4), C, and D), indicating that the catalytic activity of recombinant MAN1B1 is expendable in terms of its contribution to NHK ERAD. In further support of this novel observation, co-transfection with FL-1–240, devoid of the entire luminal catalytic domain (Fig. 3A), enhanced NHK ERAD and diminished its secretion to levels comparable to that of FL-WT and the catalytic mutants (Fig. 3, B (lane 5), C, and D).

To validate our conclusions based from steady-state observations, 35S metabolic pulse-chase radiolabeling was conducted to directly quantify and compare the rates of newly synthesized NHK in HeLa cells co-transfected with EV, FL-WT, or FL-1–240. Co-transfection with FL-WT diminished the pulse-radio-labeled intracellular and extracellular NHK levels during the chase as compared with the effects of EV, indicating an increase in the intracellular degradation of NHK (Fig. 3, E (lanes 4–6), F, and G). Importantly, co-transfection with FL-1–240 was sufficient to increase the intracellular degradation of pulse-radio-labeled NHK and suppress its secretion comparable to that of FL-WT (Fig. 3, E (lanes 7–9), F, and G). A parallel steady-state representation of the pulse-chase experiment from the same populations of cells demonstrated the intracellular concentrations of FL-WT and FL-1–240 and their similar influences on NHK (Fig. 3, H–J). Considering the importance of our observation that non-catalytic domains contribute to the roles played by MAN1B1 in the Golgi-based quality control of NHK, immunofluorescence microscopy was performed to validate that both FL-WT and FL-1–240 were delivered to the Golgi complex. HeLa cells were transfected with FL-WT or FL-1–240 and probed with a FLAG-specific antibody to detect their subcellular localization. Endogenous MAN1B1 was detected with a monoclonal antibody previously generated by our group (29). Giantin was used as a marker for the Golgi complex and calreticulin for the ER. Endogenous MAN1B1 co-localized with giantin in a juxtanuclear pattern associated with the Golgi complex and did not localize with calreticulin, the latter of which appeared distributed throughout the cell (Fig. 4A). The FLAG antibody confirmed that both recombinant MAN1B1 proteins FL-WT and FL-1–240 co-localized with giantin in the Golgi complex similarly to endogenous MAN1B1 while remaining distinct from ER-localized calreticulin staining (Fig. 4, B and C). Therefore, despite the absence of the luminal catalytic domain, FL-1–240 was delivered to the Golgi complex comparable to endogenous MAN1B1 and transfected FL-WT. These collective observations demonstrate that the catalytic domain, and therefore the inherent mannosidase activity, of human MAN1B1 are dispensable for the roles it plays in the Golgi-based quality control of NHK.

**Functional Equivalency of Catalytic and Non-catalytic Human MAN1B1**—Given our findings, we wanted to validate the functional equivalency between the WT and catalytically inactive FLAG-tagged MAN1B1 mutants. For this, the above steady-state assay was modified to determine whether there was an additive effect between the two when processing NHK. The amount of transfected FL-WT MAN1B1 construct was halved and supplemented with an equal amount of EV, FL-E330Q, or FL-1–240 for the co-transfection of HeLa cells with NHK. Samples were processed as previously indicated for steady-state analyses. Co-expression of NHK with EV led to the detection of NHK in the media and cell lysates (Fig. 5, A and B (lanes 1, and C), whereas co-transfection with an equal combination of EV and FL-WT led to a significant reduction in the two NHK populations (Fig. 5, A and B (lanes 2) and C). Consistent with our previous findings, supplementing FL-WT with either FL-E330Q or FL-1–240 resulted in an even more significant decrease (Fig. 5, A and B (lanes 3 and 4) and C), indicating that FL-WT and the catalytic mutants cooperate to enhance the quality control of NHK, rather than exert a dominant-negative effect.

**Acceleration of ERAD by Recombinant MAN1B1 Catalytic Mutants Is Sensitive to Kifunensine and Mediated by the Proteasome**—The capacity of KIF to disrupt ERAD is thought to involve either the inhibition of essential mannosidase activity or the prevention of important downstream lectin-ligand interactions (48, 49). Because KIF failed to prevent the co-immunoprecipitation of endogenous MAN1B1 and NHK (Fig. 1) and the recombinant MAN1B1 catalytic mutants exhibit compromised mannosidase activity, we asked whether their ability to accelerate NHK ERAD was sensitive to KIF treatment to clarify whether other mannosidases and/or lectin interactions remain...
critical to this process. Steady-state experiments in HeLa cells were conducted as before, except a parallel sample group was pretreated for 1 h and chased with KIF before media collection and cell lysis. KIF treatment did not significantly influence NHK secretion in cells co-transfected with NHK and EV, FL-WT, or FL-1–240. However, intracellular levels of NHK increased under each condition after KIF treatment (Fig. 5, D–F). These data indicate that although FL-1–240 is capable of accelerating NHK ERAD, other mannosidases and/or lectins are crucial in the process.

FIGURE 3. Recombinant MAN1B1 does not require mannosidase activity or a luminal catalytic domain to enhance NHK retention and ERAD. A, schematic of full-length, N-terminal FLAG-tagged WT MAN1B1 (FL-WT, top), with the indicated amino acid mutations FL-D463N and FL-E330Q that inhibit MAN1B1 catalytic activity. A truncated version of FLAG-tagged MAN1B1 (FL-1–240, bottom) includes the first 240 non-enzymatic amino acids of MAN1B1 but lacks the luminal catalytic domain. TMD, transmembrane domain. B, steady-state whole cell lysate Western blot of HeLa cells transfected with NHK and constructs encoding EV, FL-WT, FL-D463N, FL-E330Q, or FL-1–240. Twenty-four hours later, complete media were replaced with serum-free media, and secreted NHK was allowed to accumulate in the media for 3 h. Upon media collection, whole cell lysates were generated by mixing directly with sample buffer, and 8% of each lysate was resolved by 4–20% SDS-PAGE. The gels are representative of three independent experiments. C, media Western blot of the same experiment, with 30% of each media fraction resolved by 10% SDS-PAGE. D, graphic representations of NHK in whole cell lysates (WCL) and media fractions in the three steady-state experiments. E, pulse-chase analysis of HeLa cells transfected with NHK and constructs encoding EV, FL-WT, or FL-1–240. Cells were labeled for 15 min with [35S]Met and [35S]Cys, then chased at the indicated time points. Samples were resolved by 4–20% SDS-PAGE and analyzed by fluorography. The gels are representative of three independent experiments. Graphic representations of intracellular NHK (F) and extracellular NHK (G) in three pulse-chase experiments are included. H, WCL Western blot of a parallel steady-state representation of the pulse-chase experiment using passages from the same treated cells with 8% of each lysate resolved by 4–20% SDS-PAGE. I, media Western blot of the same parallel experiment, with 30% of each media fraction resolved by 10% SDS-PAGE. J, graphic representations of NHK in WCL and media fractions in the three parallel steady-state experiments. Data are reported as the mean ± one S.E. with statistical significance calculated by one-way ANOVA and Tukey’s HSD post hoc test. ns, not significant. *, p ≤ 0.05, n = 3.
already present during pretreatment and starvation periods to ensure thorough proteasomal inhibition. Similar observations were made upon reducing MG-132 concentrations and excluding the pretreatment step (data not shown). The capacity of either KIF or MG-132 to inhibit the intracellular degradation of NHK enhanced by FL-1–240 infers the involvement of additional mannosidases and/or downstream lectins, indicating that NHK disposal under these accelerated conditions likely involves a traditional ERAD proteasomal pathway.

Accelerated ERAD of NHK by Recombinant WT MAN1B1 and Catalytic Mutants Proceeds in the Absence of N-Glycans—To answer whether the N-glycans of NHK are necessary for its enhanced entrance into ERAD by recombinant WT MAN1B1, the fate of QQQ in HeLa cells was assessed in response to its co-transfection with FL-WT MAN1B1 during steady-state experiments. FL-WT caused a significant reduction in the intracellular and secreted populations of QQQ as compared with EV (Fig. 6, A and B (lanes 3) and C), an observation that for the first time portrays the ability of recombinant WT MAN1B1 to enhance the quality control of a non-glycosylated substrate. To determine whether the same phenomenon occurs for recombinant MAN1B1 catalytic mutants, QQQ was co-expressed with FL-D463N, FL-E330Q, or FL-1–240. Importantly, all of the mutants significantly reduced the levels of QQQ within the cells and media (Fig. 6, A and B (lanes 4–6) and C), enhancing the overall quality control of QQQ comparable to that of FL-WT.

As in the preceding experiments, 35S metabolic pulse-chase radiolabeling was conducted to validate our observations obtained under steady-state conditions. The intracellular degradation of QQQ was substantially diminished in response to co-transfection with FL-WT (Fig. 6, D (lanes 4–6), E, and F). Consistent with the previous data, transfection with FL-1–240 also increased the quality control of QQQ (Fig. 6, D (lanes 7–9), E, and F). A parallel steady-state representation of the pulse-chase experiment from the same populations of cells demonstrated the intracellular concentrations of FL-WT and FL-1–240 and their similar influences on QQQ (Fig. 6, G–I). Finally, the inhibitory capacity of MG-132 confirmed that the expression of FL-1–240 enhanced the ERAD of QQQ via a proteasomal pathway (Fig. 6, J (compare lanes 1–3 with lanes 4–6), K, and L). These data support the conclusion that the quantitative trait in which MAN1B1 regulates the Golgi-based quality control of NHK, via the proteasome, does not require inherent mannosidase activity or glycans attached to NHK.

A Highly Conserved, Non-enzymatic Sequence within the Luminal Stem Endows FL-1–240 with the Capacity to Regulate NHK Quality Control—Numerous in vitro and in vivo studies have demonstrated that human MAN1B1 exhibits α1,2-linked mannosase activity (18–20, 43, 44, 46). Therefore, our discovery that FL-1–240 can promote NHK quality control in the absence of catalytic activity implies that the corresponding gene has undergone a gain-of-function adaptation during evolution. To test this hypothesis, our initial strategy was to identify distinct amino acids sequences within FL-1–240 that are absent from the orthologs in lower eukaryotes but conserved among orthologs of higher species.

FIGURE 4. The MAN1B1 truncation mutant FL-1–240 successfully reaches the Golgi. Confocal images of HeLa cells fixed and immunostained 48 h after transfection. A, cells immunostained with anti-MAN1B1 clone 1D6 and anti-giantin or anti-calreticulin antibodies. Endogenous MAN1B1 is shown in green using Alexa 488-conjugated anti-mouse secondary antibodies. Giantin or calreticulin is shown in red using Alexa 568-conjugated anti-rabbit secondary antibodies. Cell nuclei were counterstained with Hoechst 33342. B, cells transfected with FL-WT and immunostained with anti-FLAG and anti-giantin or anti-calreticulin antibodies using the same color scheme as above. C, cells transfected with FL-1–240 and immunostained with anti-FLAG and anti-giantin or anti-calreticulin antibodies using the same color scheme as above.
The human MAN1B1 protein, which is a member of the glycoside hydrolase family 47, can be organized into four distinct domains: the N-terminal cytoplasmic tail, transmembrane domain, luminal stem, and C-terminal luminal catalytic domain (Fig. 7, top panel) (18). The cytoplasmic tail and luminal stem region, which are intact in FL-1–240, have expanded in length during evolution. For example, their lengths in the Saccharomyces cerevisiae ortholog, MNS1p, are 2 and 20 amino acids, respectively (18). In Caenorhabditis elegans where two orthologs exist, MANS-3 exhibits an elongated cytoplasmic tail of 42 residues and luminal stem of 28 residues, whereas MANS-4 has a cytoplasmic tail of 9 residues and a luminal stem of 54 residues (18). In the single human MAN1B1 ortholog, the cytoplasmic tail and luminal stem are expanded even further to 47 and 151 residues, respectively (18, 19). In support of this notion, our group recently identified /H9253-COP binding motifs in the human MAN1B1 cytoplasmic tail that contribute to the Golgi-based quality control of transfected NHK but are absent from the shorter-tailed orthologs of lower organisms (18, 30). In the current study in silico analysis comparing multiple sequence alignments of MAN1B1 orthologs across organisms ranging from yeast to humans led to the discovery of a decapeptide sequence (DPS) within the MAN1B1 luminal stem corresponding to human residues Val-205 to Glu-214 (VISWRGAVIE), which is absent from lower organisms but highly conserved among known vertebrate orthologs (Fig. 7). Protein BLAST results for VISWRGAVIE did not yield any known conserved domains or peptide-specific functions, and it is not present within other Golgi 1,2-mannosidases or glycohydrolase family 47 members.

To examine the utility of the DPS in MAN1B1 contribution to NHK quality control, we generated a construct encoding a modified version of FL-1–240, designated FL-1–240/DPS, in which the residues corresponding to the DPS (human residues Val-205–Glu-214) were selectively deleted. Notably, because both of the constructs encode proteins that are devoid of a catalytic domain, any alterations to NHK fate can be attributed to the loss of non-enzymatic features of the transfected recombinant proteins. As in prior assays, we first compared intracellular and secreted populations of transfected NHK in HeLa cells under steady-state conditions after co-transfection with either EV or constructs that encode FL-1–240 and NHK with or without MG-132. MG-132 treatment groups were pretreated with 25 mM MG-132 in complete media for 1 h, and treatment was continued throughout the experiment. Cells were labeled for 15 min with [35S]Met and [35S]Cys, then chased at the indicated time points. Samples were resolved by 4–20% SDS-PAGE and analyzed by fluorography. Graphic representations of intracellular NHK (H) and extracellular NHK (I) are included. Data were reported as the mean ± one S.E. with statistical significance calculated by one-way ANOVA and Tukey’s HSD post hoc test. *, p ≤ 0.05, n = 3.
with control (Fig. 8, A–C, compare lane 1 and 2), reflecting an enhancement in NHK quality control. In contrast, transfection with FL-1–240/H9004 DPS did not exert this effect but resulted in the detection of intracellular and secreted NHK populations that were similar to co-transfection with EV (Fig. 8, A–C, compare lanes 1–3), indicating that the deletion of the highly conserved DPS from FL-1–240 significantly decreased its capacity to accelerate the Golgi-based quality control of NHK. It is important to note that FL-1–240/ADPS displayed a more distinct doublet with a higher molecular weight than FL-1–240 (Fig. 8A, compare lanes 2 and 3). After reaching the Golgi, human MAN1B1 is O-glycosylated and sialylated on four threonine residues within its luminal stem, causing multiple MAN1B1 bands to appear by Western blot with the slower
migrating ones representing greater O-glycosylation/sialylation (29). The deletion of the 10-residue DPS accounted for the slight decrease in size of the smallest non-glycosylated bands between FL-1–240 and FL-1–240/ΔDPS. However, it also appeared to have increased the overall sialylation of FL-1–240/ΔDPS, leading to the appearance of a larger doublet than FL-1–240 (Fig. 8A, compare the lowest bands in lanes 2 and 3).

35S metabolic pulse-chase radiolabeling in HeLa cells was then conducted after co-transfection with the constructs described above to substantiate the validity of our findings generated under steady-state conditions. Consistent with the conclusions portrayed from the prior experiment, removal of the DPS considerably decreased the capacity in which transfected FL-1–240 promoted the Golgi-based quality control of NHK (Fig. 8, D (compare lanes 1–3 with 4–6), E, and F). A parallel steady-state representation of the pulse-chase experiment from the same populations of cells demonstrated the intracellular concentrations of FL-1–240 and FL-1–240/ΔDPS and their contrasting influences on NHK (Fig. 8, G–I) were highly comparable to the observations from the preceding steady-state experiments (Fig. 8, compare A–C with G–I). This indicated that the quantified differences in quality control were not the result of variability in transfection efficiencies or protein expression.

We next wanted to confirm that FL-1–240/ΔDPS localizes to the Golgi complex. Immunofluorescence localization studies validated that recombinant FL-1–240/ΔDPS was delivered to the Golgi complex similarly and to the same extent as recombinant FL-1–240 as indicated by co-localization with giantin (Fig. 8J, compare with Fig. 4C). This indicated that removal of the DPS from FL-1–240 did not alter its subcellular localization, and it was likely expressed and folded correctly.

To gain insight into how the DPS might contribute to MAN1B1 function, HeLa cells were co-transfected with constructs encoding NHK and either EV, FL-1–240, or FL-1–240/ΔDPS. Using FLAG antibodies, FL-1–240 and FL-1–240/ΔDPS were immunoprecipitated, and immunocomplexes were probed for the presence of NHK. NHK was detected in FLAG immunoprecipitates of cells transfected with FL-1–240 (Fig. 8K, lane 5), confirming that the mannosidase domain of recombinant MAN1B1 is dispensable for its association with NHK. Importantly, a substantial reduction in co-immunoprecipitated NHK was observed in cells transfected with FL-1–240/ΔDPS (Fig. 8K, compare lanes 5 and 6), revealing that the removal of the DPS diminishes the association between recombinant FL-1–240 and NHK. Because FL-1–240/ΔDPS properly localizes to the Golgi, the reduction in associated NHK was likely not the result of FL-1–240/ΔDPS misfolding due to DPS removal. These data provide evidence that the DPS plays an integral role in maintaining the association between recombinant MAN1B1 and NHK.

In conclusion, the localization of MAN1B1 to the Golgi complex, the lengthening of the luminal stem region and
conservation of the DPS among the vertebrate orthologs, and the functional consequences observed through the DPS deletion studies support the notion that the contribution of MAN1B1 in regulating the Golgi-based quality control system coincided with an evolutionary non-enzymatic gain-of-function adaptation.

**DISCUSSION**

Prior studies have demonstrated that MAN1B1 exhibits a quantitative trait in regard to the capacity of its intracellular concentration to regulate the efficiency of NHK quality control (20). This functional trait has been traditionally credited to the protein catalytic activity as an α1,2-linked mannosidase, similar
MAN1B1 Gain-of-function in Protein Quality Control

to what has been reported for its yeast ortholog, Mns1p (16–19, 50). However, a series of recent studies have begun to question this conclusion. The results of intracellular localization studies (29) and the existence of functional γ-COP binding sites that allow the co-immunoprecipitation of NHK (30) have begun to indicate that MAN1B1 operates within a Golgi-localized quality control module, perhaps serving as a backup system to ERAD surveillance in the ER.

In the current study several lines of experimental evidence indicated that endogenous MAN1B1 does not operate as a lectin-like cargo receptor (Fig. 1). A series of additional experiments substantiated that the capacity of MAN1B1 to quantitatively influence NHK quality control does not rely on inherent mannosidase activity or even the luminal catalytic domain. This central conclusion was most effectively established by the capacity of transfected FL-1–240, which eliminates any un-intentional mannose trimming by overexpressed MAN1B1, and focuses solely on the consequences of its non-enzymatic functions to accelerate the quality control of NHK (Fig. 3). Moreover, FL-1–240 was capable of accelerating NHK quality control even in the absence of N-linked glycans (Fig. 6). Although we cannot specifically determine whether FL-1–240 directly controls the fate of NHK by enhanced capturing or increased degradation, our observations ultimately support its ability to promote ERAD in the absence of an enzymatic domain. Subsequent studies are in progress to determine whether the dispensable nature of the MAN1B1 catalytic domain is inclusive to additional variants of α1-antitrypsin as well as other clinically relevant protein substrates.

Importantly, the quality control of overexpressed NHK is still dependent on intracellular α1,2-mannosidase activity or lectin-based interactions independent of human MAN1B1, as demonstrated by the ability of KIF to inhibit NHK ERAD in cells co-transfected with MAN1B1 catalytic mutants (Fig. 5). It is very possible that the enzymatic role of the yeast ortholog (Mns1p) was imparted elsewhere over evolution to facilitate ERAD in higher organisms. In support of this notion, mannose trimming persisted after MAN1B1 knockdown with siRNA (Fig. 2). There are three additional Golgi α1,2-mannosidases (IA, IB, IC) in mammalian cells that do not exist in yeast, and their overexpression can accelerate NHK quality control (25, 51). Furthermore, EDEM1, which is an additional glycoside hydrolase 47 member, has been reported to enhance mannose trimming during ERAD (26, 27, 52, 53). Moreover, two mammalian EDEM1 homologs, EDEM2 and EDEM3, are not present in yeast but are reported to promote ERAD (54–56). Intriguingly, it was concluded that EDEM3 possesses α1,2-mannosidase activity in addition to a KDEL sequence that endows it with the ability to traverse the ER and be retrieved from later compartments. It is noteworthy that EDEM1 and the downstream ERAD lectins XTP3-B and OS-9 were reported to interact with and enhance ERAD in a glycan-independent manner in mammalian cells (23, 24, 26, 27). Several of these proteins are up-regulated during ERAD or the unfolded protein response, so if one or more of them are capable of initiating ERAD, this could have diminished the need for MAN1B1 mannosidase activity in promoting ERAD during subsequent evolutionary events.

Given our observations that NHK glycan trimming persists after MAN1B1 knockdown and KIF inhibits NHK ERAD by FL-1–240, then KIF is likely blocking essential mannosidase activity downstream from MAN1B1. Although much of the literature supports the role of mannose trimming from misfolded proteins as critical to their recognition for ERAD, one cannot overlook the fact that much of the ERAD quality control machinery, including EDEM1, OS-9, XTP3-B, and SEL1L, are themselves N-glycosylated. Because the removal of mannose units from their N-glycans is required for ERAD (24, 57), one should consider in future studies that treatment with KIF might actually function by inhibiting critical ERAD associations with SEL1L (24, 48, 49).

Regardless of the non-enzymatic role that MAN1B1 plays in Golgi-based quality control, ample evidence indicates that it still functions as an intracellular mannosidase in which the catalytic activity is critical for the structural processing of N-linked oligosaccharides that transit through the Golgi complex. For example, mutations in the MAN1B1 gene have been linked to autosomal-recessive intellectual disability (58), with the identified mutations located within the MAN1B1 catalytic domain and proposed to reduce $k_{cat}$ or disrupt protein stability. Another study linked mutations within the MAN1B1 gene, most of which were found within the catalytic domain, to MAN1B1 protein deficiency, leading to the presentation of a new type II congenital disorder of glycosylation (28). Using patient fibroblasts, the authors concluded that the mutations diminish MAN1B1 steady-state expression levels, alter Golgi complex morphology, and delay the conversion of asparagine-linked Man$_n$GlcNAc$_2$ to Man$_n$GlcNAc$_2$. It is certainly possible that the clinical presentations resulting from the identified MAN1B1 mutations are a result of reduced or ablated catalytic activity, as these mutations exist primarily within the catalytic domain. However, the accompanying reduction in MAN1B1 expression, which diminishes both enzymatic and non-enzymatic activities, cannot exclude a potential role for the latter in disease pathogenesis.

With the discovery of a non-enzymatic role for MAN1B1, we sought to identify amino acid sequences that might be responsible for the observed gain-of-function in the human ortholog. To this end, the comparison of multiple amino acid sequence alignments allowed us to discover a highly conserved DPS, VI$\text{SWRGAVIE}$, within the luminal stem of multiple vertebrate MAN1B1 orthologs. Our functional analyses indicated that the highly conserved DPS imparts FL-1–240 with the capacity to enhance NHK ERAD (Figs. 7 and 8). Although we cannot rule out the existence and contribution of non-enzymatic activity in the yeast ortholog Mns1p toward ERAD, its ER localization, smaller non-enzymatic regions, lack of obvious features like the DPS, and synergy with the EDEM1 homolog Htm1p argues against this notion. Future studies will be designed to elucidate the underlying mechanisms in which the DPS and its individual amino acids as well as its effects on MAN1B1 O-glycosylation and sialylation contribute to the operation of the Golgi-based quality control system. Additionally, a proteomics approach will be designed to identify members of a putative cargo-capturing complex in which MAN1B1 is proposed to function. Given our findings, we propose a model for MAN1B1 contri-
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