Glucosidase II and N-glycan mannose content regulate the half-lives of monoglucosylated species in vivo

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ABSTRACT Glucosidase II (GII) sequentially removes the two innermost glucose residues from the glycan (Glc$_3$Man$_9$GlcNAc$_2$) transferred to proteins. GII also participates in cycles involving the lectin/chaperones calnexin (CNX) and calreticulin (CRT) as it removes the single glucose unit added to folding intermediates and misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase (UGGT). GII is a heterodimer in which the α subunit (GIIα) bears the active site, and the β subunit (GIIβ) modulates GIIα activity through its C-terminal mannose 6-phosphate receptor homologous (MRH) domain. Here we report that, as already described in cell-free assays, in live Schizosaccharomyces pombe cells a decrease in the number of mannoses in the glycan results in decreased GII activity. Contrary to previously reported cell-free experiments, however, no such effect was observed in vivo for UGGT. We propose that endoplasmic reticulum α-mannosidase-mediated N-glycan demannosylation of misfolded/slow-folding glycoproteins may favor their interaction with the lectin/chaperone CNX present in S. pombe by prolonging the half-lives of the monoglucosylated glycans (S. pombe lacks CRT). Moreover, we show that even N-glycans bearing five mannoses may interact in vivo with the GIIβ MRH domain and that the N-terminal GIIβ G2B domain is involved in the GIIα–GIIβ interaction. Finally, we report that protists that transfer glycans with low mannose content to proteins have nevertheless conserved the possibility of displaying relatively long-lived monoglucosylated glycans by expressing GIIβ MRH domains with a higher specificity for glycans with high mannose content.

INTRODUCTION Protein N-glycosylation involves the initial transfer of a glycan (GlclMan$_n$GlcNAc$_m$; Figure 1) from a dolichol (Dol)-P-P derivative to Asn residues in the consensus sequence Asn-X-Ser/Thr in proteins in the endoplasmic reticulum (ER). Transfer is immediately followed by the removal of the external glucose unit (residue n, Figure 1) by glucosidase I (GI) and the removal of the two remaining glucose residues (residues l and m, Figure 1) by glucosidase II (GII). One or more mannose residues may be removed in the ER by ER mannosidase(s). Both GII-mediated cleavages are determining factors in the quality control of glycoprotein folding in the ER. Monoglucosylated glycan-bearing glycoproteins may interact with calnexin (CNX) and/or calreticulin (CRT), two highly homologous ER lectin and chaperones that enhance folding efficiency by preventing aggregation and facilitating correct disulfide bond formation through their interaction with ERP57, a protein disulfide isomerase. Furthermore, the interaction of the folding intermediates and misfolded glycoproteins with the lectin and chaperones prevents exit from the ER to the Golgi. The second GII-mediated cleavage, which

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Abbreviations used: CNX, calnexin; CRT, calreticulin; Dol, dolichol; DTT, dithiothreitol; Endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; GI, glucosidase I; GII, glucosidase II; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; Man$_n$P, mannose 6-phosphate; MRH, Man$_n$P receptor homologous; NMDNJ, N-methyl 1-deoxynojirimycin; pNPG, p-nitrophenyl α-D-glucopyranoside; UGGT, UDP-Glc:glycoprotein glucosyltransferase.

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generates unglycosylated molecules, abolishes the glycoprotein-lectin and chaperone interaction, thus allowing glycoproteins to pursue their transit through the secretory pathway. If not yet properly folded, however, glycoproteins may be reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase (UGGT), a soluble ER enzyme that specifically glucosylates nonnative conformers and regenerates monoglucosylated glycans. These, in turn, interact again with the lectin and chaperones. Cycles of reglucosylation and deglucosylation catalyzed by the opposing activities of UGGT and GII continue until the glycoproteins acquire their native tertiary structure or, if unable to properly fold, are driven to the cytosol for proteasomal degradation (Caramelo and Parodi, 2007, 2008; Caramelo et al., 2010; Parodi, 2000). Cell-free assays may thus not always reflect what occurs in vivo in the processing of N-glycans in the ER.

The main purpose of this work was to examine in vivo whether variations in the opposing activities of GII and UGGT triggered by removal of mannoses favored generation of unglycosylated or of monoglucosylated N-glycans; that is, whether demannosylation resulted in the exit or a prolonged permanence of misfolded and slow-folding glycoproteins in the CNX/CRT cycles. The system used for these studies was the fission yeast Schizosaccharomyces pombe because of the availability of genetic and biochemical tools and because this microorganism displays an ER quality control mechanism of glycoprotein folding similar to that of mammalian cells (D’Alessio et al., 1999). Saccharomyces cerevisiae lacks UGGT activity and therefore also the cycles mentioned above (Fernández et al., 1994). Contrary to most other eukaryotic cells, both yeast species lack CRT but express CNX. Therefore cycles involving unglycosylated and monoglucosylated glycans in S. pombe will be referred to as CNX cycles.

Here we show that, in live S. pombe cells, the removal of mannosides from the B and/or C branches of the transferred glycan (Figure 1) results in a reduced glucose removal by GII but not in a reduced glucose addition by UGGT. We suggest that ER \( \alpha \)-mannosidase-mediated glycoprotein demannosylation would prolong the half-lives of monoglucosylated glycans, thus preventing exit to the Golgi of misfolded and slow-folding glycoproteins and increasing their possibility of forming proper native structures.

RESULTS

In vivo N-glycan deglucosylation diminishes as a function of the mannose content

To study whether a reduced mannose content in the protein-linked N-glycan affects GII glucose trimming activity in vivo, we constructed the Man6P receptor sharply reduce in vivo deglucosylation rates, thus strongly suggesting that the MRH domain somehow accelerates enzymatic reactions upon recognition of the mannose units in the glycan (Quinn et al., 2009; Stiglianò et al., 2009). As has been shown for UGGT, cell-free assays have shown that the removal of mannose residues from the glycan results in a decrease in the deglucosylation rates and in the affinity of the GII-MRH domain for glycans (Grinna and Robbins, 1980; Totani et al., 2006; Hu et al., 2009). It was suggested, therefore, that misfolded glycoproteins could exit from futile CNX/CRT cycles if a more pronounced decrease in in vivo reglucosylation than deglucosylation occurred upon ER mannosidase removal of the N-glycan mannose units (Cabral et al., 2001). Interestingly, a lectin (Yos9p in yeasts, OS-9 in humans) that is apparently involved in driving misfolded glycoproteins to proteasomal degradation also displays an MRH-like domain, but, in contrast to the MRH domain of GII\( \beta \), the Yos9p/OS-9 MRH-like domain has a higher affinity for glycans with a lower mannose content (Hosokawa et al., 2009; Quan et al. 2009, Satoh et al., 2010). This finding is consistent with a model in which long ER permanence of misfolded glycoproteins results in the generation of partially demannosylated molecules that can be driven to the cytosol.

The ER is a crowded environment in which the protein content may reach concentrations as high as 200–300 mg/ml. Mimicking crowded conditions by adding different proteins (bovine serum albumin, RNase A) or polyethylene glycol to enzymatic assays has revealed that the removal by GII of the more internal (residue \( l \), Figure 1) but not the middle glucose (residue \( m \), Figure 1) increases sharply in crowded environments. These experimental conditions also triggered a conformational change in GII. A similar activating effect was found for mannose removal by an \( \alpha \)-1,2-mannosidase (Totani et al., 2008). Cell-free assays may thus not always reflect what occurs in vivo in the processing of N-glycans in the ER.
a series of fission yeast S. pombe mutants transferring truncated N-glycans to nascent proteins. Except where otherwise stated, the yeast cells used displayed a Δalg10 mutation. Because Alg10p catalyzes the transfer of the last glucose from Dol-P-Glc to Glc2Man7 GlcNAc2–P-P-Dol, the mutants used transferred N-glycans with only two glucose units (Table 1 and Figure 1). The rationale for introducing this mutation was to eliminate any possible effect of the N-glycan mannosic content on GI activity, which could affect the relative levels of N-glycans bearing two, one, or no glucose units to be observed.

Cells were incubated with [14C]Glc for 15 min in the presence of 5 mM diithiothreitol (DTT), and whole-cell N-glycans were isolated and analyzed as described in Materials and Methods. The total incorporation of the label into the N-glycans was linear during the incubation time, and the presence of DTT prevented the passage of glycoproteins to the Golgi, hindering further extension of the recently synthesized N-glycans (Fernández et al., 1998). Addition in the Golgi of many mannose and galactose units would have prevented the measurement of the proportion of N-glycans bearing two, one, or no glucose units. The short incubation time of the cells with 5 mM DTT (a total of 20 min) ensured that the unfolded protein response triggered by the addition of the drug did not affect the levels of GI and UGGT (Pincus et al., 2010).

The N-glycan patterns obtained with Δalg10, Δalg10/Δalg12, Δalg10/Δalg9, and Δalg10/Δalg3 (i.e., transferring Glc2Man7 GlcNAc2, Glc2Man2GlcNAc2, Glc2Man2GlcNAc2, and Glc2Man5 GlcNAc2, which will be respectively abbreviated as G2M9, G2M7, G2M6, and G2M5; the structures of all glycans mentioned in this article are given in Table 1 and Figure 1) revealed that, whereas deglucosylation of G2M9 was so rapid that no glucose-containing glycans were detected, the amount of glucosylated glycans increased as the N-glycan mannosic content decreased (Figure 2, A–E). These results agree with those of previous cell-free assays in which a decrease in the glucosylated glycans during the 15-min labeling period, but a decrease in mannose content resulted in a decrease in both GI-catalyzed reaction rates (Grinna and Robbins, 1980; Totani et al., 2006). The relative rates of deglucosylation of the diglucosylated and monoglucosylated glycans cannot be calculated from the results shown in Figure 2, A–E, because of the continuous refeeding of diglucosylated glycans during the 15-min labeling period, but a decrease in mannose content clearly reduces the conversion of glucosylated glycans to unglucosylated glycans (Figure 2E).

| N-Glycans Recognized by GI | N-Glycans Recognized by GI | N-Glycans Recognized by GT |
|----------------------------|----------------------------|----------------------------|
| Mutant                     | Transferred oligosaccharide | Structure                  | Mutant                     | Transferred oligosaccharide | Structure                  | Mutant                     | Transferred oligosaccharide | Structure                  |
| Wild type                  | G3M9                       | Δalg10                    | G2M9                       | Δalg6                     | M9                         |                           |                           |                           |
| Δalg12                     | G3M7                       | Δalg10/Δalg12             | G2M7                       | Δalg6/Δalg12              | M7                         |                           |                           |                           |
| Δalg9                      | G3M6                       | Δalg10/Δalg9              | G2M6                       | Δalg6/Δalg9               | M6                         |                           |                           |                           |
| Δalg3                      | G3M5                       | Δalg10/Δalg3              | G2M5                       | Δalg6/Δalg3               | M5                         |                           |                           |                           |

In mutants lacking GIIβ gene, the glycan transferred is the same. Triangle: Glc; circle: Man; square: GlcNAc.

TABLE 1: Structure of N-glycans transferred to proteins in S. pombe mutants.

Mutants that transfer G2M8 could not be obtained because the same mannosyltransferase (Alg9p) is responsible for the addition of the seventh and ninth mannose residues to the Dol-P-P–linked derivative (Figure 1).

The effect of N-glycan mannosic content on the deglucosylation levels of mutants lacking the GII regulatory subunit (GIIβ)

The N-glycan pattern experiments were repeated with mutant strains that additionally lacked GIIβ (strains Δalg10/ΔGIIβ, Δalg10/Δalg12/ΔGIIβ, Δalg10/Δalg9/ΔGIIβ, and Δalg10/Δalg3/ΔGIIβ). The absence of the regulatory subunit resulted in a decrease in GlcNAc content as judged by Western blot analysis (Figure 3, lanes 3, 6, 9, and 12), confirming the role of GIIβ in GlcNAc ER retention (Stigliano et al., 2009). Similar N-glycan patterns were observed for mutants transferring G2M7, G2M6, and G2M5 and lacking GIIβ, demonstrating that the mannose content did not influence the deglucosylation of those glycans by GlcNAc in the absence of the regulatory subunit, but the pattern produced by the mutant transferring G2M9 indicated that the glycan containing the full complement of mannososes was deglucosylated at a slower rate because almost no glycans devoid of glucoses were observed (Figure 4, A–E). Mannose residues j and/or k (Figure 1) probably interact with either the GlcNAc active site or the glucose units, thus reducing the rates of deglucosylation.

GIIβ was not essential for the removal of the innermost glucose unit as has been previously suggested (Wilkinson et al., 2006); no such requirement was observed for G2M7, G2M6, or G2M5 (Figure 4, B–D). Furthermore, as already reported, a longer incubation of the ΔGIIβ cells transferring G3M9 led to the production of M9 (Stigliano et al., 2009). The interaction of mannososes j and/or k (Figure 1) with the GIIβ MRH domain likely results not only in the presentation of the glycan to the GlcNAc active site as previously speculated (Stigliano et al., 2009) but also in the elimination of the interaction of those mannose residues with either the active site or the glucoses as suggested earlier in text.

G2M9 to G2M5 N-glycans are recognized in vivo by the GIIβ MRH domain

We have previously reported that the poor deglucosylation levels observed in mutants lacking the regulatory subunit and transferring
G3M9 could be restored to wild-type levels by complementation with exogenous GIlβ, whereas the expression of GIlβ with mutations of residues in the MRH domain that have been shown to interact with mannoses in the Man6P receptor failed to correct the deficiency, even though wild-type GIlβ levels in the ER were totally restored (Stigliano et al., 2009). In this work we performed the same experiment but with mutants transferring G2M7, G2M6, and G2M5 (Δalg10Δalg12ΔGIlβ, Δalg10Δalg9ΔGIlβ, and Δalg10Δalg3ΔGIlβ mutants). Expression of wild-type GIlβ restored the N-glycan patterns observed in cells expressing endogenous GIlβ (compare Figure 5, A–D, with Figure 2, B–E). In contrast, expression of GIlβ with mutations Y462F/E456Q in the MRH domain (hence referred to as GIlβ-MRH*) failed to fully restore the original patterns, although the difference between mutants expressing wild type and mutant GIlβ was much higher for mutants transferring N-glycans with a higher mannose content (compare Figure 5, E–H, with Figure 5, A–D). Western blot analysis showed that the expression of either GIlβ or GIlβ-MRH* restored Glx to similar levels (Figure 3, lanes 4 and 5, 7 and 8, 11 and 12, and 13 and 14), and the results shown in Figure 5, as well as those previously reported, indicate that the MRH domain recognizes N-glycans bearing from nine to five mannose units in vivo, albeit with different affinities.

The G2B domain is involved in the GIlα–GIlβ interaction in S. pombe

In addition to the C-terminal MRH domain, a domain near the N terminus that is conserved among GIlβ subunits of different species (G2B, Supplemental Figure S1) has been reported to be involved in the interaction of both GIl subunits in mammalian cells and in the removal of the middle glucose in S. cerevisiae (Arendt and Ostergaard, 2000; Quinn et al., 2009). A series of mutations were introduced in the S. cerevisiae GIlβ G2B domain. Mutation E132A did not prevent the Glx–GIlβ interaction but did result in reduced G1M9 production in vivo. We mutated the corresponding amino acid (E114A) and amino acid E73A in the S. pombe GIlβ G2B domain (Supplemental Figure S1). Microsomes of S. pombe mutants lacking both GIlα and GIlβ (ΔGIlαβ but expressing GIlβ-E73A or GIlβ-E114A (source of GIlβ) failed to complement the trimming of G1M9 by microsomes of S. pombe cells expressing only GIlβ in the ER (ΔGIlαβ+pGIlβ.VDEL) (Figure 6A). Similar results were obtained with the same mutants expressing the mutated GIlβ MRH domain (ΔGIlαβ+pGIlβ-MRH*). In contrast, microsomes of S. pombe mutants expressing only wild-type GIlβ restored the ability of microsomes expressing only Glx to hydrolyze G1M9 (Figure 6A).

We have previously reported that measurements of microsomal Gl activity with the small molecule pNPG (p-nitrophenyl α-β-glucopyranoside) reflect ER Glx content. We have also previously shown that GIlβ is involved in Glx ER localization (Stigliano et al., 2009). Mutations in G2B but not in the MRH domains affected microsomal Gl activity when measured with pNPG as the substrate, indicating that the former, but not the latter, mutations influence ER Glx retention. In contrast, mutations in both domains influenced Gl activity when G1M9 was the substrate: Microsomes of S. pombe mutants lacking GIlβ but expressing GIlβ with mutations in the G2B domain (ΔGIlβ+pGIlβ-E114A and ΔGIlβ+pGIlβ-E73A) were inactive toward G1M9 as a
number of mannoses decreases from nine to five. It has also been

The transfer of glycans to proteins containing three glucoses
deglucosylation? (Supplemental Figure S2). We were able to deglucosylate G2M9 to G1M9 and G2M6 to G1M6

yeast as cells expressing the E114A mutant of the regulatory subunit

S2A with 4A, S2B with 4C, and S2C with 4E). Similar results were

expression of GII

levels (Figure 6C). In contrast, the expression of wild-type GII

substrate, as were the same mutants expressing GIIβ-MRH* (Figure 6B). As expected, Western blot analysis revealed that the E114A and E73A mutations in GIIβ resulted in reduced ER GIIα levels that were similar to those present in ΔGIIβ mutants, despite the fact that the G2B mutation only slightly affected GIIβ levels (Figure 6C). In contrast, the expression of wild-type GIIβ or GIIβ with mutations in the MRH domain fully restored GIIα levels. A lower band that reacted with the GIIβ antibodies appeared in cells expressing GIIβ with mutations in the G2B domain. We have not yet identified this faster migrating protein, but it likely resulted from a proteolytic cleavage of perhaps a less conformationally stable mutant subunit.

The N-glycan patterns obtained by in vivo labeling of cells transferring G2M9 or G2M6 and lacking endogenous GIIβ but expressing GIIβ with a mutation in the G2B domain (Δalg10/ΔGIIβ + pGIIβ-E114A and Δalg10/Δalg9/ΔGIIβ + pGIIβ-E114A) were similar to those produced by mutant cells transferring the same glycans and lacking the regulatory subunit (Δalg10/ΔGIIβ and Δalg10/Δalg9/ΔGIIβ) (Supplemental Figure S2; compare Figures S2A with 4A, S2B with 4C, and S2C with 4E). Similar results were obtained for the E73A mutation (unpublished results).

These results show that the G2B domain is indeed involved in the GIIα–GIIβ interaction in S. pombe. We did not obtain evidence that it also participates in the removal of the middle glucose in this yeast as cells expressing the E114A mutant of the regulatory subunit were able to deglucosylate G2M9 to G1M9 and G2M6 to G1M6 (Supplemental Figure S2).

Does the N-glycan mannose content influence GI-mediated deglucosylation?

The curious case of Leishmania mexicana

Is the prolonged existence of monoglucosylated glycans due to an ER demannosidase (GII) catalyzed deglucosylation? The curious case of L. mexicana GII. Many years ago, we described the transfer of unglucosylated glycans to proteins by trypanosomatid protozoa (M9, M7, or M6, depending on the species) (Parodi, 1993). These protozoa lack the Dol-P-Glc–snythesizing enzyme and, depending on the species, one or...
No N-glycan demannosylation was detected. Together with the low mannose content of the glycan, this result indicated that ER-demannosylation–triggered loss of GII activity did not occur in this protozoon. The *L. mexicana* genome has putative GIIα and GIIβ subunit–encoding genes, and the latter also has G2B and MRH domains (Supplemental Figure S1). Has the *L. mexicana* GIIβ MRH domain evolved to a structure with a higher affinity for smaller glycans, or has it conserved the higher affinity for M9 of the ancestor microorganism? Proteins with MRH domains with higher affinities for glycans shorter than M9 do exist. One example is Yos9p/OS-9, which displays MRH domains with a higher affinity for shorter glycans, although the glycan structure is not identical to the M6 involved in protozoon N-glycosylation. If the *L. mexicana* GIIβ MRH domain had a higher affinity for smaller glycans, this would eliminate the possibility of preventing Golgi exit of misfolded and slow-folding glycoproteins by extending the existence of monoglucosylated glycans. In contrast, maintenance of the higher affinity for larger glycans would result in a slow deglucosylation of all glycoproteins, including misfolded and slow-folding species.

A comparison of the deglucosylation of G1M9 and G1M6 having the same specific activities by an *L. mexicana* microsomal soluble fraction revealed that the parasite GIIβ MRH has maintained the original N-glycan specificity (Figure 8). Because these glycans were labeled at both the glucose and mannose residues, we confirmed that we were measuring deglucosylation and not demannosylation by adding 1-deoxymannojirimycin (an inhibitor of ER mannosidase) alone or in combination with NMDNJ to the incubation mixtures. Substrate deglycosylation was completely inhibited only when 1-deoxymannojirimycin was added in combination with NMDNJ (Figure 8). Our results suggest that the GII-mediated regulation of the existence of monoglucosylated glycans is an important factor in ER glycoprotein folding quality control.

**DISCUSSION**

The glycans with the highest in vivo deglucosylation levels in the fission yeast *S. pombe* were G2M9 and G1M9; no traces of these glycans were detected after the 15-min labeling period (Figure 2A). The deglucosylation progressively decreased in glycans displaying seven, six, or five mannoses; increasing amounts of their di- and monoglucosylated derivatives were observed as the mannose content decreased (Figure 2, B–E). Nevertheless, even the smallest glycan was recognized in vivo by the MRH domain because the expression of wild-type GIIβ, but not of GIIβ with mutations in the MRH domain, in ΔGIIβ cells fully restored the glycan patterns to that of cells expressing endogenous wild-type
GIIβ (Figure 5). This result suggests that, although the affinity of the MRH domain for G1M9 is approximately seven times higher than that for G1M5, MRH recognition of residue e in addition to residues i and k (see Figure 1 and below; Hu et al., 2009) may influence GII catalytic rates when it is exposed in a glycan, such as in G1M5. A similar influence may exist for mannose i in the case of G2M7 and G1M7 and mannose h for G2M6 and G1M6 (Figure 1).

Unexpectedly, although the in vivo deglucosylation of G2M7, G2M6, and G2M5 was similar for strains expressing GIIα but not GIIβ, that of G2M9 was somewhat lower (Figure 4). This result points to an interaction of residues j and/or k with either the catalytic site or with the glycoses that slow deglucosylation. In an operative holoenzyme, the interaction between the MRH domain and the complete glycan may not only present the latter to the catalytic site, thus somehow strongly accelerating deglucosylation, but also annul the inhibitory effects of residues j and/or k.

G2B, a domain located close to the GIIβ N terminus, has been proposed to be involved in the GIIα–GIIβ interaction or, additionally, in G2M9 deglucosylation (Arendt and Ostergaard, 2000; Quinn et al., 2009). Our results support the former role because microsomes of cells lacking endogenous GIIβ but expressing exogenous wild-type GIIβ were able to correct the poor ability of GIIα to trim glucose residues from G1M9 in the absence of GIIβ. This result was most likely due to a reduced GIIα content as judged by Western blot, supporting a role for GIIβ in which it is partially responsible for GIIα ER retention (Figure 6C). Moreover, the glycan patterns formed upon transfer of either G2M9 or G2M6 in live cells lacking wild-type GIIβ but expressing GIIβ with a G2B mutation were similar to those of cells lacking GIIβ (Supplemental Figure S2). Our interpretation is that the absence of the GIIα–GIIβ interaction results in a failure to both allow MRH-mediated deglucosylation enhancement and relieve the inhibition of deglucosylation mediated by residues j and/or k. Our results show that the G2B domain is not directly involved in G2M9 or G2M6 deglucosylation in S. pombe.

**FIGURE 5:** Glycan patterns synthesized by mutants transferring diglucosylated glycans containing seven to five mannoses and lacking GIIβ but expressing exogenous wild-type GIIβ (A–D) or exogenous GIIβ with a mutated MRH domain (E–H). (A) G2M7 (Δalg10/Δalg12/ΔGIIβ + pGIIβ); (B) G2M6 (Δalg10/Δalg9/ΔGIIβ + pGIIβ); (C) G2M5 (Δalg10/Δalg3/ΔGIIβ + pGIIβ); (D) G2M7 (Δalg10/Δalg12/ΔGIIβ + pGIIβ-MRH*); (E) G2M7 (Δalg10/Δalg12/ΔGIIβ + pGIIβ-MRH*); (F) G2M6 (Δalg10/Δalg9/ΔGIIβ + pGIIβ-MRH*); (G) G2M5 (Δalg10/Δalg3/ΔGIIβ + pGIIβ-MRH*). The structures of the glycans transferred by each mutant are indicated in the corresponding panels. (D and H) Quantification of the relative amounts of the glycans shown in panels A–C (D) and E–G (H).
If the mannose content affects GI-mediated removal of the external glucose (residue n, Figure 1) as has been claimed from cell-free assays of the mammalian but not of a plant enzyme (Grinna and Robbins, 1980; Schweden et al., 1986; Zeng and Elbein, 1998), there is either no such effect in S. pombe, or the effect is much lower than that on GII activity. Alternatively, GI activity levels are much higher than those of GII because the glycan patterns of cells transferring either three or two glucoses and seven, six, or five mannoses were similar (Supplemental Figure S3).

Contrary to what we reported previously when assaying UGGT activity using glycans linked to a single amino acid (Asn) and not denatured glycoproteins as acceptors (Sousa et al., 1992), no influence of the mannose content on in vivo UGGT enzymatic activity was observed (Figure 7). Because the mannose content influenced GI activity but not UGGT activity, no monoglucosylated UGGT-generated glycanse were observed in mutants transferring M9, but increased amounts of those compounds were observed in cells transferring M7 to M5. We can speculate that, as the demannosylation of misfolded and slow-folding glycoproteins proceeds in the ER, the content of monoglucosylated glycans increases. This increase prevents surreptitious exit to the Golgi of misfolded and slow-folding glycoproteins and enhances their probability of folding properly by interacting with CNX (Figure 9). The decrease in affinity of CRT (and probably that of CNX) for N-glycans upon mannose removal (that for G1M5 is 65% of that for G1M9) is apparently more than compensated for by the reduction of the GI-mediated rate of monoglucosylated N-glycan deglucosylation (that of G1M5 is approximately 3% of that of G1M9 in cell-free assays) (Grinna and Robbins, 1980; Spiro et al., 1996). The presence of the MRH domain in GII therefore confers upon the enzyme a major role in ER glycoprotein folding quality control. Up to four mannoses may be removed from misfolded glycoproteins in the mammalian ER. The exit of those species from futile CNX cycles most likely...

**Figure 6:** Mutations in the GII β G2B domain affect the GII α–GII β interaction. (A) Complementation assay between GII α and GII β. Microsomes from ΔGII α S. pombe cells transformed with pGII VDEL (the source of the GII α subunit) were preincubated in the presence of 1% Triton X-100 with microsomes from ΔGII α S. pombe cells expressing wild-type GII α (pGII α), GII α with a mutated MRH domain (pGII α-MRH*), or mutated G2B domain (pGII α-E73A or pGII α-E114A) (sources of the GII β subunit). GII activity assays with [14C-Glc]G1M9 were performed as described in Materials and Methods. (B) GII activity in microsomal fractions of S. pombe wild-type, ΔGII β mutant cells or the same mutants expressing exogenous wild-type GII β or GII β with mutated MRH or G2B domains. Assays were performed with pNPG or G1M9 as substrates. In both cases, the activity was stated relative to that of the wild-type strain (100%). (C) Immunodetection of GII α and GII β in microsomal fractions of S. pombe. Each lane was loaded with 250 μg of microsomal proteins from wild type, ΔGII α or ΔGII β transformed with vector alone (−), wild-type GII β (GII β), or GII β bearing mutations in the MRH (MRH*) or G2B domains (E73A and E114A). The membrane was blotted using mouse polyclonal anti-GII α (1:500) or -GII β (1:1000). Goat HRP anti-mouse 1:5000 was used as the secondary antibody. Reactions were detected by chemiluminescence.
occurs upon removal of mannose unit g in Arm A (Figure 1), the last or one of the last demannosylation events that yields glycans unable to be reglucosylated by UGGT (Frenkel et al., 2003). In vivo formation of protein-linked G1M9, G1M8, G1M7, and G1M6 has been detected in the rat liver (Parodi et al., 1984b).

FIGURE 7: Glycan patterns synthesized by mutants transferring unglucosylated glycans containing nine to five mannoses with inhibition of deglucosylation (plus 5 mM NMDNJ) (A–D) or without inhibition of deglucosylation (minus NMDNJ) (E–H). (A and E) M9 (Δalg6); (B and F) M7 (Δalg6/Δalg12); (C and G) M6 (Δalg6/Δalg9); (D and H) M5 (Δalg6/Δalg3). The structures of the glycans transferred by each mutant are indicated in the corresponding panels. (I and J) Quantification of the relative amounts of the glucosylated and nonglucosylated labeled glycans from panels A–D (I) and E–H (J). In panel E, the label in M8 was added to that of M9. The label in the unidentified peak from panel F was omitted for quantification.
The order in which mannose units are removed in the ER is not the reverse of that of mannose addition in \( \text{Glc}_2\text{Man}_9\text{GlcNAc}_2\)-P-P-Dol synthesis (outlined in Figure 1). In removal, a preferential excision of the first residue, \( \iota \), followed by that of residue \( \kappa \) has been documented in \( S. \ pombe \) as well as in other cell types (Movsichoff et al., 2005). There is evidence indicating that the glycans studied in the present work (i.e., bearing the structures of the biosynthetic intermediates) as well as those produced by ER demannosylation of the transferred compound will be deglucosylated in vivo, after they are glucosylated by UGGT, at diminishing rates as their mannose content decreases. First, as mentioned earlier in text, cell-free assays have shown that GII activity decreases as the mannose content of the glycans produced by ER processing decreases (Grinna and Robbins, 1980; Totani et al., 2006). The known affinities of the MRH domain for glycans processed by ER processing also support this hypothesis (Hu et al., 2009). For example, the affinities of the MRH domain for G1M8 lacking either residue \( \iota \) or \( \kappa \) are 36 and 22%, respectively, of that for G1M9, and the affinity for G1M7 lacking residues \( \iota \) and \( \kappa \) is approximately 7% of that for G1M9. These results indicate that the MRH domain primarily recognizes residue \( \kappa \), followed by residue \( \iota \). From these data, it may be concluded that the G1M8 normally produced in cells by glycan processing (i.e., lacking residue \( \iota \), Figure 1) will be deglucosylated at a lower rate than G1M9. The next glycan produced in vivo (G1M7 lacking residues \( \iota \) and \( \kappa \), Figures 1 and 9) will be deglucosylated at an even slower rate because it lacks both residues that are primarily recognized by the MRH domain. The G1M7 produced by ER processing (lacking residues \( \iota \) and \( \kappa \) may be deglucosylated at a slower rate than the G1M7 studied in the present work (lacking residues \( \jmath \) and \( \kappa \)) because the former, but not the latter, lacks residue \( \iota \), which is the second most important residue recognized by the MRH domain. Additional ER removal of \( \alpha_1,2 \)-linked mannose units is expected to further reduce the affinity of the MRH domain for glycans. Point mutations in the MRH domain resulted in a reduction of both its affinity for glycans produced by ER processing and in the deglucosylation rates of those glycans in cell-free assays (Hu et al., 2009). Those same point mutations also resulted in reduced in vivo glycan deglucosylation (Stigliano et al., 2009). Therefore a decrease in the affinity of MRH for glycans, such as that mentioned earlier in text for ER-produced G1M8 and G1M7, will result in reduced in vivo deglucosylation. With respect to UGGT, our results indicate that all glycans bearing residue \( \gamma \) will be equally glucosylated, irrespective of their mannose content or structure (Figure 7).

The relevance of displaying a slow deglucosylation mechanism to increase the time frame in which a particular glycan has a monoglucosylated epitope is supported by the fact that \( L. \ mexicana \), a protist transferring M6 and lacking ER mannosidase activity that has retained an ancestor-derived GIIb with an MRH domain with higher affinity for high mannose content glycans (Figure 8). Proteins with MRH domains with higher affinity for glycans shorter than M9 do exist, such as Yos9p/OS-9, which displays MRH activity that has retained an ancestor-derived glycan demannosylation and has also been reported (Trombetta and Parodi, 1992; Fernández et al., 1994; Stigliano et al., 2009).

The present studies were conducted in \( S. \ pombe \), but the conclusions drawn are assumed to also be applicable to mammalian cells. No significant differences in the specificity, kinetics, and regulatory properties of UGGT and GII from either species have been reported (Trombetta and Parodi, 1992; Fernández et al., 1994; Stigliano et al., 2009).

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**FIGURE 8:** Glucose release from either G1M9 or G1M6 by \( L. \ mexicana \) GII. In all cases, the incubation mixtures contained 1 mM 1-deoxymannojirimycin and, where indicated, the same concentration of NMDNJ. The percentage of the total glucose liberated was calculated from each glycan \([14C] \) glucose content (see Materials and Methods). The value for G1M9 was taken as 100%.

**FIGURE 9:** Model proposed for GII as an in vivo regulator of misfolded/slow-folding glycoprotein ER permanence. Misfolded/slow-folding species are characterized by an ER mannosidase(s)-catalyzed N-glycan demannosylation. A decrease in N-glycan mannose content significantly diminishes in vivo GII-mediated deglucosylation rates but does not affect in vivo UGGT-mediated glucosylation, thus increasing the possibility of displaying monoglucosylated structures able to interact with CNX/CRT for longer time periods. The exit of irreversibly misfolded glycoproteins from futile CNX cycles most likely will occur, at least in mammalian cells, upon removal of mannose unit \( g \) in Arm A (see Figure 1).
MATERIALS AND METHODS

Materials
Yeast extract, Bacto-Peptone, and yeast nitrogen base were obtained from Difco (Detroit, MI). Malt extract was obtained from Britania (Buenos Aires, Argentina). Endo-β-N-acetylglucosaminidase H (Endo H), N-glycanase (PNGase F), porcine trypsin, protease inhibitors, PNP, DTT, amino acids, and supplements for culture media were obtained from Sigma (St. Louis, MO). [3H]glucose (301 Ci/mol) was obtained from PerkinElmer Life Sciences (Waltham, MA). NMNDJ and 1-deoxymannojirimycin were obtained from Tokyo Biochemicals. Geneticin (G418) was obtained from Invitrogen (Carlsbad, CA), and nourseothricin was from WERNER BioAgents (Jena, Germany).

Strains and media
Escherichia coli DH5α was used for cloning purposes, and recombinant protein expression was performed in BL26 cells. Bacteria were grown at 37°C in LB medium (0.5 % NaCl, 1% tryptone, 0.5% yeast extract) supplemented with 200 μg/ml ampicillin or 50 μg/ml kanamycin as needed. S. pombe strains were grown at 28°C in rich YES medium (0.5% yeast extract, 3% glucose, and 75 μg/l adenine) or EMM minimal medium (Moreno et al., 1991; Alfa et al., 1993), supplemented with adenine (75 μg/l), uracil (75 μg/l), and/or leucine (250 μg/l) for selective growth. Malt extract medium (3% Bacto Malt Extract pH 5.5 supplemented with adenine, uracil, and leucine) was used for matings. Geneticin was added to the medium at 200 μg/ml for KanMX6 selection, and nourseothricin was added to the medium at 100 μg/ml for NatMX6 marker selection in rich medium. When double selection for geneticin and auxotrophic markers was needed, NH₄Cl was replaced by 0.37% monosodium L-glutamate as the nitrogen source in EMM. L. mexicana cells were grown as previously described (Parodi et al., 1984a). The S. pombe strains used are summarized in Table 2.

Genetic and DNA procedures
DNA procedures were as described previously (Sambrook and Russell, 2001). Yeast DNA extraction was performed as described previously (Hoffman and Winston, 1987). S. pombe transformations were performed by electroporation with 0.5 μg of plasmid DNA or 1 μg of linear DNA as described in Stigliano et al. (2009). Cells were recovered in 0.5 M sorbitol in YES medium for 1 h at 28°C and plated on the appropriate selective medium.

S. pombe Δalg10, Δalg12, and Δalg9 strains were purchased from Bioneer (Daejeon, Korea). S. pombe Δalg6 mutants were obtained as in Fanchiotti et al. (1998), but in a Sp61 strain genetic background. S. pombe Δalg3 mutants were constructed as follows: a disruption cassette containing the sequence of the KanMX6 selection marker flanked by 307 and 266 base pairs of the 5′ and 3′ portions of the S. pombe alg3 gene (SPAC7D4.06c) was obtained in this study (Y462F/E456Q) was described in Stigliano et al. (2009). Cells were harvested, washed with 5 mM NaN₃, resuspended in 0.25 M sucrose, 20 mM imidazole, and 5 mM EDTA with 10 repetitive cycles of 1-min vortexing on ice with glass beads in 0.25 M sucrose, 20 mM imidazole, and 5 mM EDTA with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μM.

GliIΔ mutagenesis
The gateway pDONR201 plasmid containing clone 26/D11 (S. pombe GliI subunit, which was obtained from RIKEN DNA Bank; Matsuymaya et al., 2006) was used as the template for single amino acid PCR mutagenesis of the GliI GliI domain (ET73A or E114A). The amplified mutant DNA containing both GliI and vector was phosphorylated, religated, and electroporated into DH5α cells. The primers used were the following (mutagenic codons are underlined): ET73A forward mutagenic primer 5′-GATGCCAGGTTAATAGC-3′, reverse primer 5′-TGACCGTACATGAGATT-3′; E114A forward mutagenic primer 5′-GATGCCAGGTTAATAGC-3′, reverse primer 5′-AGAACCGTACATGAGATT-3′. Wild type and mutant GliI DNA clones were transferred to the pREP1-ccdb2 Gateway-compatible S. pombe destination expression vector (RIKEN DNA Bank) by the LR recombination reaction (Invitrogen). S. pombe competent ΔGliI cells were electroporated with the pREP1-GliI episomal constructs. The MRH domain double mutant construct used in this study (Y462F/E456Q) was described in Stigliano et al., (2009) (by mistake, in that reference the numbering of the amino acids was Y463F/E457Q).

Microsomal fraction preparations
S. pombe microsomes were prepared from 250 ml of cultures at A₆₀₀ = 2. Cells were harvested, washed with 5 mM NaN₃, and broken by 10 repetitive cycles of 1-min vortexing on ice with glass beads in 0.25 M sucrose, 20 mM imidazole, and 5 mM EDTA with protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μM.
The role of the GII j subunit

L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 μM Nε-p-tosyl-L-lysine chloromethyl ketone, 10 μM leupeptin, 10 μM peptatin, and 10 μM E64, and the microsomal fraction was obtained as described (D’Alessio et al., 1999). For L. mexicana, the ER soluble content was prepared by freeze-thawing the parasites as previously described for Trypanosoma cruzi (Labriola et al., 1995). Protein concentrations were determined by the Bio-Rad Protein Assay as described by the manufacturer (Hercules, CA).

Analysis of glycans synthesized in vivo
To assess ER N-glycan composition, S. pombe cells in the exponential growth phase were harvested, extensively washed with 1% YNB medium without glucose, and resuspended in two volumes (vol/vol) of the same medium. Cells were then preincubated for 5 min in 5 mM DTT and pulsed for 15 min in 5 mM glucose with 300 μCi/ml of [14C]glucose. Further details on the labeling procedure and the preparation of whole-cell Endo H-sensitive N-glycans have been described previously (Fernández et al., 1994). For strains carrying the Δalg3 mutation that transfer a pentamannosyl glycan, whole-cell proteins were degraded with porcine trypsin instead of pronase, and the removal of N-glycans was performed with N-glycanase (glycans synthesized by strains harboring the Δalg3 mutation are resistant to Endo H, and the N-glycanase requires both the Asn amino and carboxyl groups to be substituted by amino acids for activity). For strains carrying the Δalg6 mutation, cells were preincubated for 60 min in the presence or absence of 5 mM NMDNJ. Glycans were separated by paper chromatography as described (D’Alessio et al., 2009).

Strains (nickname)  Genotype  Source
Sp61 (WT)  h+, leu1−32, ade6-M210, ura4-D18, ade1  Our stock (D’Alessio et al., 1999)
ADm (WT)  h+, leu1−32, ade6-M210, ura4-D18  Our stock (D’Alessio et al., 1999)
ADp (WT)  h+, leu1−32, ade6-M216, ura4-D18  Our stock (D’Alessio et al., 1999)
Sp611α (ΔGIIq)  h+, leu1−32, ade6-M210, ura4-D18, Δalg2α::ura4+  Our stock (D’Alessio et al., 1999)
ADm1β (ΔGIIβ)  h+, leu1−32, ade6-M210, ura4-D18, Δalg2β::ura4+  Our stock (D’Alessio et al., 1999)
SpAD11αβ (ΔGIIαβ)  h+, leu1−32, ade6-M216, ura4-D18 Δalg2α::ura4+, Δalg2β::ura4+  Our stock (Soussilane et al., 2009)
SPAC56F8.06c (Δalg10)  h+, leu1−32, ade6-M216, ura4-D18, Δalg10::KanMX4  Bioneer
SPa10−4AK (Δalg10-K)  h+, leu1−32, ade6-M210, ura4-D18, Δalg10::KanMX4  This study
SPa10−4AN (Δalg10-N)  h+, leu1−32, ade6-M210, ura4-D18, Δalg10::NatMX4  This study
SPBC1734.12C (Δalg12)  h+, leu1−32, ade6-M216, ura4-D18, Δalg12::KanMX4  Bioneer
SPAC1834.05 (Δalg9)  h+, leu1−32, ade6-M216, ura4-D18, Δalg9::KanMX4  Bioneer
ADpA3 (Δalg3)  h+, leu1−32, ade6-M216, ura4-D18, Δalg3::KanMX6  This study
SPa10A12−2B (Δalg10/Δalg12)  h+, leu1−32, ade6-M210, ura4-D18, Δalg10::KanMX4, Δalg12::KanMX4  This study
SPa10A9−7C (Δalg10/Δalg9)  h+, leu1−32, ade6-M216, ura4-D18, Δalg10::NatMX4, Δalg9::KanMX4  This study
SPa10A3−7A (Δalg10/Δalg3)  h+, leu1−32, ade6-M216, ura4-D18, Δalg10::NatMX4, Δalg3::KanMX4  This study
SPa10GIIβ−1C (Δalg10/ΔGIIβ)  h+, leu1−32, ade6-M210, ura4-D18, Δalg10::NatMX4, Δalg3::KanMX4, Δalg2β::ura4+  This study
SPa10A12GIIβ−10B (Δalg10/Δalg12/ΔGIIβ)  h+, leu1−32, ade6-M210, ura4-D18, Δalg10::KanMX4, Δalg12::KanMX4, Δalg2β::ura4+  This study
SPa10A9GIIβ−12D (Δalg10/Δalg9/ΔGIIβ)  h+, leu1−32, ade6-M216, ura4-D18, Δalg10::NatMX4, Δalg9::KanMX4, Δalg2β::ura4+  This study
SPa10A3GIIβ−14A (Δalg10/Δalg3/ΔGIIβ)  h+, leu1−32, ade6-M216, ura4-D18, Δalg10::NatMX4, Δalg3::KanMX4, Δalg2β::ura4+  This study
Sp61A (Δalg6)  h+, leu1−32, ade6-M210, ura4-D18, ade1, Δalg6::ura4+  This study
SPa6A12−3D (Δalg6/Δalg12)  h+, leu1−32, ade6-M210, ura4-D18, Δalg6::ura4+, Δalg12::KanMX4  This study
SPa6A9−5C (Δalg6/Δalg9)  h+, leu1−32, ade6-M216, ura4-D18, Δalg6::ura4+, Δalg9::KanMX4  This study
SPa6A3−8C (Δalg6/Δalg3)  h+, leu1−32, ade6-M210, ura4-D18, Δalg6::ura4+, Δalg3::KanMX4  This study

WT: wild type.

TABLE 2: Yeast strains used in this study.
chromatography using Whatman 1 papers and 1-propanol and nitromethane/H$_2$O (5:2:4) as the solvent, and the peaks were identified by standards run in parallel. To improve the resolution, the identified glycans were eluted from the papers and resolved by high performance liquid chromatography (HPLC) using a TSK-GEK Amide-80 column (4.6 mm x 25 cm; Tosoh Bioscience, Tokyo, Japan) with a mobile phase of H$_2$O/CH$_3$CN in a linear gradient from 35:65 to 55:45 over 67 min and a flow rate of 0.75 ml/min at room temperature. Due to slight variations in retention times among runs, the positions of the peaks in paper chromatography and not the retention times from HPLC were used to identify the glycans.

**Synthesis of labeled N-glycan substrates**

Glucose- and mannose-labeled [14C]Glc$_3$Man$_9$GlcNAc and [14C]Glc$_2$Man$_9$GlcNAc were obtained by in vivo labeling and N-glycan purification of S. pombe cells carrying the Δalg6 or the Δalg6/Δalg9 mutations, respectively. Cells were preincubated with 5 mM NMDN for 60 min and with 5 mM DTT for 5 min and were then incubated for 45 min in 5 mM glucose containing 500 μCi [14C] glucose. The Endo H–sensitive monoglucosylated glycans were separated from the unglucosylated compounds by two successive paper chromatographies in n-propanol and nitromethane/H$_2$O (5:2:4). The label in glucose residues was 27 and 35% of the total for [14C]Glc$_3$Man$_9$GlcNAc and [14C]Glc$_2$Man$_9$GlcNAc, respectively, as determined by total acid hydrolysis of glycans followed by paper chromatography in n-butanol and pyridine/H$_2$O (10:3:3). [14C-glucose]Glc$_3$Man$_9$GlcNAc was obtained by glucosylation of de-natured bovine thyroglobulin in the presence of UDP-[14C]Glc and rat liver microsomes, followed by glycan purification as described previously (Trombetta et al., 1989).

**GII activity assays**

GII activity using labeled glycans as substrates was assayed in S. pombe or L. mexicana microsomal fractions as described in Stigliano et al. (2009). Where indicated, 1 mM 1-deoxymannojirimycin was added with or without 1 mM NMDN. GII activity in ΔGIIβ mutants transformed with wild type or mutant regulatory subunits was assayed using 5 mM pNPGL as the substrate in 0.1 M HEPES buffer, pH 7.2. Incubations lasted for 20 min at 37°C as described in Stigliano et al. (2009). Complementation assays between GIIα and GIIβ were performed using a mixture of 125 μg of proteins from microsomes containing Glitx but not GIIβ [ΔGIIαGβ cells transformed with pREP3x-GltxαAlD5L, as described in Stigliano et al., 2009] with 125 μg of proteins from microsomes containing GIIβ but not GIIα [ΔGIIαβ mutant cells expressing either wild-type GIIβ or GIIα mutated in either the MRH or G2B domains]. The mixtures were preincubated at 30 min at 4°C in the presence of 1% Triton X-100 and then assayed for GII activity with [14C-glucose]Glc$_3$Man$_9$GlcNAc in 40 mM sodium phosphate buffer, pH 7.2, for 15 min at 30°C to test the ability of GIIβ to partially restore GII N-glycan trimming ability. The liberated glucose was separated from the remaining substrate by ascending paper chromatography using 2-propanol and acetic acid/H$_2$O (25:4:9), and the activity was determined as the percentage of total glucose released.

**Antibodies and immunodetection**

Microsomal S. pombe proteins (250 μg) were resolved by 9% SDS-PAGE, electroblotted to Immobilon-P membranes (Millipore, Billerica, MA) and incubated with mouse anti-S. pombe Glitx, mouse anti-S. pombe GIIβ, or rabbit anti-S. pombe CNX antibodies. Immunodetection was carried out using enhanced chemiluminescence (West Pico SuperSignal Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA) with horseradish peroxidase (HRP)-conjugated immunoglobulin Gs (IgGs; Sigma).

Mouse polyclonal serum raised against the S. pombe GIIβ protein was obtained as follows: a 447 base pair GIIβ DNA fragment was PCR amplified from a pDONR201 plasmid containing clone 26/111 using the primers GIIβ-NdeI forward 5'- GGAATTCATTATGCCAGAATATCGGACACATTAG 3' and GIIβ-XhoI reverse 5'- CGGCTTGGAGATTTGAACTCATCGGG 3', cloned in pET22b+ (Invitrogen) and expressed as a C-terminal His$_6$ fusion protein in E. coli BL26 cells. After a 4-h induction with 1 mM isopropyl-β-D-thiogalactoside, the protein was purified from inclusion bodies by immobilized metal ion affinity chromatography using chelating Sepharose (Amersham, Little Chalfont, UK) in the presence of 6 M urea as described by the manufacturer. Protein (20 μg) was injected intra dermally to BALB/c mice, and two boosters of 10 μg were given after 15 and 30 d. The serum was used at a 1:1000 dilution as a primary antibody for S. pombe GIIβ immunodetection. Mouse polyclonal anti-Gltx and rabbit polyclonal anti-CNX sera were obtained as described elsewhere (Stigliano et al., 2009) and were used at 1:500 and 1:100,000 dilutions, respectively.

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