

A sensitive green fluorescent protein biomarker of N-glycosylation site occupancy

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ABSTRACT N-glycosylation mediates many biological functions. Genetic defects in the N-glycosylation pathway cause >35 inherited human disorders called congenital disorders of glycosylation (CDGs). As a result, some N-glycosylation sites are unoccupied. Serum transferrin is a diagnostic marker for these patients, but there are no corresponding cellular markers to assess glycosylation competence. Therefore, we engineered a green fluorescent protein (GFP) construct to measure N-glycosylation site occupancy. We designed an endoplasmic reticulum-retained GFP biomarker whose fluorescence is lost when it is N-glycosylated due to steric hindrance by the glycan. This marker is a highly sensitive indicator of N-glycosylation site occupancy. In CDG cells carrying the GFP construct, a 25% decrease of glycosylation efficiency induces a 5-fold increase in fluorescence, while cDNA complementation of the genetic defect results in a 5-fold decrease in fluorescence. This engineered GFP detects impaired N-glycosylation in multiple cell lines, including CHO cells, HeLa cells, normal and patient fibroblasts, induced pluripotent stem cells (iPSCs), and human embryonic stem cells (hESCs). This marker is a highly sensitive tool to study N-glycosylation site occupancy. It can be used to screen for compounds that reverse poor N-glycosylation site occupancy.—Losfeld, M.-E., Soncin, F., Ng, B. G., Singec, I., Freeze, H. H. A sensitive green fluorescent protein biomarker of N-glycosylation site occupancy. *FASEB J.* 26, 4210–4217 (2012). www.fasebj.org

Key Words: congenital disorders of glycosylation • stem cells • genetic disease

CONGENITAL DISORDERS OF glycosylation (CDGs) are a family of rare inherited metabolic syndromes affecting

either synthesis, transfer, or processing of glycans (1–3). Patients with CDGs show diverse clinical phenotypes, including motor and intellectual disability and variable multisystemic symptoms (4). CDGs have been classified into two main types depending on the glycosylation pathway steps affected (5). Type I impairs early steps of glycosylation, resulting in unoccupied N-glycosylation sites. In type II, the defects do not influence site occupancy, but alter glycan processing.

Usually, patients with CDGs are diagnosed by abnormal glycosylation of serum transferrin (6, 7). Assessment of a glycan alteration and the specific genetic defects is complex, often requiring a combined biochemical and genetic approach (8). Multiple biochemical analyses, such as gene sequencing and complementation, are needed to identify and confirm the mutated gene and pathogenicity of the specific mutation. No universal N-glycosylation marker comparable to serum transferrin is available in patient cells. In some cases, adenoviral transfected DNase 1 can show underglycosylation (9, 10). However, not all gene defects are capable of being complemented due to toxicity of overexpressing the wild-type gene.

In our studies, we sought to engineer a sensitive biomarker of defective N-glycosylation site occupancy using the green fluorescent protein (GFP). The GFP structure and properties have been well characterized previously (11, 12). In brief, GFP is composed of 238 amino acids and is structurally organized into a barrel containing 11 β -sheets and 4 α -helices. The fluorophore is derived from interaction of 3 adjacent amino acids, Ser65-Tyr66-Gly67, located at the center of this barrel (13). Since its discovery, the GFP amino acid sequence has been extensively modified; for instance, changing of Ser65 to a Thr residue enhances brightness (eGFP) (14).

Previously, Contessa *et al.* (15) demonstrated that endoplasmic reticulum (ER)-located luciferase can be

Abbreviations: CDG, congenital disorder of glycosylation; CM, conditioned medium; DAPI, 4,6-diamidino-2-phenylindole; EB, embryoid body; ER, endoplasmic reticulum; ER-GFP, endoplasmic reticulum-located green fluorescent protein; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; Glyc-ER-GFP, glycosylatable endoplasmic reticulum-located green fluorescent protein; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; PMM2, phosphomannomutase 2

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used as a reporter of N-linked glycosylation inhibition by tunicamycin for cancer therapy. Here, we describe the design of an ER-localized GFP containing an N-glycosylation site that destroys GFP fluorescence when it is occupied by a glycan chain. N-glycosylation consensus sequences, Asn-X-Ser/Thr, were added at several different locations in an ER-located GFP sequence to obtain the glycosylable reticular GFP. The amino acids changed by mutagenesis have been chosen using results of Goh *et al.* (16) and Richmond *et al.* (17), where engineered GFP has been used as a biosensor for bacterial endotoxin and to monitor copper binding, respectively. Such markers can be useful to monitor glycosylation efficiency for different applications, for example, to identify unknown deficient genes in CDG cases, to screen compounds that rescue impaired glycosylation in CDG cells, or to monitor effects of drugs on glycosylation efficiency.

Here, we use fluorescence microscopy, fluorescence-activated cell sorting (FACS), and Western blot analysis of our GFP to assess N-glycosylation in HeLa cells, fibroblasts from healthy donors and patients with CDGs, human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs). We apply this technique to analysis of impaired N-glycosylation.

MATERIALS AND METHODS

DNA, site-directed mutagenesis, and construction of ER-localized GFP (ER-GFP)

Constructs were prepared using the pEGFP vector from Clontech (Mountain View, CA, USA). EGFP was cloned in pcDNA3.1 vector and engineered to have an in-frame N-terminal signal peptide of calreticulin and an in-frame C-terminal KDEL sequence (pcDNA 3.1-ER-GFP). The insertions of N-glycosylation sites were made using the QuickChange directed-mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and primers from Integrated DNA Technologies (San Diego, CA, USA).

Cell culture and transfection

Fibroblasts and HeLa cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM with 1 and 4.5 g/L Glc (Cellgro, Manassas, VA, USA), respectively, containing 1% (v/v) glutamine (Cellgro), 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) penicillin/streptomycin (Cellgro) at 37°C. Cells were transiently transfected by the different constructs by addition of 6 µl of Superfect (Qiagen, Valencia, CA, USA) per microgram of DNA. H9 hESCs (WA09) and iPSCs were grown onto BD matrigel-coated plates (BD Biosciences, San Jose, CA, USA) in mouse embryonic fibroblast (MEF)-conditioned medium (CM) composed of glutamax-containing DMEM/F12, 20% (v/v) knockout serum, 1% nonessential amino acids, 1% (v/v) antibiotic-antimycotic, 0.2% (v/v) β-mercaptoethanol (Life Technologies, Inc., Grand Island, NY, USA), and 8 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA). Medium was exposed to MEFs for 24 h, collected, and filter-sterilized, and fresh bFGF was added before use. Cells were passaged manually using Dispase (StemCell Technologies, Vancouver, BC, Canada) every 6–7

d. Cells were transiently transfected using the Neon transfection system (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. Briefly, 1×10^6 cells were collected using Accutase (Millipore, Billerica, MA, USA), transfected in 100 µl of electroporation buffer containing 2 µg of either ER-GFP or glycosylatable ER-GFP (Glyc-ER-GFP; parameters: 1 pulse at 1400 V for 20 ms) and immediately transferred into MEF-CM containing 10 µM of Rock inhibitor Y-27632 (Alexis Biochemicals, Farmingdale, NY, USA). The Rock inhibitor was withdrawn 24 h after transfection.

Fibroblast reprogramming into iPSCs

CDG-Ia #3 fibroblasts were reprogrammed using the original Yamanaka factors using retroviruses according to a modified protocol of Takahashi *et al.* (19). Briefly, we used PLAT-A cell for virus production, obviating the need to introduce mouse receptor for retroviruses (Slc7a1) prior to transduction with the reprogramming factors. In contrast to Takahashi *et al.* (19), we also used human foreskin fibroblasts (HS27, ATCC) as feeder cells during the reprogramming process. The CDG-Ia #1 fibroblasts were reprogrammed using an integration-free system from Okita *et al.* (25). At 7 d after exposure to the reprogramming factors, 1×10^5 cells were plated onto a MEF layer; at d 8, medium was switched to hESC medium. hESC-like colonies started to appear around d 33–35 and were manually picked when ready. Around p3 iPSCs were adapted to a feeder-free system for characterization and further experiments.

Materials

Tunicamycin was purchased from Sigma (St. Louis, MO, USA). [$2\text{-}^3\text{H}$] mannose and [^{35}S] Met/Cys were obtained from PerkinElmer Life Sciences, Boston, MA, USA.

Radiolabeling

HeLa cells at 70% confluency were labeled as described previously after 1 h of treatment with various tunicamycin concentrations (26).

Immunofluorescence microscopy

For tunicamycin or mannose treatment, drugs were added 24 h after transfection. Cell preparation was adapted from Peanne *et al.* (27), using 4% paraformaldehyde and 4% sucrose for fixation; cells were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). For colocalization with the GRP78, cells were incubated with anti-GRP78 (Jackson ImmunoResearch, West Grove, PA, USA) at 1:500, followed by Cy3-linked anti-mouse IgG antibody (Neomarkers, Fremont, CA, USA) at 1:1000. For immunodetection of GFP, cells were incubated with anti-GFP (Clontech) at 1:500, followed by Cy3-linked anti-mouse IgG antibody (Neomarkers) at 1:1000. Images were acquired using an Olympus BX50 microscope with a $\times 100$ zoom and using the Spot Advance software (Olympus, Tokyo, Japan). Relative fluorescence was monitored using ImageJ software [U.S. National Institutes of Health (NIH), Bethesda, MD, USA].

hESCs and iPSCs were cultured in BD-matrigel-treated plates, fixed in 4% PFA, and stained *in situ*, as described previously. Briefly, cells were blocked in donkey block (1% donkey serum and 0.1% triton X-100 in PBS) for 30 min. Primary and secondary antibodies were incubated overnight at 4°C and for 2 h at room temperature and in the dark,

respectively. Primary antibodies were as follows: anti-Oct3/4 (1:25 dilution; BD Biosciences) and anti-Nanog (1:20 dilution; R&D Systems). Secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 555 were used (1:500 dilution; Invitrogen). Samples were washed thoroughly between incubations and mounted using DAPI Vectashield (Vector Laboratories). Cells were viewed on an Olympus IX71 inverted microscope. Relative fluorescence was monitored using ImageJ software.

Western blot analyses

12% SDS-PAGE gels and blots were used to detect GFP using anti-GFP monoclonal mouse antibody (1:20,000; Clontech) followed by an HRP-linked anti-mouse IgG secondary antibody (1:7500; Amersham, Piscataway, NJ, USA). PNGase F digestion was done on 50 µg protein. Proteins were denatured at 100°C for 5 min in the presence of 0.5% SDS and 10% β-mercaptoethanol, then incubated overnight at 37°C with 1% Nonidet P-40, 50 mM sodium phosphate (pH 7.5), and 6 U of PNGase F.

FACS analyses

As described previously (28), cells were harvested in the presence of trypsin/EDTA and permeabilized in 20 mM HEPES (pH 7.2), 0.16 M NaCl, 1 mM EGTA, and 0.02% digitonin for 3 min on ice. Cells were analyzed with the FACScalibur (BD Biosciences).

Complementation-cloning of Flag-phosphomannomutase 2 (PMM2), retroviral production, and infection

Human PMM2 cDNA was cloned and engineered to have an in-frame N-terminal flag tag. The resulting Flag-PMM2 was then cloned into pBABE-Puro using *Bam*H1 and *Eco*RI restriction sites. HEK 293T cells were transfected with 10 µg of pCL-Ampho (Imgenex Corp., San Diego, CA, USA) and 10 µg of either pBABE-puro, Flag-PMM2, or empty vector. Viral supernatant was collected 48 h post-transfection and filtered, then used immediately without freezing. Patient cells were infected in the presence of 8 µg/ml polybrene (Sigma) for 24 h and then allowed to recover in fresh medium for an additional 48 h. Cells were then selected with 1 µg/ml of Puromycin (Sigma) for 3 d to create a stable cell line.

Embryoid body (EB) formation

hESCs and iPSCs were manually scored and transferred onto non-tissue-culture-treated plates in standard hES medium without bFGF, where they formed EBs. EBs were maintained in suspension for 10 d while replenishing medium every other day.

RT-PCR

EBs were collected by centrifugation, washed twice with PBS, and total RNA was extracted using the RNA extraction kit (Qiagen) according to the manufacturer's guidelines, comprising a DNase treatment step. cDNA was synthesized using SuperScript First Strand cDNA kit (Invitrogen). Samples (1 µl) of the cDNA reaction were amplified with specific primers for 35 cycles using Reddy Mix PCR mastermix (1.5 mM MgCl₂; Thermo Fisher Scientific) and visualized on 1% agarose gel containing 400 ng/ml ethidium bromide using ChemiDoc XRS and Quantity One software (Bio-Rad, Hercules, CA, USA).

RESULTS

ER-GFP and Glyc-ER-GFP validation in HeLa cells

Early steps of *N*-glycosylation occur in the ER, so GFP was targeted to the ER by adding a signal peptide from calreticulin to the N terminus and a KDEL sequence to the C terminus for ER retention (Fig. 1A). We refer to this as ER-GFP. Figure 1B shows the colocalization of the ER-GFP with GRP78, a chaperon exclusively expressed in the ER.

We carried out a set of directed mutagenesis experiments to introduce one, or in some cases two, *N*-glycosylation consensus sequences throughout ER-GFP by using two approaches. First, we made single-point mutations near preexisting Asn, Thr, and Ser residues to minimize changes to GFP structure. The second approach targeted regions near the cyclization site of the GFP. Previous studies demonstrated that different regions in the GFP can be modified without affecting GFP structure, thereby modifying its fluorescence; these include aa 145–150, 200–205, and 224. A large set of >30 mutants was designed and tested. Here we present one of the most efficient constructs obtained with the mutation p.N147T, which we named Glyc-ER-GFP (Fig. 1A).

HeLa cells were transiently transfected with the different Glyc-ER-GFP constructs to determine whether addition of the *N*-glycan decreased or abolished the fluorescence. We present only the construct showing the largest fluorescence difference.

In HeLa cells, the Glyc-ER-GFP produced very low background fluorescence (Fig. 1C) compared to ER-GFP. However, after the addition of 1 µg/ml of tunicamycin, transfected HeLa cells showed enhanced Glyc-ER-GFP fluorescence. A time course in the presence of tunicamycin showed the appearance of fluorescence by microscopy 24 h after transfection (Fig. 1C, D), and expression of GFP by immunoblotting (Fig. 1E). We observed an initial appearance of GFP glow by 8 h that increased up to 72 h of treatment (Fig. 1C, D) as newly synthesized unglycosylated Glyc-ER-GFP accumulated. Nontreated cells still did not show fluorescence after 72 h. Glycosylation of Glyc-ER-GFP was confirmed by PNGase F digestion (Fig. 1E). The HeLa cells transfected by Glyc-ER-GFP were treated with 0–2 µg/ml of tunicamycin. Expression of glycosylated and nonglycosylated GFP was checked by Western blot (Fig. 2A). Appearance of green fluorescence (Fig. 2B) was compared to incorporation of radiolabeled [³H]-mannose and [³⁵S]-labeled amino acids (Fig. 2C). We observed a decreased incorporation of [³H]-mannose but incorporation of [³⁵S]-labeled amino acids was stable. Fluorescence begins to appear at a very low concentration of tunicamycin (Fig. 2D) which had only modest effects on [³H]-mannose incorporation into glycoproteins (Fig. 2C). These results underscore the high sensitivity of this construct. CHO cells gave a nearly identical dose-response profile (Fig. 2D).

HeLa cells expressing the Glyc-ER-GFP were also analyzed by flow cytometry. Figure 2E clearly shows a

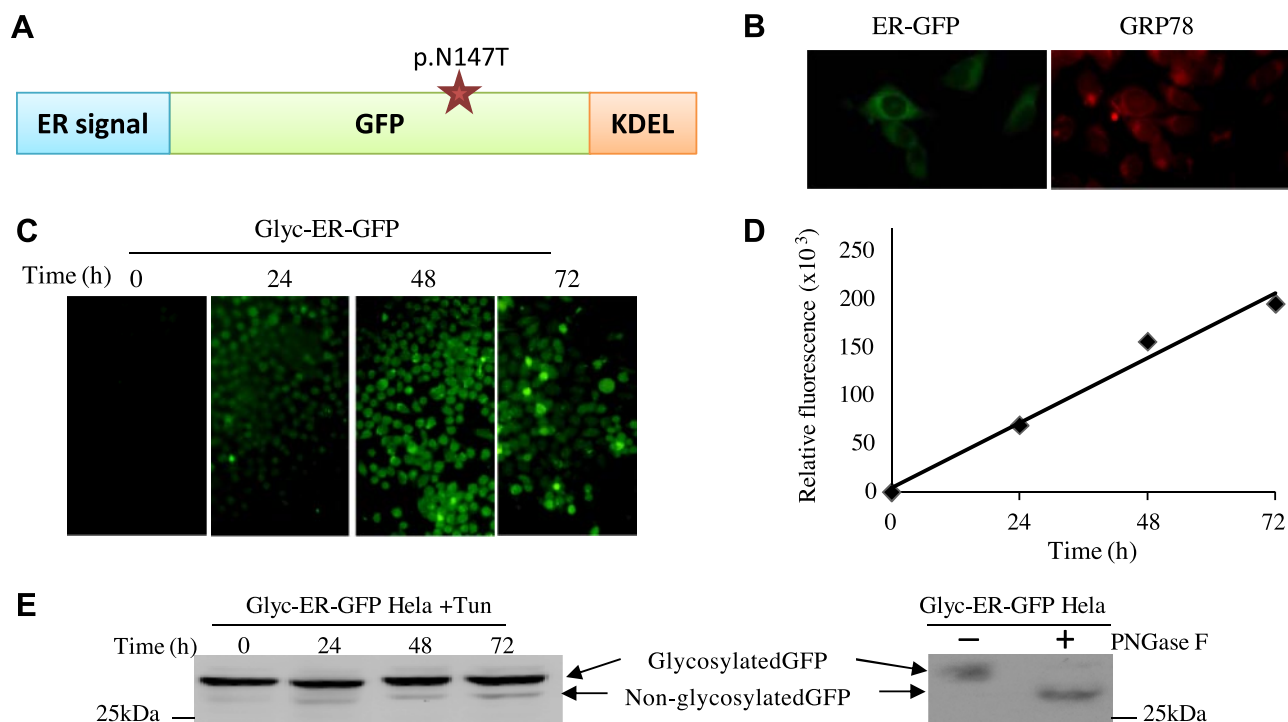


Figure 1. Localization and kinetics of ER-GFP and Glyc-ER-GFP expression. *A*) View of Glyc-ER-GFP construct. Red star corresponds to the mutation p.N147T inserted, ER signal corresponds to the calreticulin ER signal sequence, and KDEL is the ER retention sequence. *B*) Images of the green fluorescence of ER-GFP. Colocalization of ER-GFP is observed in HeLa cells with a marker of the ER (GRP78) in red. *C*) Images of the green fluorescence of Glyc-ER-GFP. Cells were transiently transfected by Glyc-ER-GFP and after 24 h were treated with 1 μ g/ml of tunicamycin for 24, 48, and 72 h. *D*) Quantification (ImageJ software) of the relative green fluorescence intensity observed in cells transiently transfected by Glyc-ER-GFP and treated with 1 μ g/ml of tunicamycin 24 h after transfection and incubated 24, 48, and 72 h. *E*) Western blot analysis of kinetics of expression of glycosylated or nonglycosylated GFP in cells transiently transfected by Glyc-ER-GFP and treated with 1 μ g/ml of tunicamycin (Tun).

large right shift when Glyc-ER-GFP cells are treated with tunicamycin, due to the production of a nonglycosylated GFP.

Glyc-ER-GFP does not fluoresce in control fibroblasts

Preliminary results using HeLa cells were extended to human control fibroblasts. Control fibroblasts A and B were transfected with the ER-GFP and Glyc-ER-GFP. After 24 h, the cells were either analyzed by microscopy or incubated with tunicamycin for 24 h (Fig. 3A). The nontreated Glyc-ER-GFP cells did not glow due to the presence of the *N*-glycan. However, both control fibroblasts transfected with Glyc-ER-GFP produced a fluorescent pattern after tunicamycin treatment. Moreover, the fluorescence observed for the tunicamycin-treated Glyc-ER-GFP cells is comparable to that seen in ER-GFP transfected cells. These results confirmed that Glyc-ER-GFP could be used in different cell lines.

Type I CDG patient cells have an increased fluorescence of Glyc-ER-GFP

We transfected different type I and type II CDG patient cells with the Glyc-ER-GFP and the ER-GFP (Fig. 3B).

CDG-Ia patient cells are deficient in PMM2 activity. Three different CDG-Ia patient fibroblast lines having

different levels of PMM2 activity (#1=37%, #2=8%, #3=15% of normal control activity) were compared (Fig. 3B and Supplemental Fig. S1). When Glyc-ER-GFP is expressed in these cells, a portion of the protein is nonglycosylated, and we can observe a green fluorescence in all three CDG-Ia cell lines, comparable to that of ER-GFP. These results confirm the ability of the Glyc-ER-GFP marker to detect even a mild glycosylation defect, similar to HeLa cells (Fig. 2).

Since GFP fluorescence is lost due to *N*-glycosylation site occupancy, we predicted that the Glyc-ER-GFP will discriminate between type I and type II CDG defects. Figure 3B shows that patient fibroblasts transfected by ER-GFP show green fluorescence independently of the CDG type, as expected. However, the cells transfected by Glyc-ER-GFP show a different pattern. The CDG-Ib and -Ic cells glow at a comparable level to ER-GFP (Fig. 3B and Supplemental S1). This fluorescence is due to an accumulation of nonglycosylated GFP in the ER of the cells deficient in the early steps of glycosylation. CDG type II cells do not glow, consistent with the fact that CDG type II does not affect addition of *N*-glycans. Thus, the Glyc-ER-GFP carries an *N*-glycan whose steric hindrance disrupts the glowing. We tested 33 patient cell lines, including CDG-Ia (6 lines), CDG-Ib (3 lines), CDG-Ic (2 lines), CDG-Ik (2 lines), unknown type I (15 lines), COG7 (1 line), COG4 (1 line), COG8 (1 line),

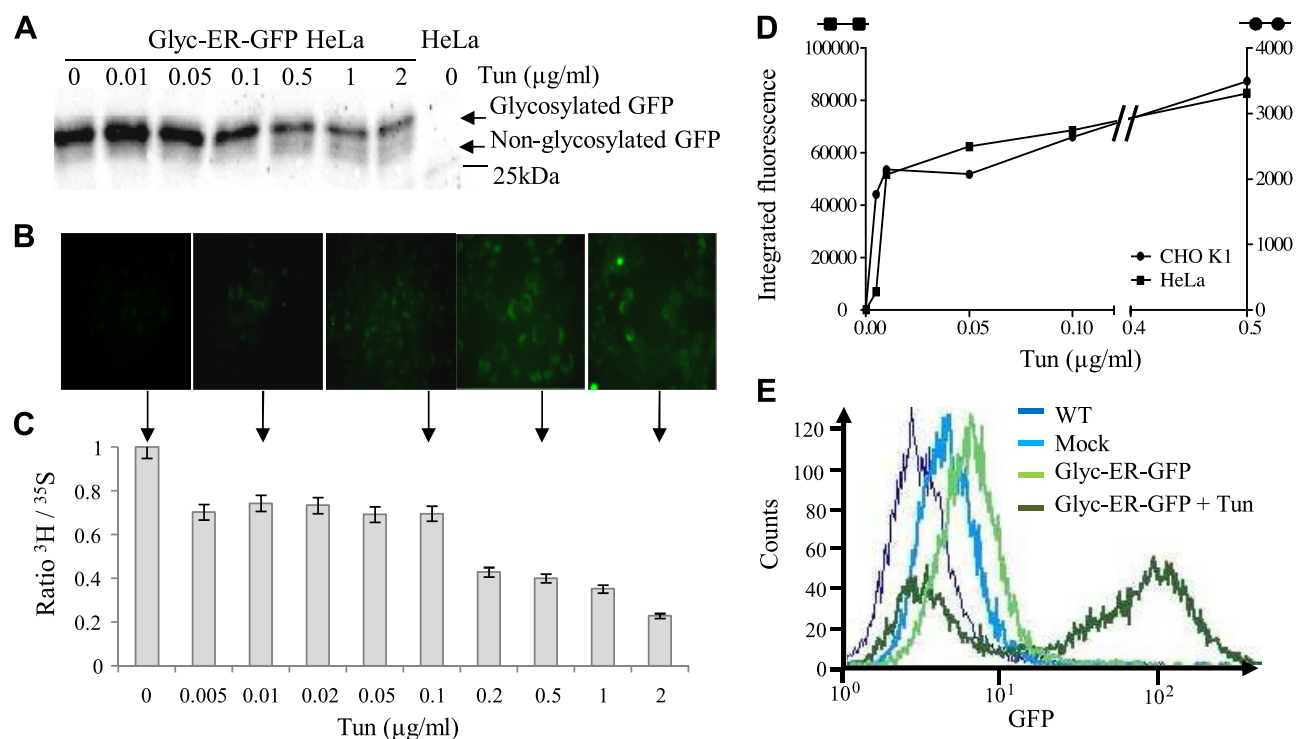


Figure 2. Dose-dependent sensitivity of HeLa cells transfected by Glyc-ER-GFP. *A*) Western blot analysis of dose-dependent sensitivity of HeLa cells transiently transfected by Glyc-ER-GFP and treated with 0 to 2 µg/ml tunicamycin (Tun) for 48 h. *B*) Images of green fluorescence observed in HeLa cells transiently transfected by Glyc-ER-GFP and treated with 0 to 2 µg/ml of tunicamycin for 48 h. *C*) Ratio of the incorporation of [^3H]-mannose and [^{35}S]-labeled amino acids in HeLa cells transiently transfected by Glyc-ER-GFP and treated with 0 to 2 µg/ml of tunicamycin for 48 h. Error bars = SD. *D*) Quantification (ImageJ software) of the relative green fluorescence intensity per cell from images in panel *B*. *E*) Monitoring of green fluorescence of Glyc-ER-GFP in HeLa cells by flow cytometry. FACS analysis showing the green fluorescence in stable HeLa cells expressing the Glyc-ER-GFP with and without 1 µg/ml of tunicamycin compared to wild-type (WT) and mock-transfected (mock) cells.

and unknown type II (2 lines). The 27 type I cell lines showed a green fluorescence, whereas the 5 type II cell lines did not glow. In summary, the Glyc-ER-GFP appears as a specific marker of glycosylation site occupancy and is capable of differentiating type I and type II CDG cells.

Fluorescence of Glyc-ER-GFP in CDG-Ib cells can be abolished by mannose treatment

As described previously, mannose corrects glycosylation deficiency and is a therapy for patients with CDG-Ib and their cells, which are deficient in phosphomannose

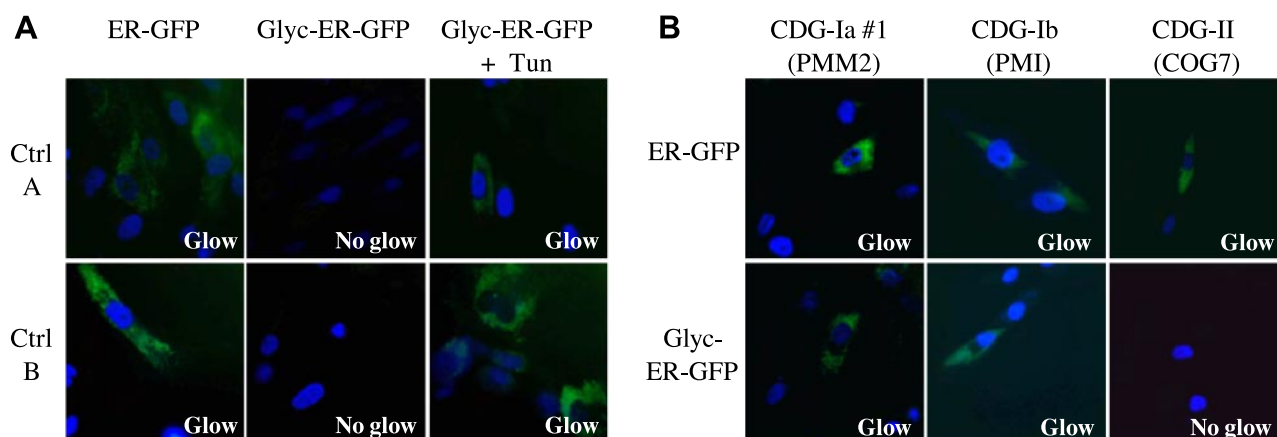


Figure 3. ER-GFP and Glyc-ER-GFP fluorescence in control and patient fibroblasts. *A*) Green fluorescence observed in different control fibroblasts transiently transfected by the ER-GFP and Glyc-ER-GFP constructs. The Glyc-ER-GFP-transfected cells were untreated or treated with 1 µg/ml of tunicamycin (Tun) for 24 h. *B*) Green fluorescence observed in 3 different patient CDG fibroblasts transiently transfected by the ER-GFP and Glyc-ER-GFP constructs after 48 h of expression.

isomerase (18). An important application of a marker is to monitor improved glycosylation in response to drug treatment or gene complementation. Transfected cells were treated for >48 h with different doses of mannose, ranging from 0 to 500 μ M. **Figure 4A** clearly shows dose-dependent decreased fluorescence after addition of mannose, confirming that Glyc-ER-GFP could be used as a drug-screening tool.

Complementation of PMM2 in CDG-Ia cells reduces Glyc-ER-GFP fluorescence

Here, we assessed the ability of the glyco-sensitive ER-retained GFP biomarker to monitor gene complementation in patient cells. To do so, 2 CDG-Ia cell lines were stably transfected with either the mock vector or the wild-type *PMM2* gene (Fig. 4C). Complementation with the *PMM2* gene presumably increases N-glycosylation. We then transfected these complemented cell lines with the ER-GFP and Glyc-ER-GFP and monitored the relative fluorescence (ImageJ software; NIH). To identify transfected cells expressing a nonglowing Glyc-ER-GFP, GFP was immunostained and detected by a secondary antibody linked to a red Cy3 fluorochrome (Fig. 4B). In Fig. 4C, we observed a decrease in the maximum fluorescence of ~79% in the cells overexpressing the wild-type *PMM2* compared to the controls. These results confirm that the Glyc-ER-GFP can be a tool for testing gene complementation.

Glyc-ER-GFP in hESCs and patient-derived CDG-Ia iPSCs

Human fibroblasts were reprogrammed into iPSCs, which resemble hESCs both morphologically and functionally (19, 20). These cells can be expanded and differentiated into various precursors of different germ layers or more

mature cell types. We wanted to determine whether the Glyc-ER-GFP marker could also be used in these cell lines, which hold great promise for future disease modeling. Initially, H9 hESCs (WA09) were transfected with either ER-GFP or Glyc-ER-GFP (with or without tunicamycin) and analyzed after 48 h. As expected, ER-GFP was fluorescent, but Glyc-ER-GFP was not (**Fig. 5A**) unless tunicamycin was added (**Fig. 5B, C**).

Two independent iPSC lines were derived from two CDG-Ia (*PMM2*-CDG) patient fibroblasts by ectopic expression of various transcription factors using a modified protocol of Takahashi *et al.* (19) and Okita *et al.* (25) (**Fig. 5D**). After establishing stable cell lines, we performed standard tests to confirm successful reprogramming and adapted to feeder-free culture conditions for further experiments. Both cell lines expressed typical pluripotency markers, such as Oct-4 and Nanog (Supplemental Fig. S2A). We also generated EBs to demonstrate the potential of these cells to differentiate into ectodermal, endodermal, and mesodermal lineages (Supplemental Fig. S2B). The established *PMM2* mutations and residual *PMM2* enzymatic activity in these patients were confirmed in the iPSCs lines. When the cells were transfected with the Glyc-ER-GFP marker, they showed green fluorescence similar to that seen in fibroblasts (**Fig. 5E**). These proof-of-principle experiments suggest that our marker can be successfully applied to iPSCs, which could be amenable to high-throughput screening of drug candidates.

DISCUSSION

Transferrin is currently the best clinical biomarker to identify putative CDGs in patients. It has also been used

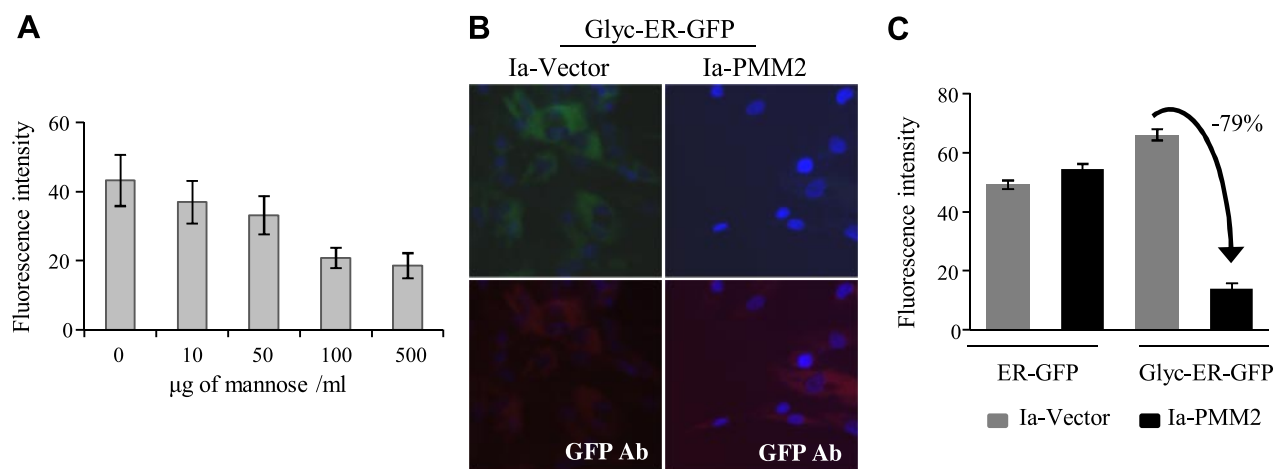


Figure 4. Effect of drug or gene complementation. A) Effect of mannose on fluorescence intensity on CDG-Ib cells transfected by Glyc-ER-GFP. Quantification (ImageJ software) of the relative green fluorescence intensity observed in CDG-Ib cells transfected with Glyc-ER-GFP and treated for 48 h with 0, 10, 50, 100, or 500 μ M of mannose. Error bars = SD. B) Green fluorescence observed in patient CDG-Ia fibroblasts stably complemented by the pBABE-puro vector (Ia-vector) or by pBABE-puro-Flag-PMM2 (Ia-PMM2) transfected by Glyc-ER-GFP. Nuclei were stained by DAPI (blue), and GFP was immunodetected with an anti-GFP antibody and a secondary linked to Cy3 (red). C) Effect of *PMM2* gene complementation of CDG-Ia cells on Glyc-ER-GFP fluorescence. Quantification (ImageJ) of the relative mean green fluorescence, normalized to GFP expression, observed in CDG-Ia cells complemented by Ia-vector or by Ia-PMM2 transfected by the ER-GFP and Glyc-ER-GFP constructs after 48 h of expression. Error bars = SD.

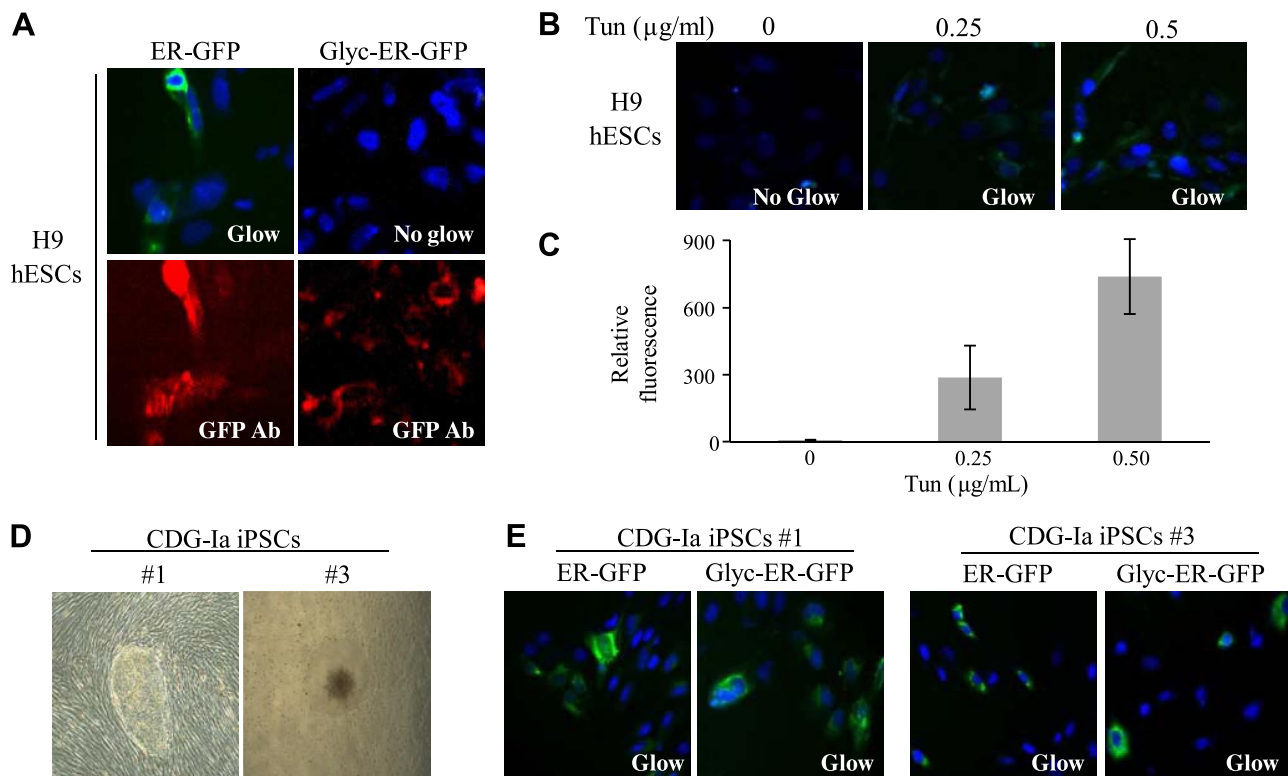


Figure 5. Glyc-ER-GFP in hESCs and patient-derived CDG-Ia iPSCs. *A*) Green fluorescence observed in H9 hESCs transiently transfected by the ER-GFP and Glyc-ER-GFP constructs. Nuclei were stained by DAPI (blue), and GFP was immunodetected with an anti-GFP antibody and a secondary linked to Cy3 (red). *B*) Green fluorescence observed in H9 hESCs transiently transfected by Glyc-ER-GFP construct and treated for 48 h with different doses of tunicamycin (Tun). *C*) Quantification (ImageJ software) of the relative green fluorescence intensity observed in H9 hESCs after tunicamycin treatment for 48 h. Error bars = sd. *D*) Representative phase-contrast images of CDG-Ia iPSC colonies on MEFs. *E*) Green fluorescence observed in CDG-Ia iPSCs transiently transfected by the ER-GFP and Glyc-ER-GFP constructs.

to indicate clinical improvement. However, no comparable markers exist for patient-derived somatic or stem cell fibroblasts. To address this problem, we designed a fluorescent marker that is sensitive to small decreases in *N*-glycosylation site occupancy to serve as an *N*-glycosylation biosensor.

Addition of a strategically located *N*-glycosylation site disrupts the marker's fluorescence when the site is occupied without modifying its fluorescence response when the site is unoccupied. We tested our Glyc-ER-GFP constructs in glycosylation-competent cells (HeLa cells, normal fibroblasts, and hESCs) with the *N*-glycosylation inhibitor tunicamycin, using fluorescence microscopy, SDS-PAGE, and FACS. The construct is an extremely sensitive marker of hypoglycosylation. We then demonstrated that Glyc-ER-GFP detects deficiency or insufficiency of *N*-glycosylation site occupancy in several types of CDG type I cells. CDG type II cells, with defects of *N*-glycosylation maturation but not glycosylation occupancy, do not glow. Finally, our marker can be used to monitor *N*-glycosylation-deficient cells treated with drugs or complemented with the appropriate cDNA.

Our unique Glyc-ER-GFP construct employs a single *N*-glycosylation site, but other mutations might have the same effect, and addition of multiple *N*-glycosylation sites may amplify detection capability of the marker.

Most notably, a marker of glycosylation efficiency like Glyc-ER-GFP is not only useful for gene complementation experiments, but can easily be adapted for high-throughput screening of compounds that can correct glycosylation defects. This technology can be adapted for a high-throughput screening assay of patient fibroblasts, pluripotent iPSCs, or more mature cell types derived from them (21–23).

Our preliminary data show that the Glyc-ER-GFP marker detects hypoglycosylation in patient-derived iPSCs, providing the first evidence for a “disease in a dish” model for CDGs. Stable iPSC lines expressing the glycosylation marker could be used to monitor glycosylation by high-content microscopy (24).

An especially important application of Glyc-ER-GFP will be to confirm the identity of putative glycosylation-related genes identified through whole genome or exome sequencing of CDG patients with abnormal transferrin. Expressing cDNA constructs of the normal allele would complement glycosylation and decrease Glyc-ER-GFP fluorescence in these cells.

Previous studies by Contessa *et al.* (15) using luciferase showed that *N*-glycosylation inhibition can be a therapeutic strategy to target EGFR and RTK pathway in cancer cells. Since Glyc-ER-GFP can be used in a wide variety of cells, from cancer cells to fibroblasts, or in other species, we can envisage using this marker in

cancer cells to determine how drug treatment can impair N-glycosylation site occupancy. **FJ**

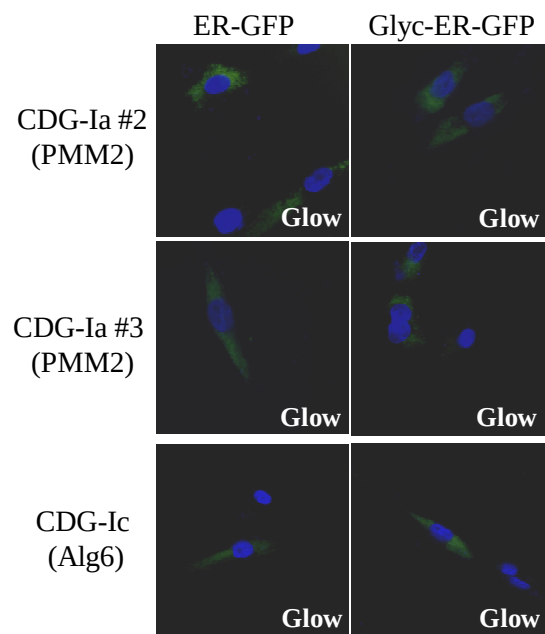
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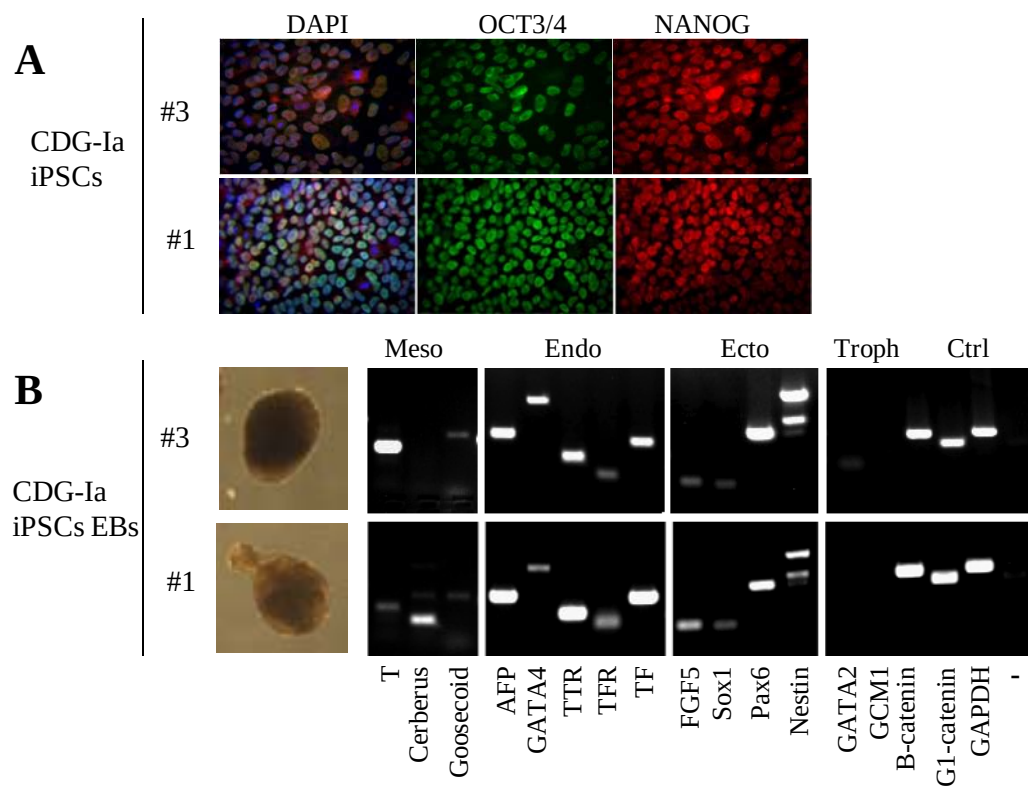
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Supplemental Figure 1



Supplemental Figure 2