

# Targeted disruption of the Walker–Warburg syndrome gene *Pomt1* in mouse results in embryonic lethality

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O-mannosylation is an important protein modification in eukaryotes that is initiated by an evolutionarily conserved family of protein O-mannosyltransferases. The first mammalian protein O-mannosyltransferase gene described was the human *POMT1*. Mutations in the *hPOMT1* gene are responsible for Walker–Warburg syndrome (WWS), a severe recessive congenital muscular dystrophy associated with defects in neuronal migration that produce complex brain and eye abnormalities. During embryogenesis, the murine *Pomt1* gene is prominently expressed in the neural tube, the developing eye, and the mesenchyme. These sites of expression correlate with those in which the main tissue alterations are observed in WWS patients. We have inactivated a *Pomt1* allele by gene targeting in embryonic stem cells and produced chimeras transmitting the defect allele to offspring. Although heterozygous mice were viable and fertile, the total absence of *Pomt1*<sup>-/-</sup> pups in the progeny of heterozygous intercrosses indicated that this genotype is embryonic lethal. An analysis of the mutant phenotype revealed that homozygous *Pomt1*<sup>-/-</sup> mice suffer developmental arrest around embryonic day (E) 7.5 and die between E7.5 and E9.5. The *Pomt1*<sup>-/-</sup> embryos present defects in the formation of Reichert’s membrane, the first basement membrane to form in the embryo. The failure of this membrane to form appears to be the result of abnormal glycosylation and maturation of dystroglycan that may impair recruitment of laminin, a structural component required for the formation of Reichert’s membrane in rodents. The targeted disruption of *mPomt1* represents an example of an engineered deletion of a known glycosyltransferase involved in O-mannosyl glycan synthesis.

Protein O-mannosylation is an important protein modification in uni- and multicellular eukaryotes (1, 2). In mammals, all O-mannosyl glycans identified so far are variations of the tetrasaccharide NeuAc $\alpha$ 2–3Gal $\beta$ 1–4GlcNAc $\beta$ 1–2Man–Ser/Thr, although only a limited number of glycoproteins that carry this modification has been described (reviewed in ref. 2). The best-studied O-mannosylated glycoprotein is  $\alpha$ -dystroglycan ( $\alpha$ -DG), a component of the dystrophin glycoprotein complex that is implicated in the interaction between extracellular matrix proteins and the cytoskeleton of muscle and neurons (reviewed in refs. 3 and 4). The O-linked carbohydrate chains are involved in the binding of  $\alpha$ -dystroglycan ( $\alpha$ -DG) to its ligands, such as neuronal and muscle laminin/merosin. In recent years, it has become evident that a group of human neuromuscular diseases associated with hypoglycosylation of  $\alpha$ -DG are the result of defects in known glycosyltransferases involved in O-mannosyl glycan synthesis (reviewed in refs. 2 and 5). These include congenital muscular dystrophies (CMDs) with neuronal migration defects, such as Walker–Warburg syndrome (WWS) (6), and muscle–eye–brain disease (MEB) (7). Other similar CMDs that feature abnormal  $\alpha$ -DG glycosylation and mutations in putative glycosyltransferases are Fukuyama congenital muscular dystro-

phy (FCMD) (8), congenital muscular dystrophy 1C (MDC1C) (9), and congenital muscular dystrophy 1D (MDC1D) (10).

Protein O-mannosylation is initiated in the endoplasmic reticulum by the transfer of mannose from dolichyl phosphate-activated mannose to serine or threonine residues in secretory proteins (reviewed in ref. 1). This reaction is catalyzed by an evolutionarily conserved family of protein O-mannosyltransferases (PMTs) (11). Although these proteins have been most extensively characterized in yeast (1, 2), PMT homologues have been identified throughout the animal kingdom (with the exception of *Caenorhabditis elegans*). Moreover, in *Drosophila melanogaster*, mutations in the PMT family members *rotated abdomen (rt)* and *twisted (tw)* affect muscle development (12, 13). In human and mouse, two PMT family members are known to exist, namely POMT1 (14) and POMT2 (13). Human POMT1 and POMT2 catalyze protein O-mannosyl transfer to  $\alpha$ -DG, which serves as a protein substrate (15). Mutations in the *POMT1* gene result in WWS, a severe muscular dystrophy that also involves structural alterations in eye and brain malformations, such as cobblestone lissencephaly (6). Most WWS patients die within the first months of life, with a few surviving to 3 years of age. Patients with mutations in *POMT1* show nuclear and extracellular matrix abnormalities in skeletal muscle, as well as defective glycosylation and the loss of laminin-binding activity in  $\alpha$ -DG (16, 17).

Here, we show that during embryonic development, murine *Pomt1* is expressed in the tissues affected in WWS patients. Targeted deletion of *Pomt1* results in early embryonic lethality due to defects in the assembly of Reichert’s membrane. Our results demonstrate the importance of O-mannosyl glycans not only in human disease, but also for critical processes during embryonic development such as the formation of early basement membranes.

## Materials and Methods

**Characterization and Mapping of the Mouse *Pomt1* Gene.** Rapid amplification of cDNA ends PCR was performed on a mouse 17-day embryo marathon-ready cDNA library (Clontech) to generate full-length *Pomt1* cDNA. For chromosomal mapping, an *HphI* polymorphism in exon 20 between *Mus musculus* and *Mus spretus* was used in both panels of backcrosses: EUCIB (BSB) and The Jackson Institute (BSS).

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Abbreviations: CMD, congenital muscular dystrophy; DG, dystroglycan; PMT, protein O-mannosyltransferase; WWS, Walker–Warburg syndrome; En, embryonic day *n*.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY494857)

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**Pomt1 Expression.** Membranes containing RNA from mouse and human tissues (OriGene Technologies, Rockville, MD) and total mouse embryo RNA (Seegene, Del Mar, CA) were hybridized with <sup>32</sup>P-labeled *Pomt1* (base pairs 449–1,193), *Pomt2* (base pairs 659–1,727), or *POMT1* (base pairs 1,398–2,184) cDNA probes. Normalization was performed by using human  $\beta$ -actin and chicken GAPDH.

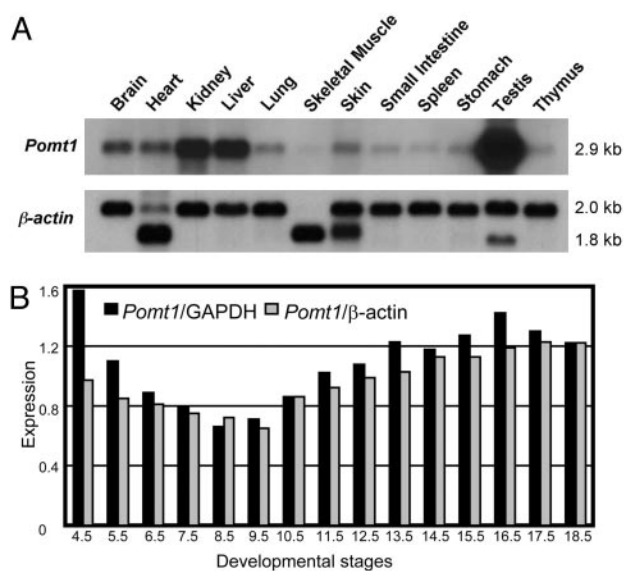
**Whole-Mount *in Situ* Hybridization and Histological Analysis.** Embryos were obtained from timed pregnancies, dissected in PBS (pH 7.3), and fixed overnight at 4°C in 4% paraformaldehyde/PBS. Sense and antisense *Pomt1* (base pairs 2,052–2,611) and *brachyury* (base pairs 1–1,765) probes were generated by using the digoxigenin RNA labeling kit (Roche Diagnostics). Whole-mount *in situ* hybridization was carried out as described (18). Stained embryos were cryoprotected overnight at 4°C in 30% sucrose/PBS, embedded in Cryoblock (Mediate Medizintechnik, Burgdorf, Germany), and sectioned (35  $\mu$ m) at –25°C. Processed sections were mounted under coverslips in Mowiol (Calbiochem).

**Gene Targeting.** A 15-kb region of the mouse *Pomt1* gene (intron 2 to exon 20) was isolated from a 129/SvJ genomic library (Mobi-Tec, Göttingen, Germany), by using mouse *Pomt1* cDNA as a probe. To construct the *Pomt1* targeting vector, the plasmid pPNT was used (19). A 4.5-kb *XhoI* fragment (introns 2–9) was cloned into the *XhoI* site of pPNT as the long arm. A 2.3-kb *KpnI-XbaI* fragment, from the intergenic sequence 5' upstream to intron 1 of *Pomt1*, was amplified by PCR from 129/SvJ genomic DNA by using primers a and b (see *Supporting Text*, which is published as supporting information on the PNAS web site). This fragment was cloned as the short arm into the *XbaI-KpnI* sites of pPNT between the neo and hsv tk cassettes in an opposite orientation.

The targeting construct was linearized and electroporated into embryonic day (E)14.1 embryonic stem (ES) cells. Homologous recombinants were identified by PCR by using primers 1 and 2 (see *Supporting Text*) and confirmed by Southern blot. Two independent targeted ES clones were obtained and injected into BALB/c blastocysts. Both clones yielded chimeras that transmitted the mutant *Pomt1* allele to their offspring. Heterozygous *Pomt1*<sup>+/-</sup> progeny were mated, and the offspring was genotyped by Southern blotting and PCR by using primers 3–5 (see *Supporting Text*). Similarly, embryos from timed pregnancies were genotyped by PCR.

**Antibodies and Immunohistochemistry.** Rabbit anti-laminin (Sigma) and rat monoclonal anti-entactin (ELM1; Abcam, Cambridge, U.K.) antibodies were used at 1:200 dilutions. Monoclonal anti- $\alpha$ -dystroglycan antibodies (VIA4–1, 1:100; and IH6, 1:200), polyclonal anti- $\beta$ -dystroglycan (AP83, 1:50), and anti- $\alpha$ -dystroglycan antibodies (GT20ADG, 1:15) were kindly provided by Kevin Campbell (University of Iowa, Iowa City).

Decidual sacs from wild-type and heterozygous crosses were collected at E6.5 and E7.5, fixed overnight at 4°C in 4% paraformaldehyde/PBS, and cryoprotected or embedded in paraffin. Staining was performed on 7- $\mu$ m sections according to the standard protocol for the M.O.M. Basic kit (Vector Laboratories). Primary antibodies were applied in PBS/mouse blocking solution for 1 h at room temperature, and anti-rabbit-, anti-rat-Alexa488 (Molecular Probes, 1:1,000), anti-goat-Cy3 (Jackson ImmunoResearch, 1:100), and anti-mouse-biotin (Vector Laboratories, 1:200) secondary antibodies were applied in PBS/mouse blocking solution for 30 min at room temperature. If necessary, a 15-min incubation with streptavidin-Cy3 conjugate (Jackson ImmunoResearch, 1:1,000) followed. Sections were mounted with ProLong Antifade kit (Molecular Probes) and viewed with an epifluorescence Zeiss Axioskop microscope.



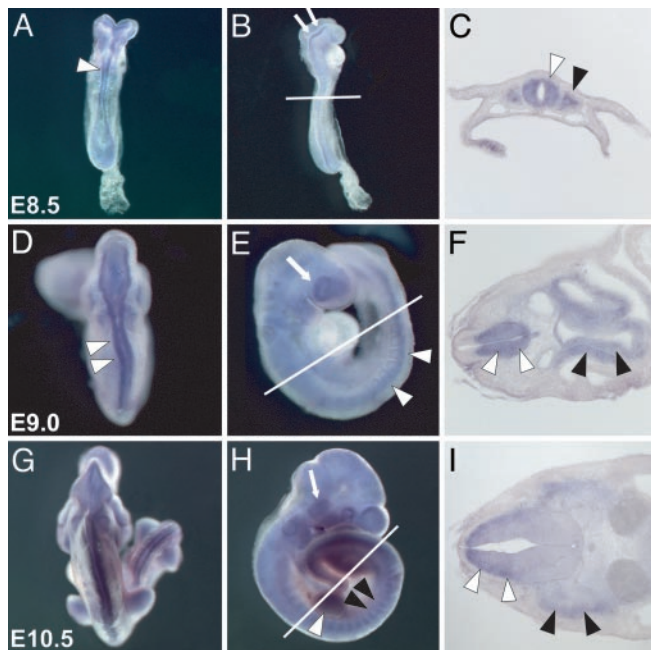
**Fig. 1.** Expression of mouse *Pomt1* gene. (A) Northern blot analysis of adult tissues hybridized with cDNA probes: *Pomt1* (2.9 kb) and  $\beta$ -actin (2.0 and 1.8 kb). (B) *Pomt1* expression during mouse embryogenesis. Original Northern blot is shown in Fig. 6C. Northern blot was normalized by using GAPDH (black columns) and  $\beta$ -actin (gray columns).

**Laser-Capture Microdissection for Genotyping.** Tissues from paraffin-embedded embryos were isolated by using a Zeiss Axiovert microscope equipped with a P.A.L.M. microbeam unit (P.A.L.M. Microlaser Technologies, Bernried, Germany). Microdissected material was lysed for 3 h at 55°C in catapult buffer containing 0.5 M EDTA, pH 8.0; 1 M Tris, pH 8.0; 0.5% Igepal CA-630; and 0.2 mg/ml Proteinase K. The heat-inactivated lysate was used as template for PCR genotyping (see above).

## Results

**Characterization of the Mouse *Pomt1* Gene.** We isolated the full-length *Pomt1* cDNA (2,885 bp; GenBank accession no. AY494857) that corresponded to a predicted protein of 724 aa from a mouse embryo cDNA library. The *Pomt1* gene extends over 18,416 bp of genomic DNA, and its organization into 20 exons is conserved between human and mouse (data not shown). The *Pomt1* gene was localized to centimorgan (cM) 18 of mouse chromosome 2 in the EUCIB backcross (BSB), between the D2Mit120 and D2Mit152 markers, and to cM 20 between the *Abl* and *Pbx3* genes in the BSS backcross panel from The Jackson Laboratory. This 2B chromosomal region had a conserved synteny with human chromosome 9q34.1 where the *POMT1* gene maps. Northern blot analysis identified a single 2.9-kb transcript in all tissues analyzed. However, the expression levels varied from the lowest levels observed in skeletal muscle to high levels of expression in testis (Fig. 1A). The same blot hybridized with *Pomt2* revealed a similar expression pattern (Fig. 6A, which is published as supporting information on the PNAS web site). A similar pattern of human *POMT1* expression was found in adult tissues (Fig. 6B). Northern blot analysis with mRNA from different developmental stages revealed that *Pomt1* is also expressed throughout embryogenesis (Figs. 1B and 6C). High levels of *Pomt1* mRNA were seen at very early stages of development, but one must take care in interpreting these results because this RNA may be the product of contamination with maternal tissues (supplier's note, Seegene, Del Mar, CA). However, the expression of *Pomt1* appeared to decrease slightly during gastrulation and to later increase at the onset of orga-

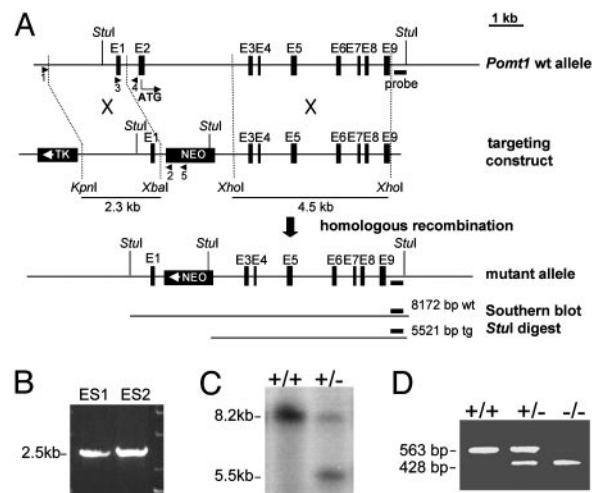




**Fig. 2.** Whole-mount expression of *Pomt1* in mouse embryos. Whole-mount *in situ* hybridizations (A, B, D, E, G, and H) and representative cryosections (C, F, and I) through the anterior neural tube at the level of the forelimb bud (indicated by a white line in B, E, and H). (A–C) At E8.5, strong *Pomt1* expression is found along the neural tube (white arrowhead) and in the dorsal aspects of the neural fold (arrows). Expression was also detected in the somites (black arrowhead). (D–F) At E9.0, strong expression was seen in the ventral part of the neural tube (white arrowheads), in the developing eye (white arrow), and in the gut endoderm (black arrowheads). (G and H) At E10.5, high levels of *Pomt1* mRNA were detected in the somites (black arrowheads), limb buds (white arrowhead), and trigeminal ganglion (white arrow). (I) Pronounced *Pomt1* expression in the mantle layer of the dorsal neural tube (white arrowheads), as well as in the dermomyotome (black arrowheads), was verified in the E10.5 section.

nogenesis at E9.5, suggesting an important role for *Pomt1* throughout embryogenesis.

**Mouse *Pomt1* Is Expressed in WWS-Related Tissues During Early Embryogenesis.** To examine the expression of *Pomt1* during mouse embryogenesis in more detail, we performed whole-mount *in situ* hybridizations on embryos from different developmental stages (E7.5–10.5). At E7.5, *Pomt1* transcripts could not be detected by *in situ* hybridization (data not shown). At later stages, low-level expression of *Pomt1* mRNA was relatively ubiquitous, whereas higher levels of *Pomt1* expression were observed in a specific and dynamic pattern. At E8.5, intense *Pomt1* expression was detected in the neuronal tissues. Transcripts were found throughout the neural tube (Fig. 2A and C) and in the dorsal aspects of the neural folds of the future midbrain region (Fig. 2B). In addition, *Pomt1* was expressed in the somites (Fig. 2C). At E9.0, pronounced expression of *Pomt1* transcripts was seen along the neural tube (Fig. 2D and E) and in the developing eye (Fig. 2E). In cross sections of E9.0 embryos, *Pomt1* transcripts were predominantly detected in the ventral domain of the neural tube, including the region from which motoneurons emerge. Furthermore, *Pomt1* was expressed in the floor plate, notochord, and gut endoderm (Fig. 2F). At E10.5, *Pomt1* transcripts were detected in the somites, the limb-bud mesenchyme, and the developing trigeminal ganglion (Fig. 2G and H). In cross sections anterior to the forelimb bud, *Pomt1* expression was observed in the mantle layer of the dorsal neural tube and the dermomyotome of the somites (Fig. 2I). This



**Fig. 3.** Targeted disruption of the *Pomt1* gene. (A) Schematic representation of the targeting strategy: the genomic locus, the targeting construct, and the expected mutant *Pomt1* allele after homologous recombination. Selectable markers: herpes simplex virus thymidine kinase (TK) and neomycin gene (NEO). The short (2.3-kb) and long (4.5-kb) arms for homologous recombination are represented. PCR primers are represented by arrows. (B) Primers 1 and 2 were used to identify two targeted embryonic stem clones after homologous recombination. (C) Southern blot analysis of genomic DNA from mouse tail tissue. Endogenous (8.2-kb) and targeted (5.5-kb) *Pomt1* alleles. (D) PCR genotyping of embryos from timed matings. Primers 3 and 4 identify the endogenous allele, whereas primers 3 and 5 identify the targeted allele.

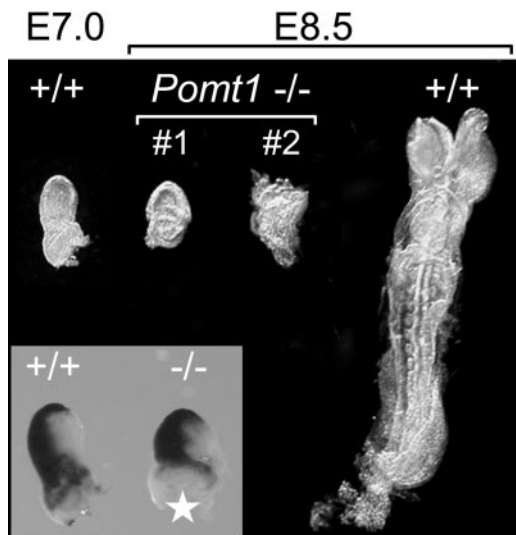
precise expression pattern suggests that *Pomt1* is involved in the development of the muscles, the nervous system, and the eye, which is consistent with the neuronal, muscle, and eye abnormalities found in WWS patients (6).

**Loss of *Pomt1* Results in Early Embryonic Lethality.** The disruption of the *Pomt1* gene was achieved by replacing exon 2, containing the start codon conserved among all vertebrates instead of the putative start codon located in exon 1, with a neomycin resistance gene (Fig. 3A). Successfully targeted clones were identified by PCR and Southern blotting (Fig. 3B and C and data not shown). Two independent embryonic stem cell clones were used to generate chimeric mice that transmitted the mutant *Pomt1* allele to their offspring. *Pomt1*<sup>+/-</sup> heterozygous mice developed normally and were fertile. The progeny of heterozygous intercrosses were 31% wild-type and 69% heterozygous *Pomt1* (Table 1), an ≈1:2 ratio that is indicative of the Mendelian inheritance for a recessive embryonic lethal trait. To determine when homozygous mutant embryos die, we isolated embryos at different stages and genotyped them by PCR (Fig. 3D). Blastocysts at E3.5 were flushed from the uterus and genotyped directly or

**Table 1. Offspring and embryo genotypes from different heterozygous matings**

Developmental stage	Total	Genotypes		
		+/+	+/-	-/-
Adult mice	81	25 (31%)	56 (69%)	0
E10.5	38	15	23	0
E9.5	48	8	32	8*
E8.5	44	9	24	11*
E7.5	26	7	12	7*
E3.5 (blastocysts)	109	23	69	17

\*As development proceeded, *Pomt1* null mutants showed progressive growth retardation and degradation.



**Fig. 4.** Morphology of wild-type and *Pomt1*<sup>-/-</sup> embryos. Representative littermates from an E8.5 *Pomt1* heterozygous intercross are shown. PCR genotyping confirmed *Pomt1*<sup>-/-</sup> null mutants. *Pomt1*-deficient embryos display severe growth retardation presumably due to a developmental block in E6–7. For size comparison, an additional E7.0 wild-type embryo is presented. (Inset) Whole-mount *in situ* hybridization with the gastrulation marker *brachyury*. The asterisk indicates a missing part of the extraembryonic ectoderm used for PCR genotyping.

after *in vitro* culture. Homozygous mutant blastocysts with normal trophoblast outgrowth were indistinguishable from wild-type and heterozygous littermates (data not shown) and were identified in the expected Mendelian ratio of 1:2:1 (Table 1). Genotyping of embryos from E7.5 to E9.5 also revealed a 1:2:1 Mendelian ratio (Table 1), although *Pomt1*<sup>-/-</sup> embryos were significantly smaller and became increasingly disorganized with age (Fig. 4). *Pomt1*<sup>-/-</sup> embryos isolated at E8.5 displayed a variable degree of morphological abnormalities. Some mutant embryos appeared to remain at the egg-cylinder stage reminiscent of E6.5 embryos; others developed beyond that stage but appeared to suffer a later developmental block (Fig. 4, #1 and #2). Gastrulation did not seem to be impaired, because whole-mount *in situ* hybridization with *brachyury* revealed no obvious abnormalities (Fig. 4 Inset). Finally, *Pomt1*<sup>-/-</sup> embryos were resorbed during pregnancy, such that at E10.5, no tissue from homozygous mutant embryos could be recovered (Table 1). Taken together, these results suggest that *Pomt1* fulfils a crucial function during early embryogenesis.

**Loss of *Pomt1* Results in the Disruption of Reichert's Membrane.** One known protein substrate of POMT1 is  $\alpha$ -DG. The posttranslational glycosylation of the  $\alpha$ -DG protein, including the addition of *O*-mannosyl glycan chains, is critical in determining its ligand-binding properties (4, 17, 20). In mice,  $\alpha$ -DG is a component of Reichert's membrane, one of the first basement membranes to form in the embryo (21, 22). We determined whether glycosylation of  $\alpha$ -DG might be affected in *Pomt1*<sup>-/-</sup> embryos in genotyped sections from E7.5 homozygous embryos. In wild-type and heterozygous embryos,  $\alpha$ -DG glyco-specific antibodies VIA4-1 and IIIH6 mainly recognized Reichert's membrane and the maternal decidual cells surrounding the embryo (Fig. 5 *Ab* and *Bb*, and data not shown). In contrast, the  $\alpha$ -DG glycoepitope was detected neither in Reichert's membrane nor in the embryonic tissue in sections of *Pomt1*<sup>-/-</sup> embryos (Fig. 5 *Af* and *Bf*), although the expression of this  $\alpha$ -DG epitope could be observed in the surrounding maternal decidual cells.

These data suggested that, *in vivo*, POMT1 contributes to the major mannosyltransferase activity required for the incorporation of *O*-mannosyl glycans into  $\alpha$ -DG. Immunofluorescence with antibodies directed against the protein core of the  $\alpha$ - and  $\beta$ -subunit of dystroglycan, respectively, revealed a reduction of both DG proteins in Reichert's membrane in *Pomt1* null mutants (Fig. 5 *Ac*, *Ag*, *Bc*, and *Bg*), indicating incorrect maturation or targeting due to hypoglycosylation.

The absence of *O*-mannosyl glycans and the reduced level of DG proteins might affect the distribution of laminin and thus the formation of basement membranes such as Reichert's membrane. Laminin, as well as the laminin-binding protein nidogen/entactin (23), was readily detected in the Reichert's membrane of wild-type embryos (Fig. 5 *Ad* and *Bd*). In *Pomt1*<sup>-/-</sup> embryos, the levels of laminin and nidogen were significantly reduced, and the distribution was disrupted, becoming discontinuous and in parts patchy (Fig. 5 *Ah* and *Bh*).

In summary, the targeted deletion of *Pomt1* in mice impairs basement membrane formation, manifested by the disruption of Reichert's membrane. The failure of this membrane to protect the embryo is reflected by the fact that maternal red blood cells can be found in the yolk sacs of *Pomt1* null mutant embryos (data not shown).

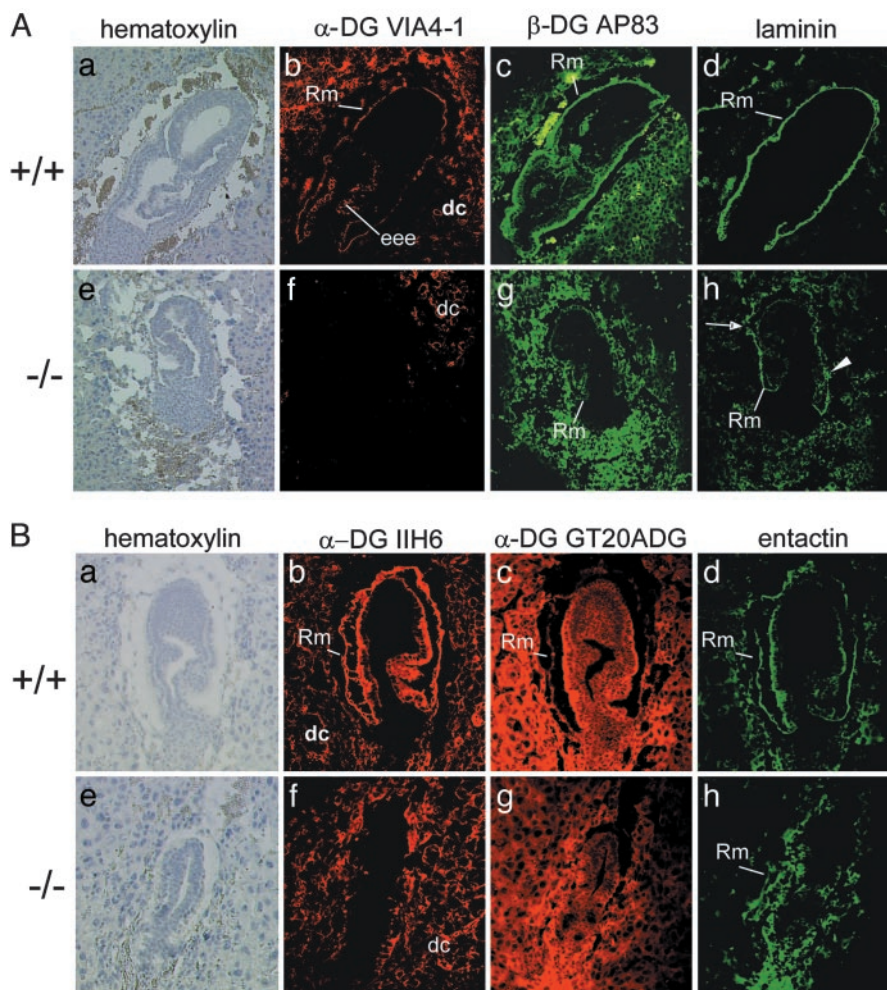
## Discussion

We have generated a knockout mouse model of a known glycosyltransferase, *Pomt1*. Several glycosyltransferases are involved in CMDs in humans. Mutations in *POMT1* are responsible for WWS, a severe recessive CMD that is combined with ocular and retinal abnormalities and brain defects that include type II lissencephaly (6). Children born with WWS generally do not survive past the age of 3 years. The expression of *Pomt1* in the neural tube, dermomyotome, and developing eye during mouse embryonic development emphasizes the importance of *Pomt1* in all of the tissues most affected in WWS patients. However, the early embryonic lethality observed in *Pomt1*<sup>-/-</sup> null mutants contrasts with the effects of the mutations found in WWS patients. The reason that the murine *Pomt1*<sup>-/-</sup> null mutation results in a notably more severe phenotype than the mutations seen in human WWS patients may well lie in the defects observed in Reichert's membrane. Although Reichert's membrane is a feature particular to rodent embryos (21, 22),  $\alpha$ -DG glycosylation might be a general and crucial prerequisite for the correct development of basement membranes also in humans. This is supported by the glia limitans defects observed in WWS patients (6).

It is widely accepted that the carbohydrate moiety in general and *O*-mannosyl glycans in particular play a crucial role in determining the ligand-binding properties of  $\alpha$ -DG. It has been reported that laminin binding to  $\alpha$ -DG is reduced or almost completely lost in muscle tissue of WWS, muscle-eye-brain (MEB) disease, Fukuyama CMD (FCMD), and congenital muscular dystrophy 1C (MDC1C) patients (9, 17, 20). Furthermore, *in vitro* correct glycosylation of DG is crucial for protein processing and targeting to the cell surface (24). *In vivo* targeting defects of  $\alpha$ - and  $\beta$ -DG due to hypoglycosylation are observed in nonmuscle tissue, such as brain of the spontaneous mouse mutant (*myd*), which represents a null mutation of the putative glycosyltransferase LARGE and shows a relevant CMD phenotype (20, 25).

Interestingly, we found a reduction of both dystroglycan subunits ( $\alpha$ - and  $\beta$ -chains) in the Reichert's membrane of *Pomt1*<sup>-/-</sup> embryos, which obviously resulted in aberrant recruitment of laminin to Reichert's membrane. In the dystroglycan null mutant embryo, the lack of laminin leads to a loss of this membranes' integrity and causes embryonic lethality early in gastrulation (22). Thus, the lethal phenotype observed in the *Pomt1* mutant embryos mimics the dystroglycan knockout





**Fig. 5.** Immunohistochemical characterization of extracellular matrix components in *Pomt1*<sup>-/-</sup> mutant embryos. Sagittal sections of paraffin-embedded E7.5 (A) or frozen E6.5 (B) embryos were stained with hematoxylin and analyzed with anti- $\alpha$ -DG antibodies directed against a glycoepitope (VIA4-1, IIH6) or the protein core (GT20ADG) and anti- $\beta$ -DG (AP83), anti-laminin, and anti-nidogen/entactin antibodies, as indicated. Wild-type embryos (A and B a-d) and independent *Pomt1* null mutants (A and B e-h) are shown. In *Pomt1*<sup>-/-</sup> embryos, the glycoepitope is missing in all embryo-derived cellular structures, although it is still present in the decidual cells. Discontinuous (arrows) and patchy (arrowhead) laminin and nidogen/entactin staining of the mutant embryos indicates a defect in the formation of Reichert's membrane. Genotypes were determined by PCR genotyping of laser-captured material. Rm, Reichert's membrane; eee, extraembryonic ectoderm; dc, maternal decidual cells.

model. Furthermore, by considering these data together, we assume that the early embryonic lethality observed in *Pomt1* null mutant mice is due to the failure of Reichert's membrane to function correctly as a filter, allowing free access of nutrients to the embryo while excluding maternal cells (21).

Because Reichert's membrane is specific to rodents, it may be difficult to establish appropriate mouse models for glycosylation disorders associated with CMDs. One possible approach to circumvent this problem would be the use of tissue- and stage-specific gene disruption, as recently attempted for  $\alpha$ -DG (26, 27). The conditional ablation of dystroglycan from the embryonic but not the extraembryonic tissues that synthesize the components of Reichert's membrane overcomes embryonic lethality. However, although live offspring are born, the pups are severely affected and poorly viable.\*\* This provides further evidence that the formation of a functional Reichert's membrane at the egg cylinder stage is the bottleneck, which mutations in dystroglycan and/or in its modifying glycosyltransferases are

unable to overcome. Indeed, another mouse mutant also suffers early embryonic lethality at around E6.5–7.5, in that the putative glycosyltransferase *fukutin* gene is disrupted (28). In humans, *Fukutin* mutations cause FCMD (8) and a more severe spectrum of WWS-like symptoms (29, 30).

It seems that POMT1 enzymatic activity is completely lost in *Pomt1*<sup>-/-</sup>-deficient embryos. In yeast, members of the PMT family form specific protein complexes (31), and it has been suggested that in mammals, both POMT1 and POMT2 have to be present to confer maximal activity (15). Our Northern blot data support this suggestion, because both *Pomt1* and *Pomt2* have similar expression patterns. This may explain why residual POMT2 activity cannot compensate for POMT1 deficiency. The binding of  $\alpha$ -DG to its ligands (e.g., laminin  $\alpha$ 2) is mediated via its sialyl-terminated carbohydrate chains (3, 32). Approximately 70% of these chains are of the *O*-mannosyl type, whereas the rest are mucin-type sugars initiated at an *N*-acetylgalactosamine sugar residue (33). Our data from mouse embryos, in conjunction with a previous study on human WWS muscle (17), emphasize that in nonmuscle tissue, *O*-mannosyl glycans are crucial for DG maturation and/or targeting to the cell surface, whereas in muscle, they mainly affect binding of  $\alpha$ -DG to laminin. Neither the residual  $\alpha$ -DG sugar moiety nor other

\*\*Barresi, R., Satz, J. S., Sawatzki, S. M., Durbeek, M., Cohn, R. D., Henry, M. D., Moore, S. A., Tallquist, M. D., Soriano, P. & Campbell, K. P. (2003) *Mol. Biol. Cell* 14, 390a (abstr.).

laminin receptors like  $\beta 1$  integrin can compensate for the loss of *O*-mannosyl glycan-mediated binding (4). The recruitment of laminin to  $\alpha$ -DG is critical for initial self assembly and cluster formation during basement membrane formation (34, 35). Laminin 1 is the major laminin found at early stages of basement membranes. The absence of the laminin 1 chain results in early embryonic death by E7 (36). Subsequently, such laminin clusters mediate the deposition of other structural extracellular matrix molecules, for example, nidogen/entactin or collagen IV (23).

Because  $\alpha$ -DG is the best-characterized *O*-mannosylated protein to date, our data on *Pomt1* expression, together with the knockout phenotype, seem to be directly related to  $\alpha$ -DG function and the observed loss of its *O*-mannosyl sugar moieties. Besides the known function of *O*-mannosyl glycans in laminin binding, our study provides *in vivo* evidence that *O*-mannosylation is important for proper maturation and/or targeting of the protein to the cell

surface. Taken together, our study demonstrates the essential role of *O*-mannosyl glycans not only in disease but also for early rodent development and the formation of early basement membranes.

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# Corrections and Retraction

## CORRECTIONS

**CHEMISTRY, BIOPHYSICS.** For the article “Relationship of Leffler (Brønsted)  $\alpha$  values and protein folding  $\Phi$  values to position of transition-state structures on reaction coordinates,” by Alan R. Fersht, which appeared in issue 40, October 5, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 14338–14342; first published September 21, 2004; 10.1073/pnas.0406091101), the author notes that Eqs. 5, 6, 8, and 9 contain typographical errors. Because the correct equations were used by the author, this correction does not affect the conclusions of the article. The correct equations appear below.

$$\Delta G^\ddagger = \lambda_2(1 - r_\ddagger)^2 + \Delta G^0 \quad [5]$$

$$r_\ddagger = \frac{-\lambda_2 + ((\lambda_1 - \lambda_2)\Delta G^0 + \lambda_1\lambda_2)^{1/2}}{\lambda_1 - \lambda_2} \quad [6]$$

$$\frac{\partial r_\ddagger}{\partial \Delta G^0} = 0.5((\lambda_2 - \lambda_1)\Delta G^0 + \lambda_1\lambda_2)^{-1/2} \quad [8]$$

$$\alpha = \lambda_1 r_\ddagger ((\lambda_1 - \lambda_2)\Delta G^0 + \lambda_1\lambda_2)^{-1/2} \quad [9]$$

[www.pnas.org/cgi/doi/10.1073/pnas.0407342101](http://www.pnas.org/cgi/doi/10.1073/pnas.0407342101)

**DEVELOPMENTAL BIOLOGY.** For the article “Targeted disruption of the Walker–Warburg syndrome gene *Pomt1* in mouse results in embryonic lethality,” by Tobias Willer, Belén Prados, Juan Manuel Falcón-Pérez, Ingrid Renner-Müller, Gerhard K. H. Przemeck, Mark Lommel, Antonio Coloma, M. Carmen Valero, Martin Hrabé de Angelis, Widmar Tanner, Eckhard Wolf, Sabine Strahl, and Jesús Cruces, which appeared in issue 39, September 28, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 14126–14131; first published September 21, 2004; 10.1073/pnas.0405899101), due to a printer’s error, the first sentence of the fourth paragraph on page 14127 incorrectly reads, “The targeting construct was linearized and electroporated into embryonic day (E)14.1 embryonic stem (ES) cells.” This sentence should be changed to read, “The targeting construct was linearized and electroporated into E14.1 embryonic stem (ES) cells.”

[www.pnas.org/cgi/doi/10.1073/pnas.0407104101](http://www.pnas.org/cgi/doi/10.1073/pnas.0407104101)

**NEUROSCIENCE.** For the article “Contribution of inhibitory mechanisms to direction selectivity and response normalization in macaque middle temporal area,” by A. Thiele, C. Distler, H. Korbmayer, and K.-P. Hoffmann, which appeared in issue 26, June 29, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 9810–9815; first published June 21, 2004; 10.1073/pnas.0307754101), the authors note that the word “baclofen” should read “saclofen” in line 18 in the right column on page 9815.

[www.pnas.org/cgi/doi/10.1073/pnas.0407382101](http://www.pnas.org/cgi/doi/10.1073/pnas.0407382101)

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## RETRACTION

**PLANT BIOLOGY.** For the article “Suppression of pathogen-inducible NO synthase (iNOS) activity in tomato increases susceptibility to *Pseudomonas syringae*,” by Meena R. Chandok, Sophia K. Ekengren, Gregory B. Martin, and Daniel F. Klessig, which appeared in issue 21, May 25, 2004, of *Proc. Natl. Acad. Sci. USA* (**101**, 8239–8244; first published May 14, 2004; 10.1073/pnas.0402344101), the undersigned authors note the following retraction. The paper is an extension of a recently retracted paper [Chandok, M. R., Ytterberg, A. J., van Wijk, K. J., and Klessig, D. F. (2003) *Cell* **113**, 469–482], which reported that the pathogen-inducible NO synthase (iNOS) in plants is a variant form of the P subunit of the glycine decarboxylase complex. Since publication of the *Cell* paper, members of the Klessig laboratory have been unable to repeat the results with recombinant variant P. Therefore, the rationale no longer exists for silencing varP in tomato and examining the effects on host defense as the authors reported in the PNAS paper. For this reason and because the undersigned authors are no longer confident in much of the data in Figs. 1, 3, and 4, they hereby retract their PNAS article. The data showing that virus-induced silencing of varP increases susceptibility to *Pseudomonas syringae* pv. *tomato* were generated by S. K. Ekengren and G. B. Martin and are not in question (Figs. 2 and 3A). However, the observed increase in disease susceptibility of varP-silenced plants was likely due to pleiotropic effects on plant defenses as a result of the decrease in glycine decarboxylase. M. R. Chandok has not approved this assessment or the retractions. The undersigned authors deeply regret this incident and sincerely apologize.

Daniel F. Klessig  
Gregory B. Martin  
Sophia K. Ekengren

[www.pnas.org/cgi/doi/10.1073/pnas.0406996101](http://www.pnas.org/cgi/doi/10.1073/pnas.0406996101)