The American Journal of Pathology, Vol. 170, No. 5, May 2007

DOI: 10.2353/ajpath.2007.061264

Molecular Pathogenesis of Genetic and Inherited Diseases

Expression of the Murine *Pomt1* Gene in Both the Developing Brain and Adult Muscle Tissues and Its Relationship with Clinical Aspects of Walker-Warburg Syndrome

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Walker-Warburg syndrome (WWS) is the most severe of a group of congenital disorders that have in common defects in the *O*-glycosylation of α-dystroglycan. WWS is characterized by congenital muscular dystrophy coupled with severe ocular and brain malformations. Moreover, in at least one-fifth of the reported cases, mutations in the *POMT1* gene are responsible for this disease. During embryonic development (E8.5 to E11.5), the mouse *Pomt1* gene is expressed in the tissues most severely affected in WWS, the muscle, eye, and brain. In this study, we show that mPomt1 expression is maintained in the muscle and eye in later developmental stages and, notably, that its expression is particularly strong in regions of brain and cerebellum that, when affected, could generate the defects observed in patients with WWS. We show that the Pomt1 protein is localized to the sarcoplasmic reticulum of muscle tissue cells in adult mice, where α-dystroglycan is *O*-glycosylated. Furthermore, the Pomt1 protein is localized to the acrosome of maturing spermatids, where α-dystroglycan is not glycosylated, so that Pomt1 might have a different target for *O*-mannosylation in the testes. This expression pattern in the testes could also be related to the gonadal anomalies observed in some patients with WWS. *(Am J Pathol 2007, 170:1659–1668; DOI: 10.2353/ajpath.2007.061264)*

Protein *O*-mannosylation is a very important post-translational modification in eukaryotes. The process takes place in the endoplasmic reticulum (ER) and is catalyzed by a family of protein mannosyltransferases that are highly conserved from yeast to humans. These mannosyltransferases transfer dolichyl-phosphate-mannose to the Ser/Thr (–OH) residues of target proteins.[1] In humans, as in other higher eukaryotes, two homologous mannosyltransferase members exist, known as protein *O*-mannosyltransferase 1 and 2 (hPOMT1 and hPOMT2).[2,3]

In mammals, *O*-mannosyl glycans are mainly found in α-dystroglycan (α-DG) from peripheral nerve, brain, and skeletal muscle. This α-DG is a central component of the dystrophin glycoprotein complex, acting as a link between extracellular matrix and the cytoskeleton of muscle cells and neurons. Indeed, α-DG represents the most extensively studied glycoprotein with a large number of *O*-mannosyl glycan residues, which mainly are of the type Sia-α2–3Gal-β1–4GlcNAc1–2Man-Ser/Thr.[1] Human POMT1 and POMT2 interact to form a complex that catalyzes *O*-mannosyl transfer to their main substrate α-DG.[4,5] In recent years, the importance of *O*-mannosylation has increased because of its novel association with human diseases.

A series of congenital muscular dystrophies (CMDs) have been described that involve brain and eye alterations of diverse severity, such as Walker-Warburg syndrome (WWS: MIM 236670), muscle-eye-brain disease (MEB: MIM 253280), and Fukuyama CMD (FCMD: MIM 253800). Two other CMDs, type 1C CMD (MDC1C: MIM

Supported by the Spanish Comisión Interministerial de Ciencia y Tecnología (SAF03-01619) and Fondo de Investigaciones Sanitarias (PI02/0715). B.P., A.P., and R.P.C. are Fondo de Investigación Sanitaria, Formación de Profesorado Universitario, and Formación de Personal Investigador fellows, respectively.

Supplemental material for this article can be found on *http://ajp.amjpathol.org*.

Accepted for publication February 6, 2007.

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606612) and type 1D CMD (MDC1D: MIM 608840), produce minor alterations. The genes principally associated with these diseases are *POMT1*, *POMGnT1*, *fukutin*, *FKRP*, and *LARGE*, respectively, and the products of these genes are involved in the *O*-glycosylation of α-DG molecules.[6–9] Indeed, the common feature of all these disorders is the hypoglycosylation of α-DG in muscle biopsies.[10]

WWS is an autosomic recessively inherited disease and is the most severe of these conditions. Affected patients show little motor activity due to the severe CMD. The brain anomalies originated by the defects in neuronal migration include type II lissencephaly typically involving the disorganization of the cortex in the cerebral hemispheres and cerebellum, agenesis of the corpus callosum, hydrocephalus, absence of septum and cerebellar vermis, agyria, fusion of cerebral hemispheres, dilation of the fourth ventricle, and occasionally, occipital encephalocele. Among the eye abnormalities, cataracts, buphthalmia, microphtalmia, and persistent hyperplastic primary vitreous have been described. Patients with WWS usually die within the first months of age, reaching 3 years in some isolated cases.[11–13]

WWS is produced by mutations in the *hPOMT1* gene in at least 20% of cases.[14,15] Other causative genes have been identified in a small percentage such as *POMT2*, *FKRP*, or *fukutin*, implying that WWS is a genetically heterogeneous disorder.[16–18] Recently, two other muscular dystrophies with mental retardation but without eye abnormalities have been reported that are caused by *hPOMT1* mutations: CMD/MR, a milder form of WWS,[15] and the autosomal recessive limb girdle muscular dystrophy (LGMD2K: MIM 609308).[19] Furthermore, mutations in *hPOMT2* gene have also been found in a milder form of CMD previously described as "MEB-like" disease, which displays similar clinical aspects to the CMD/MR caused by mutations in *hPOMT1*.[20]

Considering that several neuromuscular disorders are caused by mutations in the *hPOMT1* gene, it would be helpful to establish a relationship between POMT1 expression and the clinical phenotypes observed in these disorders. Because the human and mouse POMT1 proteins are highly homologous and their RNA expression patterns are very similar in adult tissues,[21] information obtained in the mouse could be relevant to humans.

We previously reported that during mouse embryonic development (E8.5 to E11.5), the *mPomt1* gene is expressed in those tissues most severely affected in patients with WWS. We also showed that *O*-mannosyl glycans are not only important in human disease but also in critical processes during embryonic development, such as the formation of early basement membranes. Indeed, targeted disruption of the murine *Pomt1* gene results in embryonic lethality due to the defective assembly of Reitcher's membrane.[21]

Here, we show that in advanced developmental stages, the expression of murine *Pomt1* is maintained in the tissues affected in patients with WWS. Indeed, *Pomt1* is mainly expressed in those regions critical for correct brain and cerebellum development, which when affected may give rise to the multiple brain abnormalities observed in patients with WWS. We also show that in the adult mouse, the Pomt1 protein is located, together with Pomt2, in the sarcoplasmic reticulum of muscle tissue cells, where the *O*-mannosylation of α-DG takes place. This distribution is consistent with the muscular dystrophy displayed by patients with disorders caused by mutations in *hPOMT1* or *hPOMT2* genes: WWS, CMD/MR, and LGMD2K. We have also detected the mPomt1 protein in the testes, in the acrosome of maturing spermatids, in a similar expression pattern to that described for mPomt2.[3] These expression data are in accordance with the fact that POMT1 and POMT2 act together in driving *O*-mannosylation.[4,5] Furthermore, the expression in mouse testes suggests that POMT1 could be related to the gonadal defects presented by some patients with WWS[22] and that POMT1/POMT2 may have a different *O*-mannosylation substrate because α-DG is not glycosylated in these cells.

Materials and Methods

Animals

The mice used in this study were bred and maintained on BALB/c and 129P2/Ola genetic backgrounds. All animal handling procedures were in accordance with the European Communities Council Directive (86/609/EEC) and they were approved by the Ethics Committee of the Universidad Autónoma de Madrid. We used embryos (E12.5 and E13.5), tissue extracts, or tissue sections from young adult animals (3 to 9 months old). We also used male heterozygous *Pomt1*$^{+/-}$[21] and wild-type (wt) females for the fertility test.

Embryos and Tissue Preparation for in Situ *Hybridization and Immunofluorescence*

Embryos were dissected out from the uteri of pregnant females at E12.5 and E13.5, and other tissues were obtained from young adult animals. Whole embryos or tissues were extracted in phosphate-buffered saline (PBS) and fixed overnight at 4°C in 4% paraformaldehyde in PBS. The tissues were cryoprotected overnight at 4°C in 15% sucrose/PBS and then embedded at 37°C in 15% sucrose/7% gelatin in PBS. Gelatin-embedded tissues were frozen in isopentane at −80°C and then sectioned (10 μm) at −25°C in a cryostat (Cryocut 1900; Leica Microsystems, Deerfield, IL). The resulting slices were used for *in situ* hybridization or immunofluorescence.

Whole-Mount or Tissue Section in Situ *Hybridization*

Sense and antisense Pomt1 RNA probes[21] were generated using the digoxigenin RNA labeling kit (Roche Diagnostics Corp., Indianapolis, IN). *In situ* hybridization with digoxigenin-labeled RNA probes was performed in a similar way for whole-mount embryos or cryosections.[23] Stained embryos were photographed, embedded in su-

crose/gelatin, and cryosectioned (25 μm) at −25°C. Processed embryo sections or stained tissue sections were mounted under coverslips in Mowiol (Molecular Probes).

Antibodies

The rabbit polyclonal anti-POMT1 antiserum (SP26) was produced by Eurogentec (Seraing, Belgium) using a synthetic peptide coupled to keyhole limpet hemocyanin corresponding to amino acids 510 to 524 (VEEHRYGASQEQRER) of the human POMT1 protein. The resulting antiserum was affinity-purified using a chromatography column of the antigenic peptide covalently bound to Sepharose. The affinity-purified antibody was used to probe Western blots (1:1000) or for immunofluorescence (1:25). The polyclonal anti-POMT2 antiserum[3] (immunofluorescence, 1:25) was kindly provided by Dr. Sabine Strahl (University of Heidelberg, Heidelberg, Germany). The other antibodies used here were mouse monoclonal anti α-dystroglycan antibodies VIA4–1 (Western blot, 1:1000; immunofluorescence, 1:100; Upstate Biotechnologies, Lake Placid, NY), mouse monoclonal β-dystroglycan antibodies (immunofluorescence, 1:50; Vector Laboratories, Inc., Burlingame, CA), and polyclonal anti-calnexin antiserum (immunofluorescence, 1:200; Stressgen Biotechnologies Corp., San Diego, CA).

Isolation of Protein Extracts from Mouse Tissues and Western Blot

All solutions used to obtain protein extracts contained a protease inhibitor mixture: 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), and an antiprotease cocktail (Roche Diagnostics). To obtain total extracts, mouse tissues were dissected in PBS and homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1% Triton X-100, and 0.1% sodium dodecyl sulfate. After 15 minutes at 4°C, lysates were centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the supernatant was used as total protein extract. To obtain the microsomal fraction, tissues were homogenized in membrane buffer (50 mmol/L Tris-HCl, pH 7.5, and 0.3 mmol/L $MgCl_2$) and centrifuged at 900 × *g* for 10 minutes at 4°C. The supernatant was subsequently centrifuged at 10,000 × *g* for 15 minutes at 4°C, and the resulting supernatant was subjected to ultracentrifugation at 45,000 × *g* for 1 hour at 4°C. The pellet was washed in 50 mmol/L Tris-HCl, pH 7.5, 50 mmol/L $MgCl_2$, and 250 mmol/L NaCl and resuspended in the same buffer. Protein quantification was performed by the Bradford assay.

The proteins from the total extracts (30 μg) or the microsomal fraction (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6 or 8%, respectively) and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). Membranes with total extracts were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% bovine serum albumin and probed with the primary anti-α-DG antibodies (VIA-4) in TBS-T/3% bovine serum albumin overnight at 4°C. Blots of microsomal extracts were blocked in TBS-T/5% skim milk and probed for 1 hour at room temperature with the affinity-purified anti-POMT1 antiserum in the same buffer. Incubation with anti-mouse-anti-rabbit IgGs conjugated with peroxidase (1:10,000; Nordic Immunological Laboratories, Tilburg, The Netherlands) secondary antibodies for 1 hour at room temperature followed. Proteins were visualized by enhanced chemiluminescence using the Amersham enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

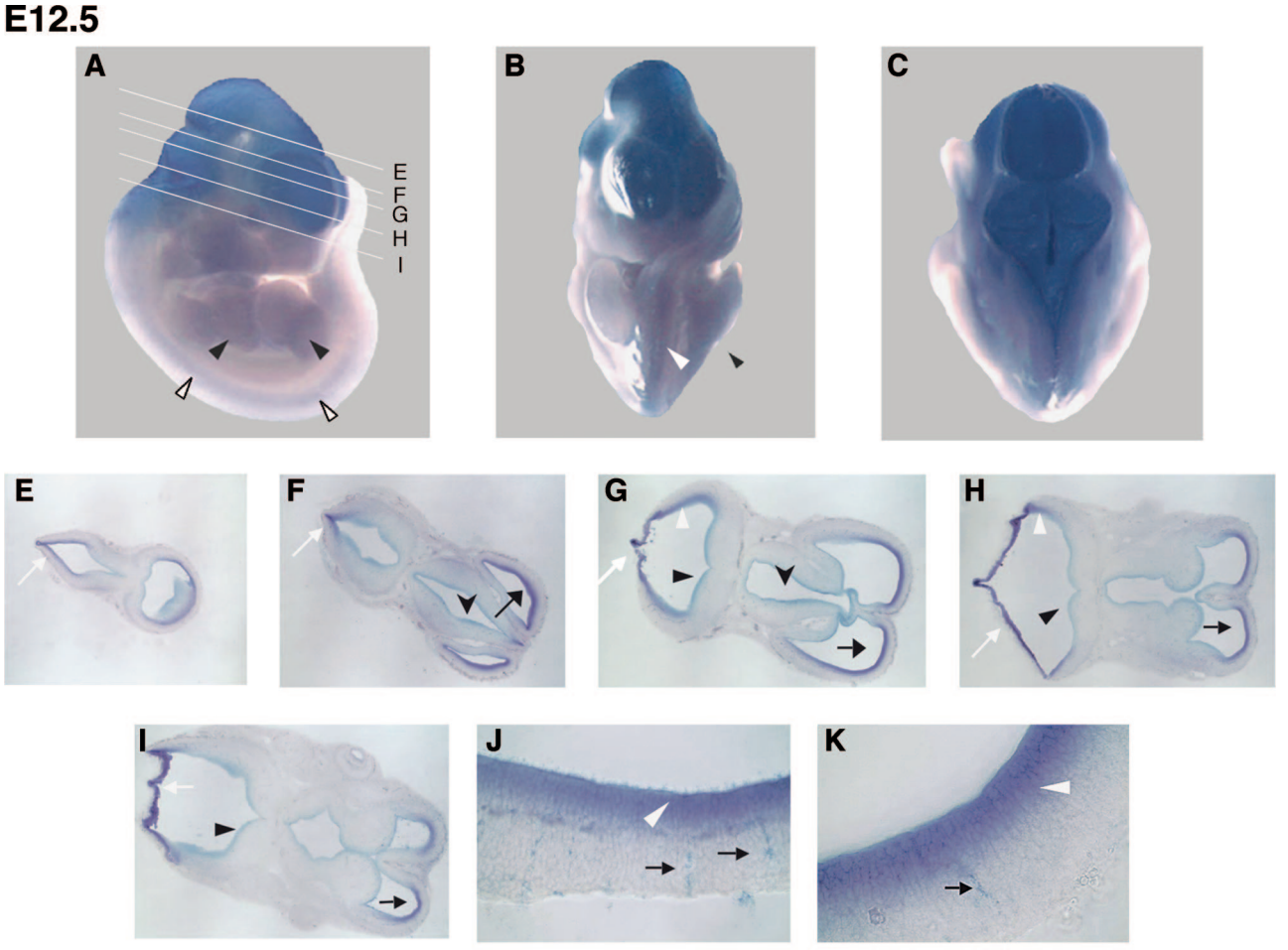
Immunofluorescence on Tissue Sections

Staining was performed on 10-μm cryosections according to the standard protocol for the mouse on mouse (M.O.M.) Immunodetection Basic kit (Vector Laboratories). Sections were incubated for 1 hour at room temperature with primary antibodies diluted in PBS/M.O.M., and then anti-mouse-anti-rabbit biotin (1:200; Vector Laboratories) or anti-mouse-anti-rabbit-Alexa 488-Alexa 594 (1:400; Molecular Probes) secondary antibodies were applied in PBS/M.O.M. blocking solution for 30 minutes at room temperature. If necessary, the sections were incubated for 10 minutes with streptavidin-Alexa 488-594 conjugates (1:400; Molecular Probes). For peanut agglutinin (PNA) detection in the testes, after incubation with the secondary antibodies, the sections were exposed to a fluorescein isothiocyanate-conjugated lectin peanut agglutinin from *Arachis hypogea* for 10 minutes (FITC-PNA, 1:1000; Sigma). In some cases, sections were stained with 1 μg/ml 4′,6-diamidino-2-phenylindol (DAPI; Molecular Probes). Stained sections were mounted in Mowiol (Molecular Probes) and viewed with a Nikon fluorescence microscope or a Leica confocal microscope.

Results

mPomt1 RNA Is Expressed in Critical Regions of the Developing Mouse Brain and Cerebellum

We previously reported that the *mPomt1* gene is expressed during early mouse embryogenesis (E8.5 to E10.5) in the developing nervous system, the muscles, and the eye.[21] To determine whether *Pomt1* expression pattern was maintained in later developmental stages, we performed whole-mount *in situ* hybridization on E12.5 and E13.5 mouse embryos. At these developmental stages, weak expression of Pomt1 mRNA was relatively ubiquitous, whereas stronger expression was observed in specific tissues. At E12.5, Pomt1 RNA was detected in the developing eye, the limb buds, and along the spinal cord (Figure 1, A and B). However, the strongest expression of Pomt1 was concentrated in the cephalic region where the brain and cerebellum develop (Figure 1, A–C). At E13.5, Pomt1 expression was weaker but maintained in the developing eye, limb buds, and spinal cord, but it was no longer so prominent in the cephalic region (Figure 1, L–N). In contrast, transcripts could now also be seen in


E12.5
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the heart (Figure 1M). In different cross sections of the rostral part of hindbrain at E12.5, Pomt1 RNA was detected in the ependymal layer of the metencephalon (Figure 1, G–I), the mantle layer of myelencephalon (Figure 1, G and H), and the roof of the fourth ventricle, where the strongest expression was observed (Figure 1, E–I). In the forebrain, Pomt1 transcripts were detected in the ependymal layer of the third and lateral ventricles, although the strongest expression was detected in the mantle layer of telencephalic vesicles (Figure 1, F–I). In the mantle layer, Pomt1 RNA was detected in proliferating neuroblasts that later migrate through the outer marginal layer of the telencephalic vesicles. Furthermore, Pomt1 transcripts appeared to be present in some migrating neuroblasts (Figure 1, J and K).

In cross sections at the cephalic level of an E13.5 embryo, Pomt1 expression was still evident in the roof of the fourth ventricle (Figure 1O) as well as in the mantle layer of telencephalic vesicles (Figure 1, O and P). In the developing eye, basal levels of Pomt1 transcripts were detected in the pigmented retinal layer. However, the strongest expression was localized to the hyaloid cavity, mainly in the vascular vessels associated with the lens and the neural retinal layer (Figure 1Q). The expression of Pomt1 RNA in the developing eye was corroborated by immunofluorescence studies on E13.5 cryosections using the affinity-purified polyclonal antiserum generated against hPOMT1 protein (anti-POMT1). This antiserum also recognizes mPomt1 protein, because the amino acid sequence in loop 5 that it recognizes is highly conserved (80% identity). The mPomt1 protein was localized in a very similar pattern to its RNA (Supplemental Figure 1, B and C, see *http://ajp.amjpathol.org*). When Pomt1 RNA expression was analyzed in the rest of the body, the strongest expression was detected in the ventricular walls of the heart (Figure 1R). These findings suggest that POMT1 could play an important role in the development of the brain, the cerebellum, and the eye and that it might be responsible for the severe brain and eye anomalies presented by patients with WWS.

Distribution of Dystroglycan and Pomt1 Protein in Adult Mouse Muscle

The best known *O*-mannosylated glycoprotein that serves as a substrate for POMT1 and POMT2 is α-DG.[4] This glycoprotein is present in several tissues, and although its amino acid sequence predicts a protein of ≈70 kd, its molecular mass varies depending on the degree of glycosylation in each tissue: 120 kd in the brain and peripheral nerves, 140 kd in heart, and 156 kd in skeletal muscle.[24,25] Western blot analysis of some adult mouse tissues using a monoclonal antibody that specifically recognizes glyco-epitopes of α-DG (VIA-4) showed that α-DG is glycosylated to a variable degree in heart, skeletal muscle, brain, and kidney. However, it is notable that it does not seem to be glycosylated in the liver and that it is weakly glycosylated in the testes (Figure 2A). We attempted to determine whether mPomt1 protein expression correlated with that of glycosylated α-DG. In Western blot analysis, the affinity-purified anti-POMT1 antiserum was only able to detect a ≈72-kd band that corresponded to the mPomt1 protein in heart and skeletal muscle (Figure 2B). Accordingly, the α-DG in these tissues must be *O*-mannosylated.

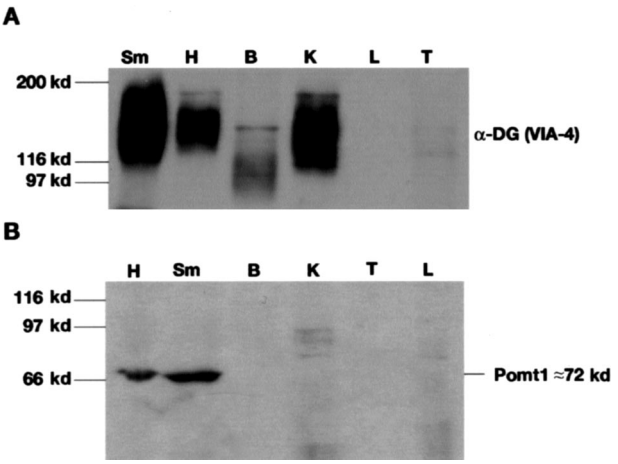

Figure 2. Western blot of protein extracts from mouse adult tissues. Total protein extracts (30 μg) were probed with VIA-4 antibodies that recognize glyco-epitopes of α-DG. The distinct molecular mass in each tissue is determined by the pattern of glycosylation (**A**). Microsomal protein extracts (50 μg) probed with anti-POMT1 antibodies. The 72-kd band detected in the heart and skeletal muscle corresponds to the mPomt1 protein (**B**). Sm, skeletal muscle; H, heart; B, brain; K, kidney; L, liver; T, testis.

To examine further the distribution of Pomt1 in mouse muscle tissue and its relationship to DG, we performed indirect immunofluorescence on cryosections of skeletal and cardiac muscle with the anti-POMT1 antiserum and anti-DG antibodies (anti-α-DG and anti-β-DG). In skeletal muscle fibers, whereas mPomt1 was localized to the sarcoplasmic reticulum, as well as to the peripheral region of cells under the sarcolemma (Figure 3A), both DG subunits were relegated to the sarcolemmic region (Figure 3, B and E).[25] In heart cryosections, Pomt1 was detected in the sarcoplasmic reticulum of cardiac cells

Figure 1. Whole-mount expression of Pomt1 in mouse embryos. Whole-mount *in situ* hybridization (**A**, **B**, **C**, **L**, **M**, and **N**) and representative cryosections of E12.5 embryos taken at the level of cephalic region indicated by the **white line** in **A** (**E**, **F**, **G**, **H**, and **I**, magnification ×4) and of E13.5 embryos taken at the level indicated by a **white line** in **L** (**O–R**; **P–R**, magnification ×10). Magnification of the telencephalic vesicles in **G** (**J**, magnification ×40; **K**, magnification ×60). At E12.5, the maximal Pomt1 expression was detected in the head (**A–C**), whereas lower levels of expression were seen in the limb buds and the spinal cord (**A** and **B**, **black** and **white arrowheads**, respectively). In the hindbrain, Pomt1 was detected in the mantle layer of myelencephalon (**G** and **H**, **white arrowheads**), as well as in the ependymal layer of metencephalon (**G–I**, **black arrowheads**). However, the strongest expression was detected in the roof of the fourth ventricle (**E–I**, **white arrows**). In the forebrain, Pomt1 was expressed in the ependymal layer of the third ventricle (**F** and **G**, **open black arrowhead**). The maximal expression was seen in the mantle layer of telencephalic vesicles (**F–I**, **black arrows**) where Pomt1 RNA was detected in mitotic neuroblasts (**J** and **K**, **white arrowheads**), as well as in neuroblasts migrating to the marginal layer (**J** and **K**, **black arrows**). At E13.5, Pomt1 expression was maintained in the developing eye (**L** and **M**, **black arrows**), the limb buds (**M**, **black arrowheads**), the spinal cord (**L–N**, **white arrowheads**), and the heart (**M**, **white arrow**). Pomt1 expression was also maintained in the roof of the fourth ventricle (**O**, **open arrow**) and in the mantle layer of the telencephalic vesicles (**O** and **P**, **arrowheads**). Pomt1 RNA was detected in the pigmented retinal layer of the developing eye (**Q**, **white arrowhead**) and in the hyaloid vessels of hyaloid cavity (**Q**, **black arrows**). Pomt1 transcripts were also seen in the walls of the heart (**R**, **arrowhead**).

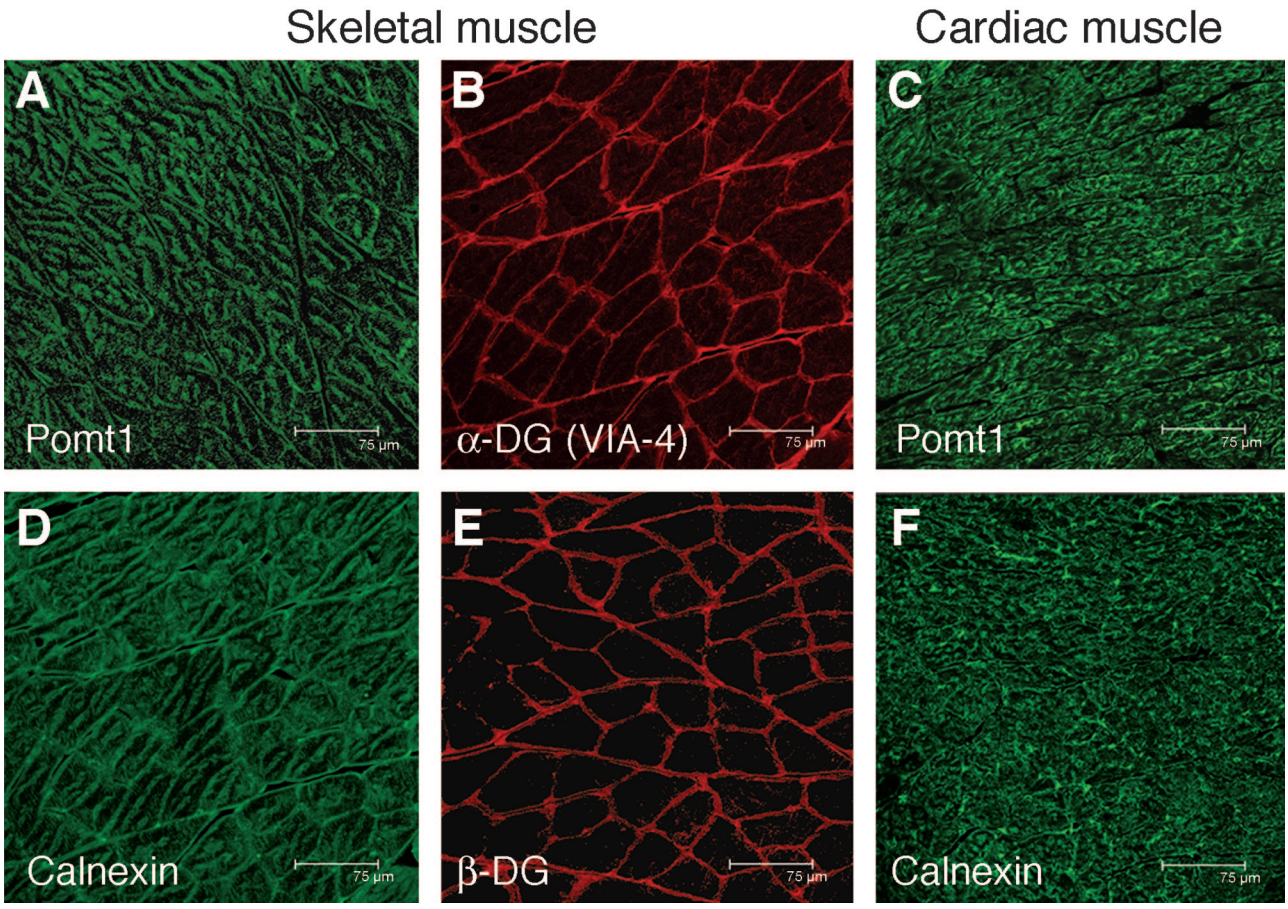

Figure 3. Pomt1 and DG immunodetection on muscle tissues. Immunodetection of mPomt1, DG, and the ER marker calnexin in skeletal and cardiac muscle sections. Incubation with anti-POMT1 (**A** and **C**), anti-α-DG, VIA-4 (**B**), anti-β-DG (**E**), and anti-calnexin (**D** and **F**). Pomt1 was localized in the sarcoplasmic reticulum of skeletal and cardiac cells (**A** and **C**), as well as in the subsarcolemmic region of skeletal muscle cells (**A**) displaying a very similar distribution to the ER maker calnexin in both skeletal (**C**) and cardiac (**F**) muscle cells. The glyco-epitopes of α-DG and β-DG subunits were localized in the sarcolemma (**B** and **E**). Fluorescence microscopy images, magnification ×40.

(Figure 3C), displaying a similar pattern of expression to the ER marker calnexin in both skeletal and cardiac muscle (Figure 3, D and F).

POMT1 and POMT2 seem to act together to produce maximal enzymatic activity.[4] Since the RNA expression pattern of both POMT1 and POMT2 is very similar in adult mouse tissues,[21] it seems likely that both proteins will be present in the same tissues. Immunofluorescent staining of muscle tissue with anti-POMT2 antibodies corroborated this hypothesis, since this protein appeared to be localized to the same compartment as mPomt1 in skeletal and cardiac muscle (Supplemental Figure 2, A and B, see *http://ajp.amjpathol.org*).

mPomt1 RNA Is Expressed in the Diploid Phase of Spermatogenesis, and the Protein Is Mainly Localized to the Acrosome of Maturing Spermatids

We had previously observed the strongest expression of Pomt1 mRNA in mouse testes,[21] and here we have further examined the expression of the *mPomt1* gene within the testes by performing *in situ* hybridization on sections of this tissue. Pomt1 mRNA was mainly detected in the diploid phase of spermatogenesis, in spermatogonia, and primary spermatocytes. No significant expression of Pomt1 mRNA was detected in postmeiotic cells such as the secondary spermatocytes, maturing spermatids, or sperm (Figure 4, A and B).

Immunofluorescence studies with the anti-Pomt1 antiserum on sections from the testes were performed to verify whether the localization of the mPomt1 protein correlated with that of mPomt1 RNA. The Pomt1 protein was clearly detected in the acrosome of spermatids in all stages of maturation, either in the round spermatids in the cap phase (Figure 4C) or as elongated spermatids in the acrosome and maturation phase (Figure 4, D and E). Because the acrosome is a sperm-specific organelle and POMT1 is an integral membrane protein of the ER (data not shown),[5] we wanted to confirm this localization by co-immunofluorescence of Pomt1 and the acrosomic marker lectin PNA. A perfect correlation was detected in the distribution of both proteins in the acrosome of round (Figure 5C) and elongated spermatids (Supplemental Figure 3, C and D, see *http://ajp.amjpathol.org*). However, a weak Pomt1 signal was also detected in the spermatogonia and primary spermatocytes (Figure 5, B and C),

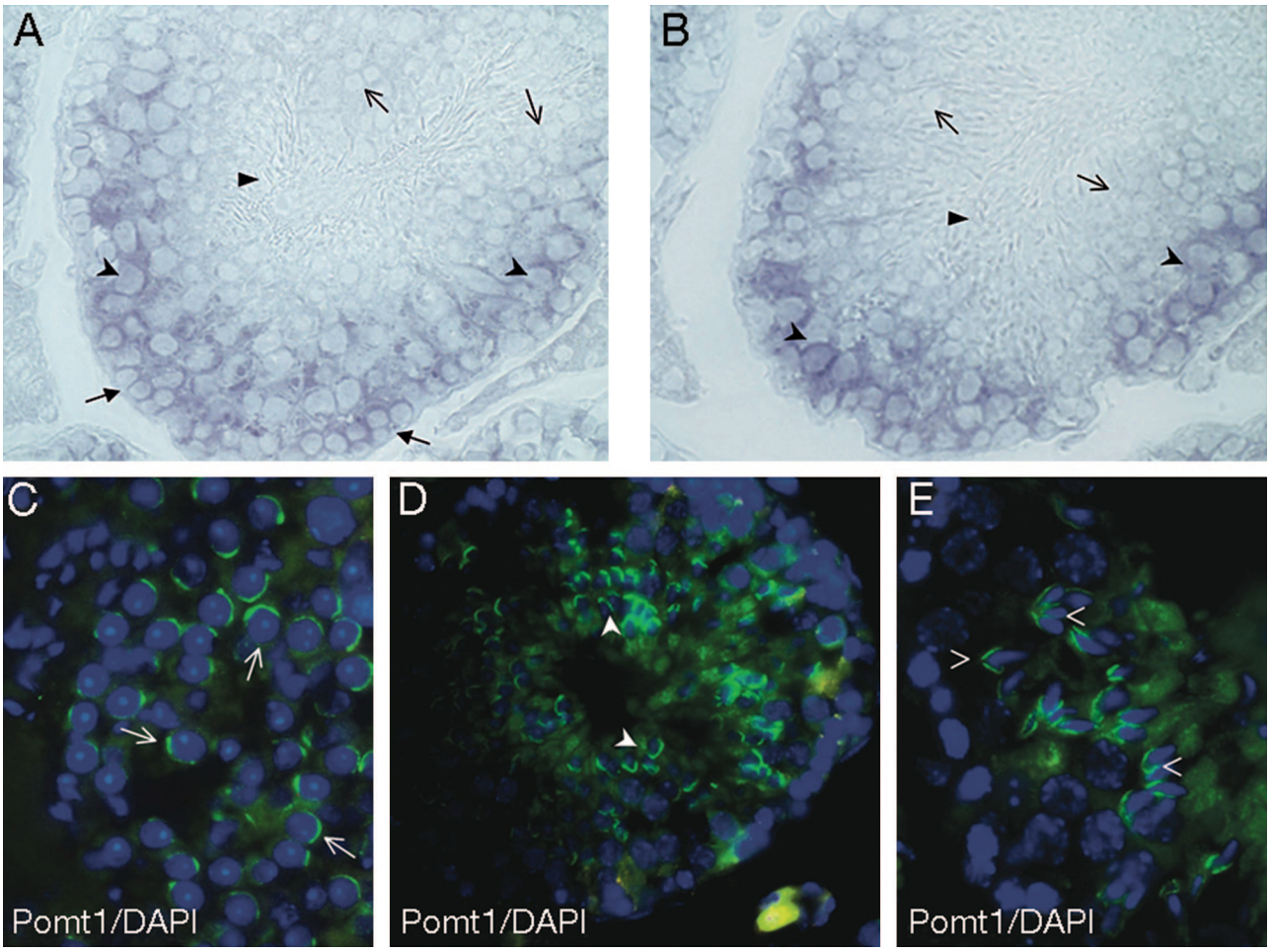

Figure 4. Pomt1 RNA and protein expression in mouse testes. *In situ* hybridization on testes sections showing the expression of Pomt1 RNA in spermatogonial cells next to the wall of seminiferous tubules (**black arrows**) and in primary spermatocytes (**A** and **B**, **open black arrowheads**). No mRNA was apparent in spermatids (**open black arrows**) or mature sperm (**black arrowheads**). Fluorescence microscope images of Pomt1 immunodetection in testis sections (**C** and **E**, magnification ×100; **D**, ×60) where Pomt1 (green) was localized in the acrosome of spermatids at all stages of maturation: cap phase round spermatids (**C**), elongating spermatids in the acrosome phase (**D**) and elongated maturing spermatids. 4,6-Diamidino-2-phenylindole stained the nuclei (blue).

although Pomt1 was confined to the ER in these cells because it displayed a very similar distribution to the ER marker calnexin (Figure 5D). Very similar RNA and protein expression patterns have been reported for Pomt2.[3]

We examined whether mouse Pomt1 might play a role in male fertility by programming crosses between female wt mice and male heterozygous *Pomt1*$^{+/-}$ mice.[21] However, the progeny of these crosses were wt and heterozygous in a ≈1:1 ratio as would be expected by Mendelian inheritance (data not shown). These data suggest that Pomt1 may play a role in spermatogenesis, although it seems not to be essential for male fertility in the mouse.

A Novel O-Mannosylation Substrate for Pomt1 and Pomt2 Might Exist in the Testes

Because α-DG is the main *O*-mannosylation substrate for both POMT1 and POMT2, we determined whether DG proteins were found in the same cell types as POMTs. Previous studies showed that both the α- and β-DG subunits were present almost exclusively in the basement membrane of the seminiferous tubules,[25] even though the antibodies used in these studies detected both the α- and β-subunits of dystroglycan protein and not the glycosidic residues of α-DG. Indirect immunofluorescence with anti-β-DG antibodies corroborated that the β-DG subunit was present in the basement membrane (Figure 6A). Our Western blot data indicated that α-DG was only weakly glycosylated in the testes (Figure 2A). Indeed, immunofluorescence studies with anti-α-DG (VIA-4) antibodies suggested that α-DG was only glycosylated in the basement membrane of the seminiferous tubules (Figure 6B). No glycosylated α- or β-DG subunits were found in the maturing spermatids where Pomt1/Pomt2 were located, suggesting that both Pomt proteins might have a different *O*-mannosylation substrate in these cells.

Discussion

Defects in the *O*-glycosylation of α-DG have been associated with a group of neuromuscular diseases that are caused by mutations in genes that encode glycosyltransferases or putative glycosyltransferases.[1,6–8] WWS is the

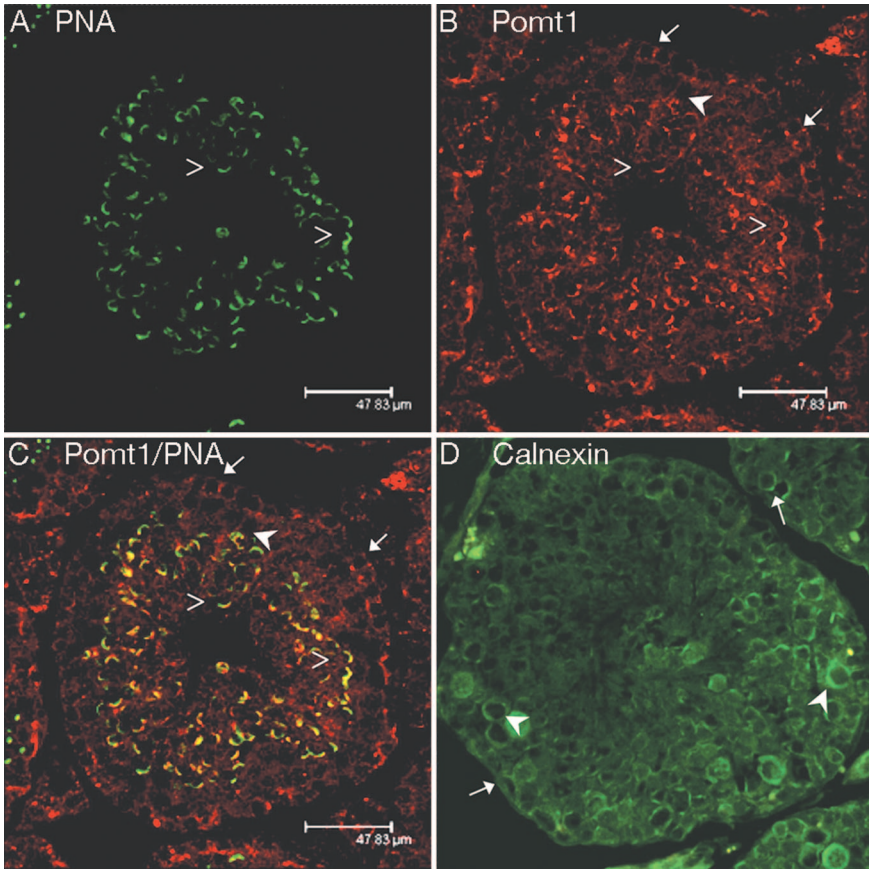

Figure 5. Co-localization of Pomt1 and the acrosomal marker PNA in seminiferous tubules. Localization of the PNA acrosomal marker (**A**) and Pomt1 (**B**, **open arrowheads**) to the acrosome of round spermatids in confocal microscopy images, magnification ×40. Co-localization of peanut agglutinin and Pomt1 (**C**). Pomt1 was expressed more weakly in spermatogonia (**B** and **C**, **white arrows**) and primary spermatocytes (**B** and **C**, **white arrowheads**), even though in these cells Pomt1 co-localized with the ER marker calnexin (**D**).

most severe of these disorders, a recessive CMD that combines ocular abnormalities and multiple brain malformations, including type II lissencephaly. WWS is mainly caused by mutations in the *hPOMT1* gene. Moreover, *hPOMT1* mutations have also been found in milder forms of muscular dystrophies like CMD/MR and LGMD2K.[15,19]

Given that mutations in the *hPOMT1* gene are responsible for a group of neuromuscular disorders with different degrees of severity, it is of interest to establish a relationship between POMT1 expression and the clinical aspects observed in these diseases. We have previously shown that Pomt1 RNA is expressed during mouse embryonic development and suggested that Pomt1 is important for the development of the nervous system, the muscles, and the eye, which are the tissues most severely affected in patients with WWS.[21] In this study, we confirmed that this pattern of RNA expression is maintained in later developmental stages (E12.5 and E13.5). In the hindbrain at E12.5, we show that mPomt1 transcripts are found in the ependymal and mantle layer of the metencephalon and myelencephalon, respectively, regions that will become the cerebellum and medulla oblongata. Furthermore, we found maximal mPomt1 expression in the roof of the fourth ventricle, where the choroid plexus produces the cerebrospinal fluid in this region of the brain. The cerebrospinal fluid flows through the ventricular system to the central canal of the spinal cord, passing through the foramina of Luschka and Magendie situated in the roof of the fourth ventricle.[26] When these pores are obstructed, the ventricles fill with cerebrospinal fluid and hydrocephalus is produced.[27,28]

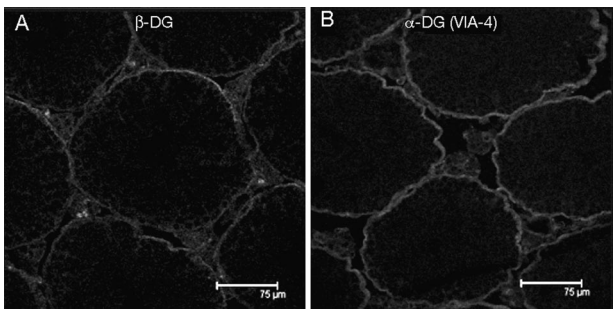

Figure 6. Immunodetection of α- and β-DG in mouse testes. The β-DG subunit was found in the basal membrane region of the walls of the seminiferous tubules (**A**). The α-DG subunit was also found in the basal membrane region, and it was *O*-glycosylated, because the VIA-4 antibodies recognize the α-DG glycoepitopes (**B**). No signal of α- or β-subunits was detected in any type of germ cell. Confocal microscopy images, magnification, ×40.

In the forebrain, the main mPomt1 expression was detected in the mantle layer of lateral ventricles or telencephalic vesicles, especially in the neuroblasts of the mantle layer. At E12.5 to E13, the postmitotic neurons

start to migrate to the marginal layer constituting the neopallial cortex that becomes the cerebral cortex.[26] We have detected Pomt1 transcripts in what seem to be neuroblasts migrating to the marginal layer.

Together, these data suggest that *Pomt1* expression in the hindbrain and forebrain could be related to the brain anomalies found in patients with WWS.[11–13] Furthermore, the Pomt1 expression in the metencephalon and myelencephalon could be related to cerebellar hypoplasia, absence of the septum and cerebellar vermis, and other cerebellar defects; the expression of Pomt1 in the fourth ventricle could be related to both the hydrocephalus and the dilation of the fourth ventricle. And finally, the presence of Pomt1 in the telencephalic vesicles is particularly relevant if we consider that neuronal migration defects in humans produce severe anomalies in the cerebral cortex such as cobblestone cortex or type II lissencephaly.[29]

The eye abnormalities found in patients with WWS involve cataracts, retinal detachment or dysplasia, and persistent hyperplastic primary vitreous, among others.[11,13] We have shown that in the developing eye, both the Pomt1 RNA and protein were expressed in the pigmented retinal layer as well as in the hyaloid vessels of hyaloid cavity. These vessels form a vascular network on the posterior surface of the lens, supply the neural retina with blood, and are responsible for the formation of the vitreous humor.[30] These data suggest that POMT1 is implied in the development of the eye and that it could be related to some of the ocular abnormalities detected in patients with WWS.

In the adult mouse tissues analyzed, we mainly detected the Pomt1 protein in skeletal and cardiac muscle tissue. We did not find Pomt1 in the adult brain, which suggests that POMT1 mainly acts during brain development. This fact may imply that the *O*-mannosyl glycans are most important when the brain structures that require them are forming.

In muscle tissues, we found that α-DG is *O*-glycosylated. As expected, given that POMT1 is an integral ER protein, we specifically detected mPomt1 in the sarcoplasmic reticulum of skeletal and cardiac fibers. The mPomt1 protein was also localized to the sarcolemma of skeletal muscle cells, as were the glycosylated α-DG and the β-DG subunit. Nevertheless, the Pomt1 detection in this region corresponds to the sarcoplasmic reticulum located just under the sarcolemma. We also found that Pomt2 protein localizes to the sarcoplasmic reticulum in skeletal and cardiac muscle cells.

These findings are consistent with the muscle phenotype found in the diseases caused by *POMT1* and/or *POMT2* mutations: WWS, CMD/MR, and LGMD2K.[15,16,19,20] Furthermore, the detection of mPomt1 in adult muscle suggests that POMT1 not only fulfills a role in the development of these tissues but that it also plays an important role, together with POMT2, in helping to maintain these structures in the adult, probably by *O*-mannosylating α-DG when muscle fiber regeneration requires it.

We previously reported that Pomt1 mRNA expression was maximal in adult testes.[21] Spermatogenesis takes place in the seminiferous tubules and involves mitotic proliferation of spermatogonia, meiosis, and spermiogenesis, in which the haploid spermatids differentiate into sperm.[31] Here, we show that Pomt1 RNA was present in spermatogonia and primary spermatocytes, whereas the protein was mainly localized to the acrosome of haploid spermatids at all stages of maturation, although some Pomt1 was detected in the ER of diploid cells. Similar RNA and protein expression were described for mPomt2 in testes,[3] suggesting that both POMTs might fulfill a similar role in this tissue and could be involved in murine spermatogenesis. The acrosome is a specific organelle of maturing spermatids and sperm that plays an important role during fertilization.[32] Here, we have shown that *Pomt1*-null sperm are able to fertilize wt eggs, because the offspring of male *Pomt1*$^{+/-}$ mice with wt females produced 50% wt and heterozygous animals. Together, these findings suggest that, whereas Pomt1 might be involved in fertilization, it is not essential for this process in the mouse. Further analyses will be needed to define what function POMT1 fulfills in fertilization.

With regard to the main *O*-mannosylation substrate for POMTs, α-DG seems to be relegated to the basement membrane of seminiferous tubules together with the β-subunit, being the only site where it is *O*-glycosylated. If mouse Pomts act as *O*-mannosyltransferases in maturing spermatids and sperm, a novel *O*-mannosylation substrate might exist because α-DG is not present in these cells.

It is important to note that apart from the typical involvement of the brain and eye, some male patients with WWS display genital anomalies, and it has been suggested that the gene(s) responsible directly affect testicular development.[11,22] The results reported here may establish a closer relationship between POMT1 and the muscular, brain, ocular, and genital abnormalities that characterize the WWS. Tissue-specific deletion of *Pomt1* in those tissues or organs affected in neuromuscular diseases caused by *POMT1* mutations will help us to elucidate the pathomechanism by which *O*-mannosyltransferases cause such severe phenotypes.

Acknowledgments

We thank Raúl Borrego and Saleta Dolores for excellent technical assistance, and we are grateful to Dr. Sabine Strahl for generously providing us with the POMT2 antibody. We thank M. Sefton for critical reading of the manuscript.

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