

Expression in retinal neurons of fukutin and FKRP, the protein products of two dystroglycanopathy-causative genes

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Purpose: Dystroglycanopathies are a heterogeneous group of recessive neuromuscular dystrophies that affect the muscle, brain and retina, and are caused by deficiencies in the O-glycosylation of α -dystroglycan. This post-translational modification is essential for the formation and maintenance of ribbon synapses in the retina. Fukutin and fukutin-related protein (FKRP) are two glycosyltransferases whose deficiency is associated with severe dystroglycanopathies. These enzymes carry out in vitro the addition of a tandem ribitol 5-phosphate moiety to the so-called core M3 phosphotrisaccharide of α -dystroglycan. However, their expression pattern and function in the healthy mammalian retina has not so far been investigated. In this work, we have addressed the expression of the *FKTN* (fukutin) and *FKRP* genes in the retina of mammals, and characterized the distribution pattern of their protein products in the adult mouse retina and the 661W photoreceptor cell line.

Methods: By means of reverse transcription (RT)-PCR and immunoblotting, we have studied the expression at the mRNA and protein levels of the fukutin and FKRP genes in different mammalian species, from rodents to humans. Immunofluorescence confocal microscopy analyses were performed to characterize the distribution profile of their protein products in mouse retinal sections and in 661W cultured cells.

Results: Both genes were expressed at the mRNA and protein levels in the neural retina of all mammals studied. Fukutin was present in the cytoplasmic and nuclear fractions in the mouse retina and 661W cells, and accumulated in the endoplasmic reticulum. FKRP was located in the cytoplasmic fraction in the mouse retina and concentrated in the Golgi complex. However, and in contrast to retinal tissue, FKRP additionally accumulated in the nucleus of the 661W photoreceptors.

Conclusions: Our results suggest that fukutin and FKRP not only participate in the synthesis of O-mannosyl glycans added to α -dystroglycan in the endoplasmic reticulum and Golgi complex, but that they could also play a role, that remains to be established, in the nucleus of retinal neurons.

Dystroglycanopathies (DGPs) are a group of minority congenital neuromuscular dystrophies caused by deficiencies in the complex process of O-mannosyl glycosylation of dystroglycan (DG). They are clinically and genetically heterogeneous diseases that are inherited in an autosomal recessive fashion, and whose symptoms involve a broad spectrum of clinical manifestations mainly affecting the skeletal muscle and central nervous system (CNS), with the latter including the brain and retina [1-3]. Recently, these diseases have been jointly designated in the OMIM database under the term “Muscular dystrophies-dystroglycanopathies (congenital with brain and eye anomalies),” which are abbreviated as MDDGs.

DG is the main component of the so-called dystrophin-glycoprotein complex (DGC), a multiprotein assembly composed of peripheral and integral membrane proteins and responsible for linking the cytoskeleton of muscle and nerve cells to the extracellular matrix (ECM) of their resident tissue [4,5]. The DGC is thus crucial for the correct structure and function of muscle and nervous systems from early embryogenesis in mammals [6,7]. DG is a glycoprotein composed of two subunits: alpha (α -DG), which is extracellular, and beta (β -DG), which is transmembrane and cytoplasmic. These two polypeptides remain non-covalently linked and associated with the plasma membrane [8-10]. DG is widely distributed in a variety of cell types, and usually associated with basement membranes, such as muscle, nervous tissue, epithelial tissue and vascular endothelium [11-13]. The α -DG polypeptide is heavily and heterogeneously glycosylated by the addition of N- and (especially) O-glycans to its central, mucin-like domain [14]. Its O-linked glycan chains are essential for the interaction of α -DG with other ECM proteins, such

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as laminin, agrin and perlecan in general [4,5], neurexin [15] and slit [16] specifically in the brain, and pikachurin exclusively in the retina. The interaction between the latter and DG has been proven to be essential for the formation and function of ribbon synapses established at the outer plexiform layer (OPL) between photoreceptors (cones and rods) and their postsynaptic, bipolar and horizontal neurons [17]. Interactions between DG and ECM proteins are also crucial for the proper formation by Müller glia of the inner limiting (basement) membrane separating the neural retina from the vitreous humor [18,19]. Retinal symptoms derived from the loss of α -DG glycosylation may consequently involve chorioretinal atrophy, retinal dysplasia and detachment, and/or vitreoretinal dysgenesis [20-24].

A total of 18 genes have been hitherto identified in which mutations cause different types of DGPs with varying degrees of clinical severity. With the exception of *DAG1* (Gene ID 1605; OMIM 128239), which codes for DG itself, most of these genes encode protein glycosyltransferases whose loss of function causes α -DG hypoglycosylation and thereby affects its function as a receptor for its ECM ligands [1,25]. The nomenclature for these enzymes that was recently adopted by Yoshida-Moriguchi and Campbell [10] is used in this work. The first genetic alteration identified as causative of DGPs, namely Fukuyama congenital muscular dystrophy (FCMD), was detected in Japan as an ancestral founder mutation [26] and was subsequently mapped on the *FKTN* gene (Gene ID 2218, OMIM 607440), encoding the 461 amino-acid enzyme called fukutin [27]. Thereafter, a significant number of non-Japanese patients with mutations in the *FKTN* gene have been reported, some with FCMD phenotype and others with more severe DGPs, such as Walker-Warburg syndrome (WWS) or muscle-eye-brain disease (MEB) [23,28,29], all of them designated as type MDDGA4 (OMIM 253800). However, mutations in *FKTN* can also cause milder DGPs that do not involve mental retardation, dubbed as types MDDGB4 (OMIM 613152) and MDDGC4 (OMIM 611588), the latter previously designated as limb-girdle muscular dystrophy (LGMD) type 2M [23,24,30]. Soon thereafter, a gene encoding the 495 amino-acid enzyme called fukutin-related protein (FKRP; Gene ID 79147, OMIM 606596) was identified [31,32], which can also cause different forms of DGPs that are either severe with anomalies in the brain and eye (MDDGA5; OMIM 613153), such as WWS or MEB [22,24], or milder with or without mental retardation, dubbed as types MDDGB5 (OMIM 606612) and MDDGC5 (OMIM 607155), the latter also known as LGMD type 2I [30-32].

Fukutin and FKRP proteins are structurally and functionally related glycosyltransferases involved in the biosynthetic pathway leading to the extension of the α -DG O-mannosyl glycan core structure called M3, i.e., the phosphorylated O-mannosyl trisaccharide GalNAc(β 1-3)GlcNAc(β 1-4; P6)Man. This pathway has so far been identified only for specific O-mannose residues previously added by the POMT1/POMT2 heterodimer to Ser/Thr residues in the mucin-like domain of α -DG, and involves the addition by the enzyme POMGNT2 of a molecule of N-acetylglucosamine (GlcNAc), covalently linked by a β 1-4 bond to the O-mannose residue, presumably in the endoplasmic reticulum (ER) [33,34]. Next, the B3GALNT2 protein adds one unit of N-acetylgalactosamine (GalNAc), with subsequent phosphorylation at the 6-position of the O-mannose residue by the enzyme POMK [34]. α -DG is then thought to be transported to the Golgi complex, where fukutin and FKRP act to sequentially add to the GalNAc molecule two ribitol 5-phosphate (Rbo5P) units in tandem from cytidine-diphosphate-ribitol (CDP-Rbo) [35,36]. Thereafter, the proteins products of other DGP-associated genes contribute to add to this tandem the so-called matriglycan polysaccharide, whose presence, and especially its number of repeats, is essential for the binding of α -DG to its ECM ligands [3,10,34,37,38].

Fukutin and FKRP are believed to perform their Rbo5P transferase function in the Golgi complex, where they have been detected in human skeletal muscle cells [39,40] and mouse liver [35]. To study the role of DGP-associated proteins in vivo, several mouse models have been generated and characterized, which exhibit null mutations in particular genes encoding glycosyltransferases acting on α -DG [18,19,41]. These included chimeric knockout (KO) mice for the fukutin-encoding gene [42] and knock-down mice for the gene encoding FKRP [43,44]. In all these models, a partial or total absence of α -DG glycosylation has been unveiled, together with associated ocular structural and functional alterations resembling those existing in patients with DGPs. Nevertheless, the precise role of fukutin and FKRP proteins in the adult visual system is largely unknown, and their distribution pattern in the retina remains to be established. In this work, we have analyzed the expression of *FKTN* and *FKRP* at the mRNA and protein levels in the neural retina of several mammalian species and in cultured photoreceptors of the 661W cell line. Interestingly, in addition to being located in the ER and the Golgi complex, respectively, both fukutin and FKRP accumulate in the nucleus in this cell line, where they could play a hitherto unreported function.

METHODS

Animals: The adult mammalian species studied in this work included mice (*Mus musculus*, strain C57BL/6J), rats (*Rattus norvegicus*, strain Sprague-Dawley), cows (*Bos taurus*), and cynomolgus monkeys (*Macaca fascicularis*). All animal handling was performed in compliance with the rules set by the National Institutes of Health (USA), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the European Directive 2010/63/EU. Rodents were held at the Universidad de Alicante animal-care facility and euthanized upon CO₂ inhalation, followed by cervical dislocation. Their eyes were enucleated and stored in RNAlater solution (Ambion; Austin, TX, USA). Bovine eyes were freshly obtained from the Alicante municipal slaughterhouse. Monkeys were kept as described [45] at the animal-care facility of the Universidad de Murcia (Spain), and their eyes were kindly donated by Dr. M.T. Herrero. All protocols and primate-handling procedures used were approved by the university bioethics research committee. Young adult monkeys were anesthetized with ketamine (10 mg/kg, i.m.) and then administered a lethal injection of pentobarbital (50 mg/kg, i.p.), and their eyeballs were immediately enucleated following sacrifice. All animal eyes were kept at -80°C until the retinas were dissected for RNA or protein isolation. For immunohistochemistry, mouse eyes were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 (PB) for 1 h and then subjected to sucrose cryoprotection [46].

Cell culture: The 661W photoreceptor cell line was kindly provided by Dr. M. Al-Ubaidi (Department of Biomedical Engineering, University of Houston, TX). It was cloned from a retinal tumor of a transgenic mouse line that expressed the artificial *HITI* transgene, containing the SV40 large T antigen coding sequence under the control of the human inter-photoreceptor retinol-binding protein gene (*IRBP/RBP3*; Gene ID 5949, OMIM 180290) promoter [47]. These immortalized cells express cone molecular markers, such as opsins, transducin and cone arrestin, and lack proteins specific to rods or other retinal cell types, constituting a light-sensitive, homogeneous cell line [48,49]. The 661W cells were authenticated by means of short tandem repeats (STR) genotyping, and their genetic profile was verified to be consistent with a mixed FVB × C57BL/6 mouse strain of origin, as expected, and to carry the *HITI* transgene that makes this cell line unique (Appendix 1) [47,50]. The cells were grown on Dulbecco's modified Eagle medium (DMEM) from HyClone (Logan, UT), and supplemented with 10% fetal bovine serum from Gibco (Grand Island, NY), 2 mM L-glutamine, and 1% penicillin/streptomycin (HyClone) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were expanded on 75

cm² flasks, and after 5–10 passages under culture conditions they were frozen at -80°C until RNA or protein extraction was performed, or else they were seeded on 24-well culture plates and used for immunocytochemistry when they were nearly confluent.

Reverse transcription (RT)-PCR: The neural retina was dissected out, free from the retinal pigment epithelium (RPE), from eyes stored in RNAlater. Total RNA was extracted from retinal tissue and 661W photoreceptors using the TRIzol reagent (Invitrogen; Carlsbad, CA) and then treated with DNase I using the TURBO DNA-free kit (Ambion). RNA from normal human retina (29 donors, both sexes, 20–60 years at death) was purchased from Clontech BD (Mountain View, CA). RT reactions were performed using the RETROscript kit (Ambion), and subsequent PCR amplification of cDNA segments was performed essentially as described [50–52]. Primers specific to genes *FKTN* and *FKRP* of the different species (Table 1) were designed to bear a T_m of 60°C and flank at least one intron of the corresponding gene. PCR reactions (40 µl) contained 20 ng of cDNA, forward and reverse primers at 0.4 µM each, the four deoxynucleotides (dNTPs) at 0.2 mM each, and 1 U of GoTaq DNA polymerase (Promega; Madison, WI). PCR products were run on 2% agarose gels with added SYBR Green I (Sigma-Aldrich; St. Louis, MO) and photographed under ultraviolet (UV) light.

Immunoblotting: Total proteins were extracted from neural retinas and 661W cells, as previously described [51]. Briefly, a volume of 20 µl of lysis buffer supplemented with protease inhibitors was added per 5 mg of tissue or 10⁶ cells, and after incubation on ice for 15–20 min, the supernatant obtained upon centrifugation at 16,000 ×g at 4°C for 10 min constituted the total protein fraction. To obtain the cytoplasmic and nuclear protein fractions, the NE-PER kit from Pierce (Rockford, IL) was used, as previously detailed [50]. Proteins were resolved by sodium dodecyl sulfate–PAGE (SDS–PAGE) on 5%–20% polyacrylamide-gradient gels, and after electrotransfer to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK), they were stained with SYPRO Ruby Protein Blot Stain (Molecular Probes; Eugene, OR) to ensure even protein loading and transfer. Immunodetection was performed essentially as described [45,50–52]. The membranes were probed sequentially with primary antibodies (Table 2) at 4°C overnight and with horseradish peroxidase-conjugated anti-IgG secondary antibodies at a 1:10,000 dilution for 1 h, and detection was achieved by means of enhanced chemiluminescence. The absence of cross contamination between the cytoplasmic and nuclear fractions was assessed by immunoblotting using antibodies to proteins

TABLE 1. PRIMERS USED FOR RT-PCR IN THIS WORK.

Gene	GenBank accession number	Species	Primer sequences (5'-3')	Exon	PCR product size (bp)
<i>FKTN</i>	NM_001079802	Hu	F: GGGAGTACCATTCTGGCTGA	8	150
	NM_001257495	Mk	R: GGAAGTCCTGCATCCTGAAA	9	
	XM_002689900	Bv	F: GGGAGTGC GGTTCTGGCT	8	150
			R: GGGAGTCCTGCATCTTGAAA	9	
	NM_001108667	Rt	F: GGGAGTGCCCTTCTGGCT	8	150
	NM_139309	Mo	R: GGAAGTCCTGCATCCTGAAA	9	
<i>FKRP</i>	NM_024301	Hu	F: GACCTGGGCATCTACTTGGA	4	150
			R: CAAGTGGTTGCTTTCGCTGT	4	
	NM_001258048	Mk	F: GACCTGGGCATCTACTTGGA	4	150
			R: GAGGTGGTTGCTTTCGCTAT	4	
	NM_001099714	Bv	F: TGGGACTACGATGTGGACCT	4	163
		R: GGTGGTTGCTCTCGCTGTA	4		
	NM_001025678	Rt	F: GATCTGGGCATCTACCTGGA	4	245
	NM_173430	Mo	R: AAGTGCTCTGGAACTCAACA	4	

The accession numbers indicated correspond to mRNA sequences deposited in the [GenBank](#) database. The sequences of forward (Fw) and reverse (Rv) primers used in this work are given, with indication of the exon to which each primer aligns. The PCR product size is indicated in the last column for each primer pair. Hu, human; Mk, monkey; Bv, bovine; Rt, rat; Mo, mouse. For the 661W cell line the mouse primers were used.

exclusively found in these compartments, namely β -tubulin III and lamin A/C, respectively.

Immunohistochemistry: Cryostat vertical sections (14–16 μ m thick) were obtained and processed for immunohistochemistry, essentially as described [46,50–52]. Sections were subjected to single or double immunostaining with primary antibodies (Table 2) in PB supplemented with 1% (v/v) Triton X-100 (PBX) at room temperature overnight. Thereafter, the

samples were incubated in the presence of donkey secondary antibodies to rabbit or mouse IgG conjugated to Alexa Fluor 488 (green) or 546 (red) from Molecular Probes, at a 1:100 dilution in PBX. 4',6-Diamidino-2-phenylindole (DAPI) was simultaneously added at 10 μ g/ml, and incubation took place for 1 h at room temperature with gentle shaking in the dark in a humidified chamber. Control samples in which primary antibodies were omitted and the corresponding secondary

TABLE 2. PRIMARY ANTIBODIES USED IN THIS WORK.

Protein	Antibody	Company	Catalog no.	Working dilution		
				IB	IHC	ICC
Fukutin	Rabbit, polyclonal	Proteintech ^a	18,276-1-AP	1:1,000	1:50	1:100
FKRP	Rabbit, polyclonal	Origene ^b	TA347645	1:1,000	–	–
FKRP	Rabbit, polyclonal	AbboMax ^c	620–830	–	1:50	1:100
β -Tubulin III	Rabbit, polyclonal	Sigma-Aldrich	T3952	1:1,000	–	–
Lamin A/C	Mouse, 4C11	Sigma-Aldrich	SAB200236	1:10,000	–	–
Calreticulin	Mouse, 1G6A7	Thermo Scientific ^d	MA5–15382	–	1:1,000	–
KDEL	Mouse, 10C3	Abcam ^e	ab12223	–	–	1:250
GM130	Mouse, 35	BD Biosciences ^f	610,822	–	1:50	1:250
β -Dystroglycan	Mouse, 43DAG1/8D5	Abcam	ab49515	–	1:50	–

The two first columns enlist the antigen and animal origin of each antibody, and the clone code is specified for monoclonal antibodies. The two central columns indicate the commercial company and antibody catalog reference. The three last columns specify the dilution at which each antibody was used on immunoblotting (IB), immunohistochemistry (IHC) or immunocytochemistry (ICC). ^aProteintech (Manchester, UK), ^bOrigene (Rockville, MD), ^cAbboMax (San Jose, CA), ^dThermo Scientific (Waltham, MA), ^eAbcam (Cambridge, UK), ^fBD Transduction Labs. (Franklin Lakes, NJ).

antibodies were included were processed in parallel. Fluorescence was detected using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems; Wetzlar, Germany).

Immunocytochemistry: 661W photoreceptors were seeded on 24-well culture plates at 75,000 cells per well and grown on a 5% CO₂ atmosphere at 37°C for 24 h. Then the cells were fixed with 4% paraformaldehyde in Dulbecco's PBS (DPBS) for 10 min. Thereafter, they were permeabilized in situ with 0.2% Triton X-100 in DPBS for 10 min, and non-specific binding was prevented by incubation with 1% BSA in DPBS (blocking solution) for 1 h. Subsequently, the cells were incubated with primary antibodies at the dilutions indicated in Table 2 in blocking solution in a humidified chamber at 4°C overnight. Incubation with Alexa Fluor-conjugated anti-IgG secondary antibodies (plus 10 µg/ml DAPI when indicated), controls and fluorescence detection under confocal microscopy were performed as indicated above. Colocalization was assessed with the JACoP plugin developed for the NIH ImageJ software. Pearson's coefficient (PC) values were each calculated as the average ± SD of ten cells from micrographs obtained in duplicate experiments. The 661W nuclei or Golgi complexes were cropped before analysis. PC values can range from 1 to -1, with 1 standing for a completely positive correlation and -1 for a negative correlation, and with 0 standing for no correlation (mid-range coefficients, of -0.5 to 0.5, do not allow conclusions to be drawn) [53].

RESULTS

The expression profile of genes encoding fukutin (*FKTN*) and FKRP is fairly unknown in the mammalian retina. To evaluate whether these genes are transcribed in this tissue, eyes from different species were enucleated, and total RNA was extracted from their dissected neural retinas. Total RNA was also isolated from cultures of the 661W mouse cell line of cone photoreceptors. By means of RT-PCR using specific primers, we determined that the *FKTN* and *FKRP* genes were expressed at the mRNA level in the retinas of all mammals studied, including rodents (mouse and rat), bovine and primates (monkey and human) retinas, as well as in the 661W cell line (Figure 1A). All PCR products obtained exhibited the sizes expected from the experimental design performed, and their DNA sequencing confirmed that they corresponded to the predicted cDNA fragments in all cases.

We also demonstrated by immunoblotting that the protein products of these two genes, fukutin and FKRP, were synthesized in the retina and in 661W cells, consistently with positive results on mRNA expression. As shown in Figure 1B, the fukutin and FKRP enzymes were detected in total protein

extracts from the retina of all mammalian species analyzed, as well as from 661W photoreceptors. Fukutin migrated with an apparent molecular mass of 60 kDa, corresponding to the size reported by other authors [39,54,55]. Regarding FKRP, it migrated with an apparent molecular mass of 55 kDa, compatible with the canonical form of the protein in the different species. We next investigated the intracellular localization of fukutin and FKRP using protein extracts from the cytoplasm and nucleus of the adult mouse neural retina and 661W cell line. As shown in Figure 1C, immunoblotting analysis revealed that fukutin was found in both cytoplasmic and nuclear fractions of the mouse retina, whereas FKRP was detected exclusively in the cytoplasm. However, the two proteins were found in both the cytoplasm and nucleus of 661W photoreceptors. By using specific antibodies against β-tubulin III (an exclusively cytoplasmic protein) and lamins A and C (two exclusively nuclear proteins), we were able to evidence that in our hands, the fractionation procedure followed did not result in cross-contamination between the two subcellular fractions (Figure 1C).

Since both fukutin and FKRP were found to be expressed in the mouse retina and 661W photoreceptors, we subsequently addressed their distribution pattern in retinal sections and cultured 661W cells by means of confocal immunofluorescence microscopy. In mouse cryostat sections immunostained with an antibody specific for fukutin, we found that this protein was located in different retinal layers (Figure 2A,C), although it was mainly present in the inner segments (IS) of photoreceptors and the outer plexiform layer (OPL). We also observed a weaker immunoreactivity of fukutin in the outer nuclear (ONL) and inner plexiform (IPL) layers, as well as in the inner nuclear (INL) and ganglion cell (GCL) layers, where it accumulated in the nuclei of several cells, but without colocalizing with the DNA-binding dye, DAPI (Figure 2B,C). This analysis confirmed that in addition to the cytoplasm, fukutin was present in the nucleus of some retinal cell types, in keeping with the results obtained by immunoblotting (Figure 1C). Yet, the cytoplasmic distribution pattern of fukutin did not resemble that of the Golgi complex, as could be expected (see above). Furthermore, when we performed a double immunostaining for fukutin and GM130, a structural protein of the Golgi widely used as a marker for this organelle (Figure 2D), we did not observe colocalization, as illustrated in Figure 2E-G. Since endogenous fukutin had been previously located in the ER in a limited number of tumor cell lines [55,56], we next performed a double immunostaining using antibodies against fukutin and calreticulin, a chaperone aiding in the correct folding of glycosylated proteins in the ER that is often used as a marker for this organelle (Figure 2H). As a result, we detected an extensive colocalization of

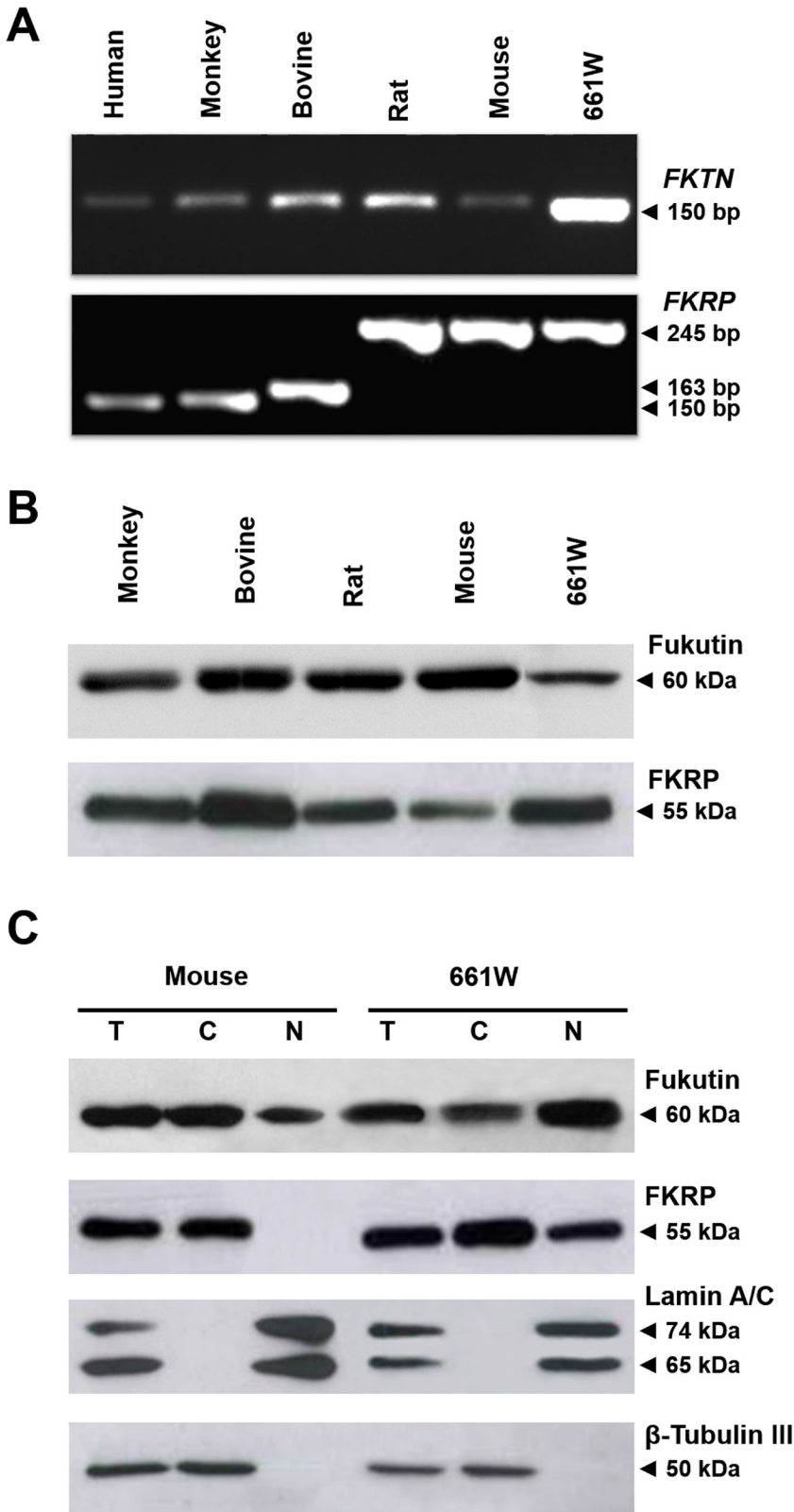


Figure 1. Expression of *FKTN* and *FKRP* at the mRNA and protein levels in the mammalian retina and 661W photoreceptor cell line. A: Total RNA was extracted from the neural retina of the indicated species and 661W cells, and the expression of the mRNAs encoded by the two genes was detected by RT-PCR using specific primers. PCR products were verified by DNA sequencing. B: Immunoblotting analysis of fukutin and FKRP expression was performed on total protein extracts (100 μ g/lane) of the neural retina of the indicated species and 661W cells. C: Immunoblotting analysis of total (T), cytoplasmic (C) and nuclear (N) protein fractions extracted from the mouse neural retina and 661W cells (50 μ g/lane) was performed using antibodies against fukutin, FKRP, β -tubulin III (a cytoplasmic control protein), and lamin A/C (two nuclear control proteins). The sizes of the bands obtained are given on the right side of each panel.

both proteins (Figure 2I, yellow), both in the IS of photoreceptors (Figure 2J) and their presynaptic terminals in the OPL (Figure 2K), i.e., the two areas of photoreceptors where the ER is known to be mainly present [57].

We then investigated the fukutin intracellular distribution pattern in 661W photoreceptor cells. As shown in Figure 3A,B, in this cell line fukutin was present both in the cytoplasm and the nucleus, supporting the results obtained by immunoblotting (Figure 1C). In the nucleus of 661W cells, fukutin exhibited a dense immunoreactivity, appearing as a dotted pattern, but again without colocalization with DAPI (Figure 3B,C; $PC=0.16\pm 0.03$). This protein was found to be widely distributed in the cytoplasm of 661W cells, but without colocalizing with the Golgi marker GM130 (Figure 3D), as found in double immunostaining experiments performed on cultured cells (Figure 3E,F; $PC=0.11\pm 0.05$). Instead, and as in retinal sections, fukutin displayed a location that was compatible with that of the ER. To investigate this possibility, we performed a double immunostaining using antibodies specific to fukutin and KDEL (Figure 3G), a well-known signal sequence retaining numerous proteins in the ER that is hence frequently used as a marker for this organelle. As a result, we detected a significant colocalization of fukutin and KDEL (Figure 3H,I; yellow; $PC=0.58\pm 0.02$), indicating that part of the fukutin protein was concentrated in the ER in this cell line.

We next addressed the expression pattern of FKRP in the mouse retina and the 661W photoreceptor cell line. We observed that FKRP was present in different layers of the retina (Figure 4A,C), accumulating in the IS of photoreceptors and additionally in the OPL, INL and GCL. This protein appeared excluded from the nuclei of all retinal cell types, which are shown stained with DAPI in Figure 4B,C. This analysis confirmed that FKRP was expressed exclusively in the cytoplasm in the mouse retina, in agreement with our results obtained by immunoblotting in this tissue (Figure 1C). Since this protein had been previously found in the Golgi complex in human skeletal muscle [39,40] and mouse liver [35], we next performed a double immunostaining using antibodies against FKRP and the Golgi marker GM130 (Figure 4D). The results obtained revealed a colocalization of these two proteins (Figure 4E, yellow), both in the IS of photoreceptors (Figure 4F, arrows) and in virtually all cells in the INL (Figure 4G, arrows) and in the GCL (Figure 4E, arrows). No colocalization was seen for FKRP with KDEL or calreticulin (data not shown), which made it unlikely that FKRP resided in the ER in addition to the Golgi. To shed light on the localization of the FKRP enzyme in the OPL, we performed a double immunostaining with antibodies against

FKRP and β -DG (Figure 4H-J), the latter being known to be present in the presynaptic membrane of photoreceptors [58,59]. As shown, a strong colocalization was found between these two proteins at the axon terminals of photoreceptors (Figure 4J), which indicated that FKRP resided in their plasma membrane, possibly in close proximity to β -DG.

FKRP was also visualized in both the cytoplasm and nucleus of 661W cells, supporting the results obtained by immunoblotting (Figure 1C). Like fukutin, this enzyme exhibited a significant intranuclear immunoreactivity (Figure 5A), appearing as a dotted pattern without colocalization with DAPI (Figure 5B,C; $PC=0.18\pm 0.04$). In the cytoplasm, FKRP distributed in highly immunopositive elements clustered in one pole of every nucleus stained with DAPI (Figure 5B,C), i.e., in a localization compatible with that of the Golgi complex. To investigate this possibility, we performed a double immunostaining using specific antibodies against FKRP and the Golgi marker, GM130 (Figure 4D). The results obtained revealed a significant colocalization of both proteins (Figure 5E,F; $PC=0.59\pm 0.04$), indicating that part of the FKRP protein concentrated in the Golgi in this cell line. In contrast, double immunolabelings for FKRP and the ER markers KDEL or calreticulin revealed a total absence of colocalization in the 661W cell line (data not shown).

DISCUSSION

FKTN and *FKRP* genes encode two related protein glycosyltransferases that act sequentially to transfer two Rbo5P molecules from CDP-Rbo to the phosphorylated core M3 glycan of α -DG. In this process, fukutin adds the first Rbo5P unit and FKRP the second, the two Rbo5P units becoming linked between them and to the GalNAc residue of core M3 by phosphodiester bonds [35,36]. Subsequently, alternating Xyl and GlcA residues are repetitively appended to the tandem Rbo5P scaffold to constitute the so-called matriglycan, i.e., the α -DG polysaccharide moiety directly involved in ligand binding [10,36]. These pathways of α -DG O-glycosylation are, however, essentially unknown in the retina, and in particular only mRNA expression of the *FKTN* gene has been demonstrated by means of in situ hybridization in the human fetal retina [21]. Yet, we have recently reported in the adult retina of several mammalian species the expression, at the mRNA and protein levels, of DGP-causative genes encoding POMT1, POMT2 and POMGNT1 [50], three glycosyltransferases acting on α -DG. POMGNT1 is responsible for catalyzing the second step in the synthesis of α -DG glycans designated as cores M1 and M2 (but not M3) by covalently attaching, with a β 1–2 linkage, one unit of GlcNAc to O-mannose residues previously added by the POMT1/POMT2 heterodimer to Ser/

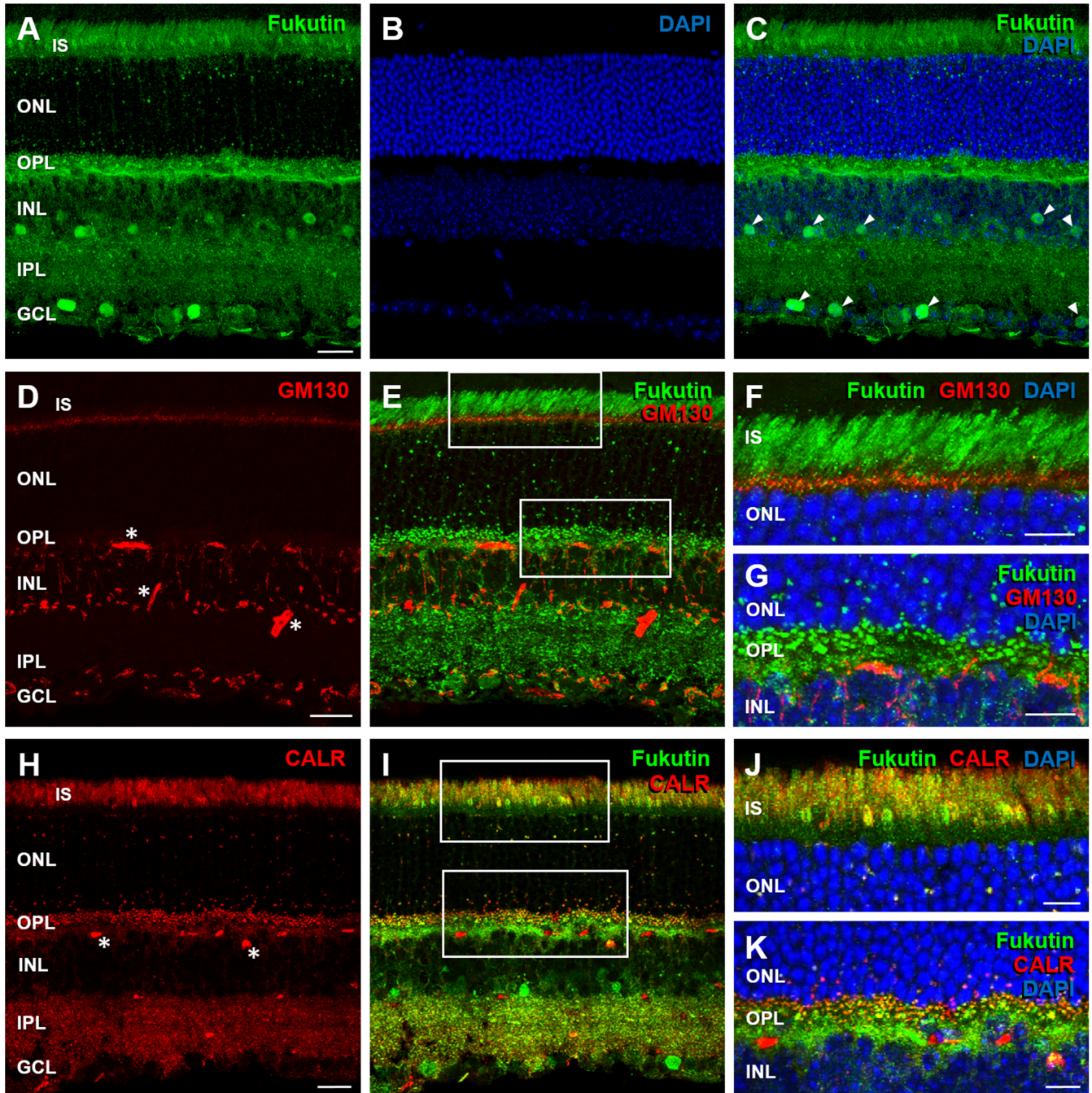


Figure 2. Immunolocalization of fukutin in the mouse retina. Double immunostainings were performed on mouse retinal sections for fukutin (A, C, E, I; green), the specific Golgi complex marker GM130 (D, E; red), and the specific ER marker calreticulin (CALR; H, I; red). Enlarged views in F and G correspond to boxed areas in E, and enlarged views in J and K to boxed areas in I. Nuclei stained with DAPI are shown in blue (B, C, F, G, J and K). Fukutin immunoreactivity was found in the cytoplasm and nucleus of cells in the INL and the GCL of the mouse retina, but without colocalizing with DAPI (C, arrowheads). However, colocalization of fukutin with GM130 (E-G). Asterisks in D and H indicate non-specific immunostaining of retinal vessels with secondary antibodies to mouse IgG. IS=inner segments; ONL=outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer; IPL=inner plexiform layer; GCL=ganglion cell layer. Each bar equals 20 μ m, except in F, G, J and K: 10 μ m.

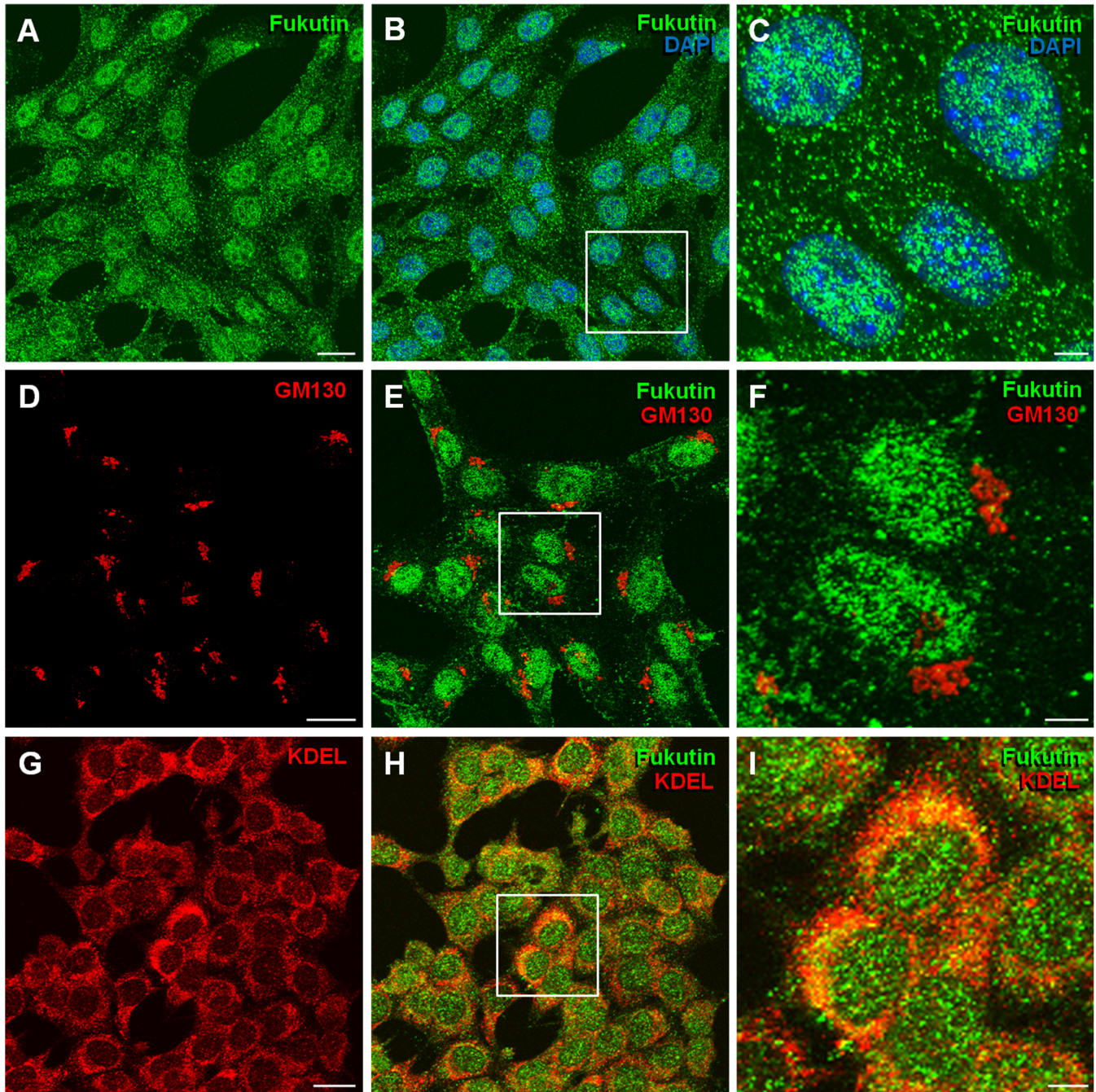


Figure 3. Immunolocalization of fukutin in the 661W photoreceptor cell line. Cells were immunostained with specific antibodies against fukutin (**A**, **B**, **E**; green), the Golgi marker GM130 (**D**, **E**; red), and the ER marker KDEL (**G**, **H**; red). Nuclei stained with DAPI are shown in blue (**B**). Enlarged views in **C**, **F** and **I** correspond to boxed areas in **B**, **E** and **H**, respectively. Fukutin immunoreactivity was found in the cytoplasm and nucleus of 661W cells, and colocalized with KDEL in the cytoplasm (**H**, **I**, yellow), but not with GM130 (**E**, **F**). Each bar equals 20 μm, except in **C**, **F** and **I**: 5 μm.

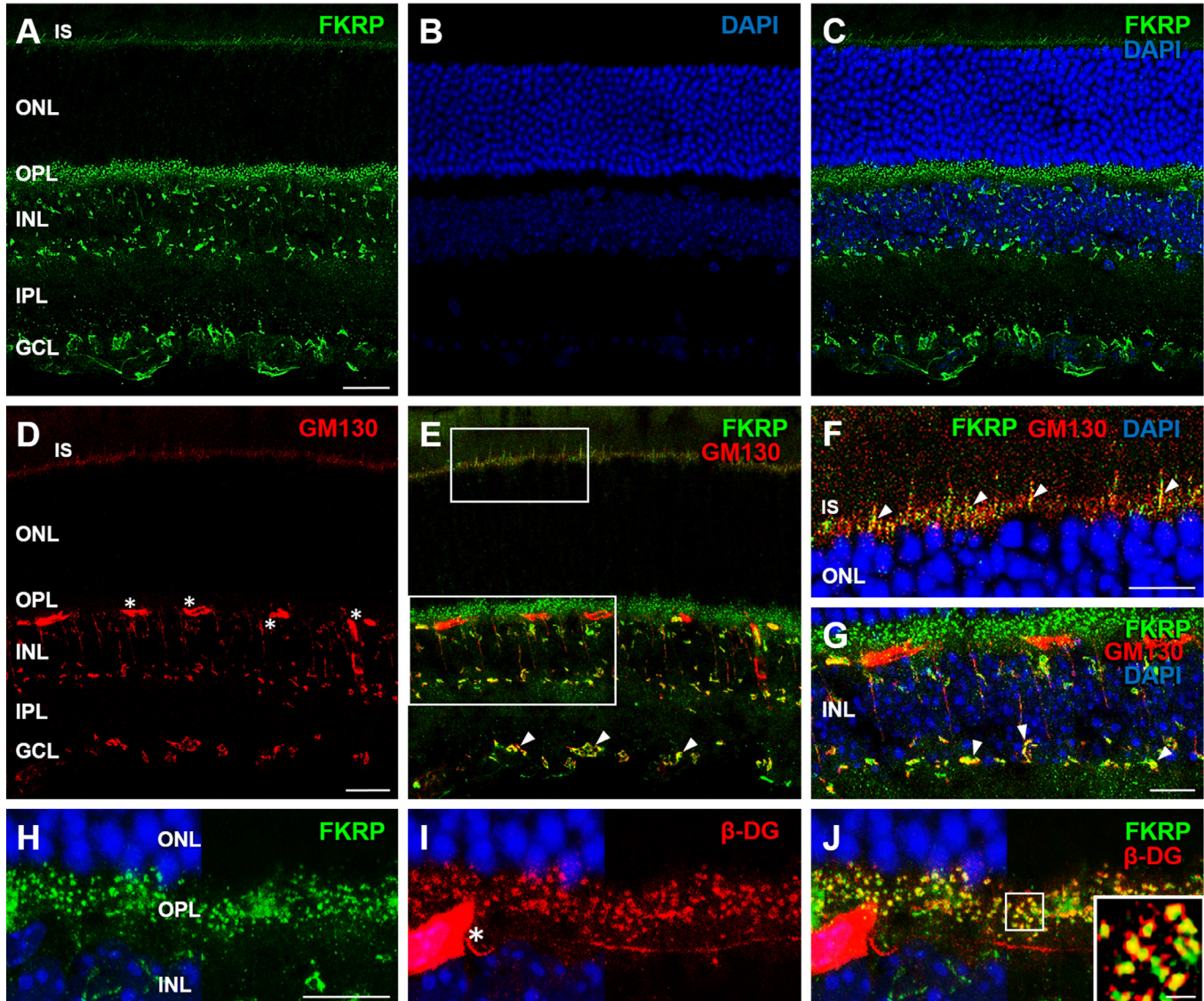


Figure 4. Immunolocalization of FKRP in the mouse retina. Double immunostainings were performed on mouse retinal sections for FKRP (A, C, E, H, J; green) and the Golgi complex marker GM130 (D, E; red) or β -dystroglycan (β -DG; I, J; red). Enlarged views in F and G correspond to boxed areas in E, and the boxed area in J is shown enlarged in the lower right inset. Nuclei stained with DAPI are shown in blue (B, C, F-J). FKRP showed immunoreactivity in the photoreceptor IS and in the OPL, INL and GCL (A, C, E). The double immunolabeling for FKRP and GM130 revealed colocalization in the photoreceptor IS, INL and GCL (E-G, arrowheads), whereas the double immunostaining for FKRP and β -DG showed colocalization in the OPL (J; yellow). Asterisks in D and I indicate non-specific immunostaining of retinal vessels by secondary anti-mouse IgG. Abbreviations are given in the legend of Figure 2. Each bar equals 20 μ m (A-E), 10 μ m (F-I) or 1 μ m (J, inset).

Thr residues [10]. We have recently located these two proteins in the ER of 661W cells (data not shown), whereas POMGNT1 was found to reside in the Golgi complex of photoreceptors in the mouse retina and 661W cell line [50].

In the present work, we evidenced expression of the mRNAs of the *FKTN* and *FKRP* genes and their protein products in the neural retina of all mammalian species studied, ranging from rodents to primates, as well as in the 661W

photoreceptor cell line (Figure 1). Expression of the *FKTN* gene mRNA has been reported in a wide variety of fetal and adult human tissues, with higher levels in the normal heart, brain, skeletal muscle and pancreas [26,27,54,60-62]. At the protein level, fukutin has been previously detected in multiple human organs, such as skeletal muscle, the brain and spinal cord in the CNS, and also in the skin, heart, liver, pancreas, kidney and uterine cervix [39,54,55,60,61]. Additionally, in

cultured cells, fukutin expression has been evidenced endogenously in human lymphoblasts and various established cell lines of different origins [55,62-64]. In this work, fukutin was detected by immunoblotting in both cytoplasmic and nuclear fractions of the adult mouse retina and of 661W cells, exhibiting an apparent molecular mass of 60 kDa. This was higher than the mass calculated from its amino acid sequence (54 kDa) or that reported in the mammalian brain and muscle and in HeLa cells [62,64], although consistent with the size reported by other authors in a wide list of human organs [54,55] and cell lines [55,63]. Such a discrepancy could be explained in terms of alternative processing of the *FKTN* gene mRNA, which has been reported to be quite an extensive phenomenon, at least in humans [27,62], and also possibly in terms of post-translational modification(s), such as glycosylation, hitherto unknown for this protein.

The *FKRP* gene has been seen expressed in several organs, with higher levels in skeletal muscle, placenta and

heart [31], and, in this work, in the mammalian retina. We also detected the FKRP protein by immunoblotting in the cytoplasmic fraction of the mouse retina, as well as in both the cytoplasm and nucleus in 661W photoreceptors, in both cases with a molecular mass of 55 kDa compatible with that calculated from its amino acid sequence. At the protein level, FKRP has been found in human skeletal and cardiac muscle [39,40,65] and in mouse liver [35], as well as endogenously in a variety of established cell lines [39,65]. It follows that despite affecting mainly the brain and muscle, fukutin and FKRP are present in many other organs, where their function has not been addressed, including the retina, as shown in this work.

For both proteins, the results we have obtained by immunoblotting have been corroborated by confocal immunofluorescence microscopy in mouse retinal sections and 661W photoreceptors. In this light, fukutin was detected in the cytoplasm of cells in all nuclear layers of the mouse retina,

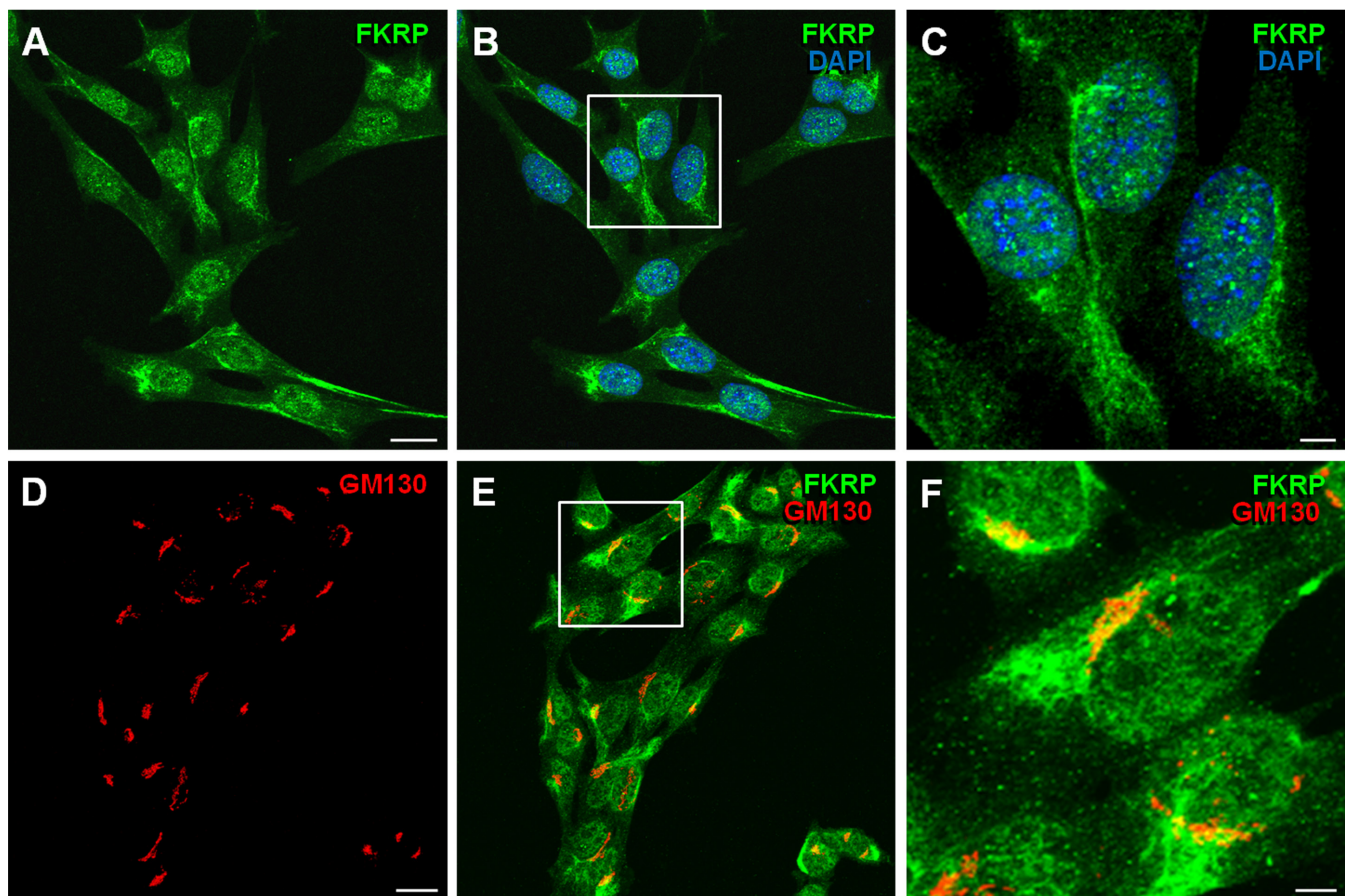


Figure 5. Immunolocalization of FKRP in the 661W photoreceptor cell line. Cells were immunostained with specific antibodies against FKRP (A, B, E; green) and the Golgi marker GM130 (D, E; red). Nuclei stained with DAPI are shown in blue (B). Enlarged views in C and F correspond to boxed areas in B and E, respectively. FKRP immunoreactivity was found in the cytoplasm and nucleus of 661W cells, and colocalized with GM130 in the cytoplasm (E, F; yellow). Each bar equals 20 μ m, except in C and F: 5 μ m,

additionally accumulating in the nucleus of particular cell types (Figure 2). However, FKRP was found to be present as well in the cytoplasm of cells in all three retinal nuclear layers, but additionally in the OPL, and was excluded from the nucleus of all retinal cells (Figure 4). Regarding 661W photoreceptors, the presence of fukutin and FKRP in both the cytoplasm and nucleus detected by immunoblotting was ascertained by immunocytochemistry in this cell line (Figure 3 and Figure 5, respectively).

We also evidenced a significant colocalization of fukutin with calreticulin in the mouse retina (Figure 2) and with KDEL in 661W cells (Figure 3), both two widely used ER markers. As well, we found colocalization of FKRP with the Golgi marker GM130 in both biological samples (Figure 4 and Figure 5). On the other hand, no colocalization was seen between fukutin and GM130, or with FKRP with calreticulin or KDEL in either the retina or 661W cells. These results allow us to conclude that fukutin and FKRP are located in retinal cells, at least in part, in the ER and Golgi, respectively, at variance with the previous notion of both proteins residing in the Golgi, as reported for human skeletal muscle [39,40] and mouse liver [35], as well as endogenously in a wide variety of established cell lines [55,65]. In this context, it must be mentioned that fukutin has been detected in the ER, rather than in the Golgi, in cultures of human astrocytoma 1321N1 and cervical carcinoma HeLa cell lines [55,56]. Hence, a scenario can be envisioned in which, in retinal neurons and 661W photoreceptors, fukutin would attach in the ER the first unit of the phosphorylated pentose alcohol, Rbo5P, to the α -DG core M3 glycan, and FKRP would subsequently act in the Golgi to link the second Rbo5P molecule. An ensuing addition to the tandem Rbo5P acceptor of the Xyl/GlcA repeat by LARGE and other enzymes in this organelle would then generate the functional receptor for α -DG ECM ligands, i.e., the matriglycan structure [10], a process that remains to be investigated in the retina. Modulation of the intracellular localization of fukutin and FKRP (i.e., ER versus Golgi) through tissue-dependent N-glycosylation has been suggested, based on their mislocalization being observed as a result of pathogenic mutations affecting particular sites of these proteins [35,62,66-68].

In the mouse retina, both proteins were also visualized in the OPL as distributing in a dotted pattern, with fukutin colocalizing with calreticulin (Figure 2) and FKRP colocalizing with β -DG (Figure 4). In this layer, they could be anchored to the presynaptic membrane of photoreceptors cells, or even secreted into the ECM of the synaptic cleft. This possibility cannot be ruled out since fukutin has been detected in the

culture medium of COS-7 cells transfected with a human *FKTN* cDNA [26]. In addition, FKRP is known to be secreted into the extracellular medium by means of exocytic vesicles formed by the Golgi in transfected CHO cells [68]. FKRP has also been reported in association with the DGC at the sarcolemma in the mouse, suggesting a new function for this protein at the skeletal muscle membrane [69].

In addition to their localization in the ER (fukutin) and Golgi complex (FKRP), we found that both fukutin and FKRP were located in the nuclear fraction of 661W photoreceptors, and fukutin as well in the nuclear fraction from the mouse retina, although at a higher abundance in the cell line (Figure 1). These results were corroborated by immunofluorescence microscopy, thus revealing the presence of both proteins in the nucleus of 661W cells (Figure 3 and Figure 5), and of fukutin also in the nucleus of several cells in the INL and GCL of the mouse retina (Figure 2). The nuclear concentration of these proteins in 661W photoreceptors could be attributable to their absence of normal cell polarity, which is derived from their lack of outer segments and interaction with RPE cells. This loss of polarity has been posited to cause misplacement of some proteins with respect to their normal location in the retina, as is the case for red/green and blue cone opsins [48] and the ceramide kinase-like protein, CERKL [70]. In addition, unlike photoreceptors in the retina, 661W is an immortalized cell line with an unlimited capacity for cell division [47]. Yet, it must be emphasized that this is not the first time that DGP-associated proteins have been detected in the nucleus of given cell types or established cell lines. Indeed, we have recently reported the accumulation of POMGNT1 in the nucleus of 661W cells, where it colocalized with POMT1 and POMT2, and these two proteins in turn with euchromatin areas [50]. Fukutin has also been found to be present in both the cytoplasm and the nucleus of human 1321N1 and HeLa cell lines [55,56], and of most neurons of the human adult brain, both excitatory and inhibitory, including the cerebral cortex and cerebellum, where it coexists with glycosylated α -DG [61]. Though highly speculative, the possibility thus exists that α -DG or other nuclear proteins could act as a substrate for both fukutin and FKRP, as well as for other DGP-associated glycosyltransferases, as we have recently proposed [50]. Yet, it cannot be ruled out that fukutin and FKRP could play in the nucleus roles that still need to be unraveled, which may or not be related to α -DG glycosylation, such as the modulation of cell proliferation, as suggested by other authors for fukutin [55,56,61] and by our group for POMGNT1 [50]. This is an issue that merits further investigation.

APPENDIX 1. STR ANALYSIS

STR analysis of the 661W cell line. To access the data, click or select the words “[Appendix 1](#)”

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