Published in final edited form as:

Vis Neurosci. 2008 ; 25(4): 563–574. doi:10.1017/S0952523808080723.

Membrane Frizzled Related Protein is necessary for the normal development and maintenance of photoreceptor outer segments

Jungyeon Won¹, Richard S. Smith¹, Neal S. Peachey^{2,3}, Jiang Wu³, Wanda L. Hicks¹, Jürgen K. Naggert¹, and Patsy M. Nishina^{1,*}

¹The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609

²Research Service, Cleveland VA Medical Center, Cleveland, OH 44106

³Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

Abstract

A 4 base pair deletion in a splice donor site of the *Mfrp* (membrane-type frizzled-related protein) gene, herein referred to as *Mfrp*^{rd6/rd6}, is predicted to lead to the skipping of exon 4 and photoreceptor degeneration in *retinal degeneration 6* (*rd6*) mutant mice. Little, however, is known about the function of the protein or how the mutation causes the degenerative retinal phenotype. Here we examine ultrastructural changes in the retina of *Mfrp*^{rd6/rd6} mice to determine the earliest effects of the mutation. We also extend the reported observations of the expression pattern of the dicistronic *Mfrp/C1qtnf5* message and the localization of these and other retinal pigment epithelium (RPE) and retinal proteins during development and assess the ability of RPE cells to phagocytize outer segments in mutant and WT mice. At the ultrastructural level, outer segments do not develop normally in *Mfrp*^{rd6/rd6} mutants. They are disorganized and become progressively shorter as mutant mice age. Additionally, there are focal areas in which there is a reduction of apical RPE microvilli. At P25, the rod ERG a-wave of *Mfrp*^{rd6/rd6} mice is reduced in amplitude by ~50% as are ERG components generated by the RPE. Examination of β -catenin localization and *Fos* and *Tcf-1* expression, intermediates of the canonical Wnt-pathway, showed they were not different between mutant and WT mice, suggesting that MFRP may operate through an alternative pathway. Finally, impaired outer segment phagocytosis was observed in *Mfrp*^{rd6/rd6} mice both in standard ambient lighting conditions and with bright light exposure when compared to WT controls.

Keywords

Mfrp; *C1qtnf5*; Retinal Pigment Epithelium; Phagocytosis; Outer Segments

INTRODUCTION

Mouse models with spontaneous forms of photoreceptor degeneration have served as tools for providing insights into disease progression, molecular function, and pathology. While a model organism may not capture the entire phenotype observed in humans, studies of these mutants are invaluable for providing information about the function of the molecules involved or the pathway that is impacted by the mutant allele.

Copyright 2008 Cambridge University Press

*Correspondence to: Patsy M. Nishina, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, Tel.: +1 207 288 6383, Fax: +1 207 288 6077. E-mail address: patsy.nishina@jax.org (P. M. Nishina).

The final published version may be found at: <http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=2176776>

The *retinal degeneration 6 (rd6)* mouse is a model of photoreceptor degeneration, which was first detected by indirect ophthalmoscopy. In this model, white spots are observable in the fundus as early as 8 weeks of age (Hawes *et al.*, 2000). Homozygous B6.C3Ga-*rd6/rd6* mice show a slow, progressive photoreceptor degeneration. By 29 months of age, all photoreceptor cell bodies are degenerated (Hawes *et al.*, 2000). Histological examinations reveal numerous aberrant cells in the subretinal space that are filled with membranous material. These aberrant cells are stained by a macrophage specific marker, MOMA-2, and were suggested to correspond to the white spots observed upon fundus examination (Hawes *et al.*, 2000). The disease-causing mutation, which maps to chromosome 9, was identified as a 4 bp deletion in a splice donor site in the *Mfrp* gene (Kameya *et al.*, 2002) that leads to the skipping of exon 4.

The deduced amino acid sequence of MFRP indicates a transmembrane domain with high homology to the frizzled family of proteins containing two cubilin domains (CUB), a low-density lipoprotein receptor a (LDLRa) domain, and a cysteine-rich domain (CRD). *Mfrp* is hypothesized to express as a dicistronic transcript with *C1qtnf5* (Kameya *et al.*, 2002). C1QTNF5 is a small secreted protein containing a complement C1q domain. The dicistronic message is expressed in retinal pigment epithelium (RPE) cells and in the ciliary epithelium, as well as the lens (Mandal *et al.*, 2006a, b). Recent studies have shown that MFRP and C1QTNF5 co-localize in the RPE and ciliary bodies, and that they are likely to directly interact (Mandal *et al.*, 2006a, b; Shu *et al.*, 2006).

Human patients that carry a Ser163Arg mutation in the *C1QTNF5* gene develop late-onset retinal degeneration (L-ORD), a retinal disorder that includes the development of drusen-like deposits (Hayward *et al.*, 2003). In another patient cohort, carrying the same *C1QTNF5*^{Ser163Arg} mutation, early onset long anterior lens zonules was also reported (Ayyagari *et al.*, 2005; Subrayan *et al.*, 2005). In a recent study, patients with familial retinitis pigmentosa were shown to carry a base pair insertion in exon 5 in the human *MFRP* gene, which is predicted to lead to an early truncation of the protein if translated (Ayala-Ramirez *et al.*, 2006). Posterior microphthalmos, foveoschisis, and optic nerve drusen were also observed in these patients. Additionally, several truncating alleles of human *MFRP* were found in nanophthalmic patients (Sundin *et al.*, 2005). Nanophthalmos, a rare disorder characterized by a thickened choroid and sclera, results in severe hyperopia. Interestingly, photoreceptor degeneration was not observed in these patients although it is the primary pathology observed in human retinitis pigmentosa and in *Mfrp*^{rd6/rd6} mutant mice.

In order to further understand the pathological consequences of the *Mfrp*^{rd6} allele, we have compared the spatial and temporal expression of MFRP and C1QTNF5 in control and *Mfrp*^{rd6/rd6} mutant mice. We also examined the early pathological changes in the *Mfrp*^{rd6/rd6} mouse model and used a number of photoreceptor and RPE markers to determine if particular pathways were affected by the *Mfrp*^{rd6} mutation during retinal development. Our results suggest that the loss of exon 4, which includes a portion of the transmembrane domain, results in the loss of MFRP and the loss of MFRP leads to an impairment of phagocytosis in *Mfrp*^{rd6/rd6} mutant mice. Finally, we noted that while both MFRP and C1QTNF5 localize to the RPE during normal early retinal development, C1QTNF5, which contains a signal peptide, appears to migrate in the interphotoreceptor matrix to the external limiting membrane (ELM) and outer nuclear layer (ONL).

METHODS

Mouse husbandry

Mice were bred and housed in the Research Animal Facility at The Jackson Laboratory. Mice were provided NIH31 4% fat diet and acidified water in a room with a 12 h light/ 12 h dark cycle. C57BL/6J and B6.C3Ga-*Mfrp*^{rd6/J} mice were used in this study.

Histological and immunohistochemical analysis

The protocols for histological and immunohistochemical assays were previously described (Lee *et al.*, 2005). Briefly, mice were killed by carbon dioxide asphyxiation. The harvested eyes were placed in cold acetic acid/ methanol solution overnight followed by paraffin embedding using standard procedures. Tissues were cut into 6 μ m sections, stained by hematoxylin and eosin (H & E) and examined by light microscopy. Deparaffinized sections were incubated with anti-hMFRP (R&D), anti-C1QTNF5 (rabbit polyclonal antibody raised against the peptide sequence SFFQYFGGWPKPASL), anti-rhodopsin (Leico Technologies), anti-blue opsin (Chemicon), anti-red/green opsin (Chemicon), anti- β -catenin (Santa Cruz Biotechnology), and anti-ezrin (Santa Cruz Biotechnology). Specific proteins were fluorescently labeled by cy3-conjugated secondary antibodies (Jax Immunoresearch) and visualized by fluorescence microscopy.

The phagocytosis assay was performed 1 h after light onset. For the light-stimulated phagocytosis experiments, the eyes of wild type and *Mfrp*^{rd6/rd6} animals were dilated with 1% atropine ophthalmic drops. The dilated eyes were exposed to 12,000 lux of white light in a reflective chamber for 15 min. After 1 h, tissues were harvested. To eliminate the autofluorescence of the pigment in the RPE, chemical bleaching was performed in deparaffinized sections with solutions containing 5% formamide and 5% hydrogen peroxide in 1 $\times$ SSC. For each treatment condition, rhodopsin positive phagosomes were counted in 15 separate fields from each of 3 different animals.

Electron Microscopy

The eyes for ultrastructural analyses were fixed in an ice cold phosphate buffered glutaraldehyde/paraformaldehyde solution for 3 h. The anterior segment was removed and the posterior segment cut into 1 $\times$ 2 mm blocks. Additional fixation with fresh fixative was performed for 2–8 h followed by post-fixation with 1% osmium tetroxide. The dehydrated blocks were embedded in plastic. Tissue sections were cut and stained by uranyl acetate and lead citrate and examined under a transmission electron microscope.

Electroretinography

After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), the corneas were anesthetized (1% proparacaine HCl), and the pupils were dilated (1% mydriacyl, 2.5% phenylephrine HCl, 1% cyclopentolate). Mice were placed on a temperature-regulated heating pad throughout each recording session. *Mfrp*^{rd6/rd6} mice and control littermates (*Mfrp*^{rd6/+} or *Mfrp*^{+/+}) were tested at P25.

To measure ERG components generated by the outer neural retina, responses were recorded from the corneal surface using a stainless steel electrode that was wetted with 0.7% methylcellulose. Needle electrodes placed in the cheek and the tail served as reference and ground leads, respectively. Responses were differentially amplified (0.3–1500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). Responses were first obtained to stimuli presented in the dark. Flash stimuli were presented in an LKC ganzfeld and ranged in intensity from -3.6 to $2.1 \log \text{cd s/m}^2$; interstimulus intervals increased from 4 s at the lowest flash intensity to 61 s at the highest stimulus levels. Stimuli were presented in order of increasing intensity and at least 2 successive responses were averaged for each stimulus condition. A steady rod-desensitizing adapting field was then presented within the ganzfeld bowl. After allowing a 7 min period of light adaptation (Peachey *et al.*, 1993), cone ERGs were recorded to flashes superimposed upon the adapting field. Flash intensity ranged from -0.8 to $1.9 \log \text{cd s/m}^2$ and responses to 50 flashes presented at 2.1 Hz were averaged at each intensity level.

The amplitude of the a-wave was measured 8 ms after flash onset from the pre-stimulus baseline. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave or, if no a-wave was present, from the pre-stimulus baseline. Implicit times were measured from the time of flash onset to the a-wave trough or the b-wave peak.

To measure ERG components generated by the RPE, responses were recorded from the corneal surface using 1-mm-diam glass capillary tubes with filament (BF100-50-10, Sutter Instruments; Novato, CA), that were filled with HBSS to make contact with a Ag/AgCl wire electrode with an attached connector. Both electrodes were shielded in a black tube and responses were differentially amplified (DP-301, Warner Instruments, Hamden, CT; dc-100 Hz; gain = 1000 x) digitized at 20 Hz and stored using LabScribe Data Recording Software (iWorx, Dover, NH).

White light stimuli were derived from an optical channel using a Leica microscope illuminator as the light source and delivered to the left eye with a 1-cm diameter fiber optic bundle; the right eye was shielded from light stimulation with a baffle constructed from black electrical tape. Mice were tested once using a single stimulus conditions that elicits a maximal response (Wu *et al.*, 2004a, b). Stimulus intensity was controlled at 4.4 log cd/m² (corresponding to 4.8 log photoisomerizations per rod/s; (Wu *et al.*, 2004a) using a neutral density filter (Oriol Instruments, Stratford, CT). Stimulus duration was controlled at 7 min using a Uniblitz shutter system.

The amplitude of the c-wave was measured from the pre-stimulus baseline to the peak of the c-wave. The amplitude of the fast oscillation (FO) was measured from the c-wave peak to the trough of the FO. The amplitude of the light peak (LP) was measured from the FO trough to the asymptotic value. The amplitude of the off-response was measured from the tail of the response to the peak of the off-response, which could be either negative or positive in polarity, depending on flash intensity (Wu *et al.*, 2004a, b).

Real time PCR

The protocol for real time PCR with Taqman (Applied Biosystems) was described previously (Collin *et al.*, 2005). Eyes were collected and placed in two separate pools (each pool consisted of n=4 to 23 eyes). Total RNA from eyes was isolated using the Trizol method (GIBCO). After removal of DNA by DNase I (Ambion) digestion, each pool was divided into three aliquots and cDNA was generated by RT-PCR (Ambion). Primers for probing the *Mfrp* coding region (spanning exon 8 and 9) and *C1qtnf5* (spanning exon 13 and 14) were manufactured by Applied Biosystems. Each pool of cDNA was tested in triplicate. The absolute quantification was performed with ABI7900HT system (Applied Biosystems).

Specific primers were used for relative quantification of canonical Wnt target gene expression; Fos-F: 5' – ATGCGAAGGGAACGGAATAA -3', Fos-R: 5' - TGCAACGCAGACTTCTCATC -3'; Tcf-1-F: 5' - CCGTGGTGGAGTCACTTCTT -3'. Tcf-1-R: 5' - GATGTTGTGCTGCTGCAAGT -3'. The expression of hypoxanthine phosphorybosyl-transferase (HPRT) was used to normalize the assay results (HPRT-F: 5' - GCTCGAGATGTCATGAAGGAGA-3', HPRT-R: 5' – AACTTTTATGTCCCCGTTGACT-3'). The assay was performed using the SYBR Green PCR system (Applied Biosystems) and the quantification was performed using the ABI7900HT SDS system using the same RNA sample described in the absolute quantification above.

Immunoprecipitation (IP) and Western analysis

Total protein from wild type and *Mfrp*^{rd6/rd6} eyes was manipulated by standard methods in RIPA buffer (1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate in PBS) or NP-40 buffer (1% NP-40, 2 mM EDTA, 200 μ M sodium vanadate, and 50 mM NaF in PBS) containing a protease inhibitor cocktail (Roche). *Mfrp*^{rd6} cDNA was kindly provided by Dr. Shu (Shu *et al.*, 2006) and subcloned into pCMV-3Tag-2A (Stratagene) to generate a N-terminal c-myc fusion protein (Myc-MFRP^{rd6}), which was expressed in COS-7 cells after transfection with FuGENE6 reagent (Roche). 100 μ g of total protein was separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was stained with anti-hMFRP (goat polyclonal raised against a fragment of the human MFRP, R&D), anti-mMFRP (rabbit polyclonal antibody raised against peptide sequence NRLPVAASLEACSQP), anti-C1QTNF5, anti-c-Myc (Zymed), anti-integrin α 5 (R&D) and β 5 (Abcam), and anti-Mer (R&D). Anti-actinin 4 (Alexis) and anti- β -actin (Sigma) were used for loading controls. Phospho-MERTK was detected by immunoprecipitation with anti-Mer antibody followed by immunostaining with anti-phospho Tyrosine (pY: Upstate). All bands were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies followed by the ECL detection system (Perkin-Elmer). Band intensity of phospho-MERTK, measured using ImageJ software, was normalized to the intensity of MERTK bands obtained by restaining the same membrane with anti-Mer.

RESULTS

Mfrp and *C1qtnf5* expression is reduced and temporal expression delayed in *Mfrp*^{rd6/rd6} mice relative to age-matched controls

Mfrp expression was assessed by real time PCR of RNA extracts of whole eyes from control (C57BL/6J) and *Mfrp*^{rd6/rd6} mutants ranging in ages from e14.5 to P28. Similar to previous reports of control mice (Mandal *et al.*, 2006b), we find that *Mfrp* expression is relatively low during embryogenesis but increases markedly after birth (Fig. 1A), with highest levels observed at P14. In *Mfrp*^{rd6/rd6} mice, *Mfrp* expression progressively increased but was lower than controls between e14.5 and P14. It eventually peaked near P28, later than observed for WT control mice. In both control and *Mfrp*^{rd6/rd6} samples, the time course of *C1qtnf5* expression (Fig. 1B) was similar to that of *Mfrp*.

Absence of MFRP but maintenance of C1QTNF5 cellular localization in *Mfrp*^{rd6/rd6} mutant mice relative to control

To determine the localization of mouse MFRP, immunohistochemistry was performed on sections of whole eyes of age-matched *Mfrp*^{rd6/rd6} and control mice using two antibodies (Fig. 2A–H): anti-mMFRP -against 15 residues of the mouse MFRP C-terminus, and anti-hMFRP -against recombinant human MFRP. Both antibodies gave similar results. As reported previously (Mandal *et al.*, 2006b), in control mice, MFRP is expressed in the epithelium of the ciliary body (data not shown) and RPE cells (anti-hMFRP: Fig. 2A, C, E, G, anti-mMFRP data not shown). However, unlike previous reports, staining was only observed in the apical RPE membranes (Fig. 2L). Both antibodies failed to detect MFRP in eyes from *Mfrp*^{rd6/rd6} mutant mice (anti-hMFRP: Fig. 2B, D, F, H, anti-mMFRP data not shown).

In order to determine if MFRP was simply mislocalized or absent from the eyes from *Mfrp*^{rd6/rd6} mutant mice, we performed Western blot analyses of whole eye lysates from control and mutants. A single ~100 kDa band was detected with both anti-MFRP antibodies in control mice. However, the band was not detectable in whole eye lysates from 2-month-old *Mfrp*^{rd6/rd6} mutant mice (Fig. 2I, J). The molecular weight of this band was larger than the predicted size of 65 kDa, suggesting that post-translational modification(s) of the MFRP protein occur. Lower molecular weight bands were also noted in both control and

Mfrp^{rd6/rd6} retinas with anti-mMFRP (Fig. 2I), but were not detectable with anti-hMFRP (Fig. 2J), suggesting that they might reflect nonspecific recognition by the anti-mMFRP antibody. To ascertain that both MFRP antibodies could indeed detect mutant MFRP, Western analysis was performed against mutant protein overexpressed in COS-7 cells. Since both antibodies were raised against the C-terminal portion of MFRP, a construct of the mutant gene with a N-terminal Myc-tag was made. Myc-MFRP^{rd6} was detected with both MFRP antibodies as well as c-Myc antibody, confirming the absence of MFRP in *Mfrp*^{rd6/rd6} animals (Fig. 2K).

In control mice, C1QTNF5 was expressed in the ciliary body (data not shown) and both the apical and basal membranes of RPE cells (Fig. 3A, C, E, G). As reported previously (Mandal *et al.*, 2006a), staining of the apical RPE membrane was stronger than at the basolateral membrane. Interestingly between P7 and P14, C1QTNF5 staining appears to decline in the RPE while it appears in both the ONL and the ELM (Fig. 3A, C). C1QTNF5, which contains a signal peptide, has been shown by *in vitro* methods to be secreted from COS-7 cells (Mandal *et al.*, 2006a). Our results suggest that while C1QTNF5 is synthesized in the RPE during retinal development, it is secreted from the RPE cells and appears to migrate into the interphotoreceptor retinal space to the region of the external limiting membrane. The absence of functional MFRP from the RPE, however, did not appear to affect the localization or secretion of C1QTNF5 (Fig. 3A–H). We performed Western blot analysis on control and mutant mice whole eye lysates to determine C1QTNF5 expression levels (Fig. 3I). Comparable bands near ~20 and ~40 kDa were detected in both control and *Mfrp*^{rd6/rd6} mice, indicating that the *Mfrp*^{rd6} mutation affects neither C1QTNF5 expression level nor dimer formation.

Morphological changes in the RPE and photoreceptor occur prior to the loss of photoreceptor cell bodies in *Mfrp*^{rd6/rd6} mutant mice

Previous reports have shown that the photoreceptor degeneration in *Mfrp*^{rd6/rd6} mice is slowly progressive (Hawes *et al.*, 2000). In order to determine the earliest pathological lesions observable in *Mfrp*^{rd6/rd6} mice, histological analyses were carried out on P7 through P28 eyes (Fig. 4). At P7 (Fig. 4A, B), just prior to photoreceptor outer segment development, the *Mfrp*^{rd6/rd6} retina appeared similar to age-matched control. By P14 (Fig. 4C, D), however, the outer segments (OSs) of *Mfrp*^{rd6/rd6} mice were shorter than those of in control animals. However, there were no apparent differences in the number of photoreceptor cell bodies contained in the ONL of control and *Mfrp*^{rd6/rd6} mice. By P21 (Fig. 4E, F), disorganization of OSs was observed in mutant mice, whereas the thickness of ONL is comparable to WT controls. By P28 the thickness of the ONL is reduced to 7–8 nuclei with observable OS thinning (Fig. 4G, H). Cells that were previously shown to be MOMA-2 positive (Hawes *et al.*, 2000) were observed at later time points when photoreceptor degeneration was observed (Fig. 4J).

In order to gain better insight of changes that occur in cellular morphology as a result of the mutation, ultrastructural analysis was performed by electron microscopy on control and *Mfrp*^{rd6/rd6} retinas at P2, P7, P14, and P21 (Fig. 5). At P2, outer retinal development is similar in control and *Mfrp*^{rd6/rd6} mice (Fig. 5A, B). At P7, when early OS formation is absent and RPE microvilli are more easily discernable, RPE microvilli were abundant in control retinas. In *Mfrp*^{rd6/rd6} retinas, however, there were focal areas in which microvilli were disorganized and reduced in number, while neighboring areas appeared normal (Fig. 5C, D). By P14 (Fig. 5E, F), the OSs of *Mfrp*^{rd6/rd6} mice were poorly oriented with ill-defined lamellae. At P21, control mice show mature OSs and an RPE with prominent microvilli. This is in contrast with the abnormal OSs and microvilli observed in *Mfrp*^{rd6/rd6} mice (Fig. 5G, H).

Functional deficits in rods accompany the morphological changes in *Mfrp*^{rd6/rd6} mutant mice

Fig. 6A presents responses obtained from a P25 control mouse (left) and an *Mfrp*^{rd6/rd6} littermate (right) in response to strobe flashes presented to the dark-adapted eye. In comparison

to control responses, ERGs of *Mfrp^{rd6/rd6}* mice have a relatively normal waveform, but were reduced in overall amplitude. Fig. 6B presents average ($\pm$ s.e.m.) intensity-response functions for the a- and b-waves of the dark-adapted ERG. Across flash intensity, a- and b-waves of *Mfrp^{rd6/rd6}* mice were reduced to $42.3 \pm 4.3\%$ and $62.9 \pm 7.9\%$ (mean $\pm$ s.d.) of control, respectively. The reduction was fairly consistent across stimulus intensity, and the positions of the a- and b-wave intensity-response functions along the intensity axis were comparable for *Mfrp^{rd6/rd6}* and control mice.

Fig. 6C presents ERGs obtained from a control mouse (left) and a *Mfrp^{rd6/rd6}* littermate (right) in response to strobe flashes superimposed on a rod-desensitizing adapting field. In comparison to control responses, cone ERGs of *Mfrp^{rd6/rd6}* mice had a relatively normal waveform, but were slightly reduced in overall amplitude. Fig. 6D presents average ($\pm$ s.e.m.) intensity-response functions for the cone ERG. The position of the *Mfrp^{rd6/rd6}* and control cone ERG intensity-response functions was comparable, but there was a consistent reduction in the amplitude of *Mfrp^{rd6/rd6}* cone ERGs. Across flash intensity, cone ERGs of *Mfrp^{rd6/rd6}* mice were reduced to $73.7 \pm 5.4\%$ (mean $\pm$ s.d.) of control. Fig. 6E plots the relative amplitude of the cone ERG against that of the rod ERG for each *Mfrp^{rd6/rd6}* mouse tested. In this plot, all data are expressed relative to the respective control mean, and each data point represents an individual *Mfrp^{rd6/rd6}* mouse. The dashed diagonal line illustrates the relationship expected if rod and cone responses are affected equally. All of the data points fall above this line, consistent with the impression that cone function is relatively spared in *Mfrp^{rd6/rd6}* mice at P25.

In order to further investigate the apparent sparing of the cone function relative to rods, retinas from P25 *Mfrp^{rd6/rd6}* were stained with anti-rhodopsin, anti-blue cone opsin and anti-green/red cone opsin (Fig. 7). Punctate staining of rhodopsin was observed in eyes from P25 mutant mice (Fig. 7A, B), in which severe OS and ONL degeneration was observable by light microscopy (Fig. 7G, H). In comparison, both blue-opsin and green/red-opsin staining of *Mfrp^{rd6/rd6}* retinas were similar to age-matched controls (Fig. 7C–F), consistent with the results indicating relative sparing of the cone ERGs.

The aberrant dc-ERGs observed in *Mfrp^{rd6/rd6}* mice are attributable to rod photoreceptor loss and not to a functional change in the RPE

Fig. 8A compares average dc-ERGs obtained from *Mfrp^{rd6/rd6}* and control mice in response to a 7 min duration stimulus. All major response components were present in *Mfrp^{rd6/rd6}* mice. In comparison to control, the overall response was smaller in *Mfrp^{rd6/rd6}* mice. Fig. 8B presents average measures for each of the major response components that comprise the dc-ERG. In comparison to control, *Mfrp^{rd6/rd6}* mice had significant reductions in the amplitudes of the c-wave, FO and LP, but there was no difference for the off-response component.

These changes in the dc-ERG could reflect an abnormality in RPE function associated with the *Mfrp^{rd6}* mutation, but could also be secondary to the reduction in rod photoreceptor activity. To distinguish these, we also examined a mouse model of photoreceptor degeneration due to a photoreceptor-specific gene defect. The *Prph2^{rd6}* mutation involves a null mutation in peripherin 2 (*prph2*) (Travis *et al.*, 1989), a structural component of rod and cone outer segments (Molday, 1994), which is not expressed in the RPE (Farjo *et al.*, 2006). We examined *Prph2^{rd6/+}* heterozygotes, which develop a gradual loss of rod photoreceptors (Cheng *et al.*, 1997), and recorded dc-ERGs at an age where the amplitude of the a-wave was reduced to $56.4 \pm 7\%$ of control, a level similar to that seen in P25 *Mfrp^{rd6/rd6}* mice. Each dc-ERG component affected in *Mfrp^{rd6/rd6}* mice was also reduced in *Prph2^{rd6/+}* animals. Taking into account the a-wave reduction, the magnitude of the reduction of the c-wave, FO and LP was comparable in *Mfrp^{rd6/rd6}* and *Prph2^{rd6/+}* mice. While it is possible that RPE function is compromised in the *Mfrp^{rd6/rd6}* RPE and that the *Prph2^{rd6/+}* RPE develops a comparable defect, a decrease in

the magnitude of the rod photoresponse may represent a more parsimonious explanation for the dc-ERG reductions observed in *Mfrp*^{rd6/rd6} mice.

MFRP does not appear to function via the canonical Wnt/frizzled pathway in the early pathology of *Mfrp*^{rd6} mutant

In order to further examine the pathways that might be impaired as a result of the *Mfrp*^{rd6} mutation, a number of biomarkers were tested by immunohistochemistry on sections obtained from *Mfrp*^{rd6/rd6} and control mice. Because MFRP has a conserved CRD domain observed in frizzled proteins that act in the Wnt signaling pathway, we examined the effect of the mutation on β -catenin by immunohistochemistry, and on *Fos* and *Tcf-1* expression by real time PCR. As can be seen in Fig. 9A, β -catenin is normally localized to the basolateral membrane of the RPE and to the ELM. No difference in staining was observed in either location for β -catenin in *Mfrp*^{rd6/rd6} mice (Fig. 9A, B). Another marker, ZO-1 that also localizes to the basolateral membrane of RPE and is thought to act in an RPE junctional complex with β -catenin (Russ et al., 1998) was examined (Fig. 9C, D). No evidence of mislocalization was observed. Finally, expression levels of *Fos* and *Tcf-1* were similar between mutant and WT mice (Fig. 9E). Taken together with our previous report that showed no differences in the Wnt and frizzled biomarkers examined (Kameya et al., 2002), this suggests that MFRP does not function through the canonical Wnt/frizzled pathway at the ages examined.

OS phagocytosis is reduced in *Mfrp*^{rd6/rd6} mutants compare to control mice

The appearance of macrophages in the subretinal space in *Mfrp*^{rd6/rd6} mice may indicate a defect in clearance of cellular debris. Clearance of photoreceptor OSs by phagocytosis peaks approximately 1 h after light onset (Besharse et al., 1977; Nandrot et al., 2004). In order to determine if OS uptake is impaired in *Mfrp*^{rd6/rd6} mice, we examined rhodopsin staining as a marker for ingested OS in RPE cells 1 h after the onset of light and under light-stimulated conditions. One hour after the onset of light, rhodopsin staining in RPE cells was reduced in *Mfrp*^{rd6/rd6} eyes (Fig. 10A, B). This difference was more pronounced in light-stimulated conditions (Fig. 10C, D). While an increase in rhodopsin staining was observed in RPE cells of control mice after 15 min of 12,000 lux light exposure, rhodopsin staining of RPE cells did not increase after light-stimulation in *Mfrp*^{rd6/rd6} animals (Fig. 10E). The levels of several proteins related to phagocytosis were examined by Western analysis of WT control and *Mfrp*^{rd6/rd6} retinal lysates. No differences in the levels of integrin- α 5 and β 5, and MERTK were observed. However, the level of phosphorylated MERTK was reduced by 38% of control in *Mfrp*^{rd6/rd6} retina (Fig. 10F). This further substantiates the observed impairment of the phagocytic process in *Mfrp*^{rd6/rd6} animals.

DISCUSSION

Our studies demonstrate that the *Mfrp*^{rd6} mutation is ostensibly a null mutation of MFRP and provide evidence for a role for MFRP in normal development of photoreceptor OSs and in RPE phagocytosis. It does not appear, however, given the conditions and ages examined, that MFRP functions via the canonical Wnt signaling pathway, despite the similarity to the conserved CRD domain observed in frizzled-related proteins.

In *Mfrp*^{rd6/rd6} mice, mutant MFRP was not detected in immunohistochemical studies or Western analysis with two independent anti-MFRP polyclonal antibodies. The *Mfrp*^{rd6} mutation is predicted to cause a skipping of exon 4 that leads to an in-frame deletion of 58 amino acids. Shu et al. (2006) demonstrated that recombinant mutant MFRP had a greater tendency to aggregate, suggesting that the deletion within MFRP may cause a misfolding or conformational change in MFRP that leads to its aggregation and subsequent rapid removal. Both anti-MFRP antibodies stained a band in WT mice at about 100 kD by Western blot

analysis, which is far larger than the predicted molecular weight of ~65 kD. This observation suggests that MFRP is modified post-translationally.

With respect to the cellular localization of MFRP and C1QTNF5, the most remarkable finding of our study is that C1QTNF5 is also found in the ONL and ELM, most probably in the interphotoreceptor space, of adult mice. These observations suggest that C1QTNF5, which contains a secretion signal peptide, is synthesized in the RPE and ciliary body and released into the interphotoreceptor space. Interestingly, the localization of C1QTNF5 does not seem to be affected by the *Mfrp*^{rd6} mutation despite their apparent direct interaction (Mandal *et al.*, 2006b; Shu *et al.*, 2006).

The importance of the RPE to the health of the overlying retina is well documented (Strauss, 2005). Examples of retinal pathology that occur as a result of mutant alleles of RPE specific molecules such as RPE65, RLBP1, and RGR have been reported in the literature (Strauss, 2005). In many of these situations, although the RPE cells appear morphologically intact, they are functionally impaired. In this study, we examined the early pathologic effects of the *Mfrp*^{rd6} mutation. Morphologically, the apical processes of the RPE in *Mfrp*^{rd6/rd6} mutants appeared to be affected focally, with some areas having fewer and shortened microvilli. These morphological defects in the RPE cells that may lead to OS disorganization appear to be the primary events that lead to the progressive photoreceptor OS degeneration, resulting in a significant loss of photoreceptor cell bodies by P21. It is interesting to note that a RPE phenotype is observed in *Vil2*^{tm1Aim} mice (Bonilha *et al.*, 2006) and that Villin 2 (also called Ezrin), that normally localizes to the apical border of the RPE, is important in the development and/or maintenance of the apical processes. The apical membrane of the RPE is composed of numerous processes, which extend and interdigitate with the photoreceptor OS providing retinal adhesion and physical support. This contact between the RPE and photoreceptor OS is important for transfer of material to and from the neural retina, including the removal of shed OS tips. Previous observations (Hawes *et al.*, 2000) have shown that *Mfrp*^{rd6/rd6} mice have large cells in their subretinal space that are filled with membranous profiles, lipofuscin-like material and pigment granules. These cells, positive for mouse macrophage-associated antigen, an intracellular marker of macrophages and monocytes, were observed in *Mfrp*^{rd6/rd6} mice as young as P21 (Hawes *et al.*, 2000). These types of phagocytic cells are not unique to *Mfrp*^{rd6/rd6} mice and are often observed in models of retinal degeneration in which cellular debris accumulates, such as the *mer1k*^{tm1GKM} mouse (Duncan *et al.*, 2003). The abnormalities in the RPE apical processes may underlie the reduced phagocytosis by RPE cells observed in *Mfrp*^{rd6/rd6} mice. Alternatively, the loss of MFRP could lead to a functional defect in the phagocytic pathway. Although we do not know whether the observed reduction in the phosphorylated form of MERTK in *Mfrp*^{rd6/rd6} mice is a primary or secondary effect of the MFRP deficiency, a reduction in activated MERTK could potentially contribute to the observed diminished phagocytic capacity of the RPE cells (Duncan *et al.*, 2003).

Predictably, ERG studies at P25 indicate that rod photoreceptor function is impaired in *Mfrp*^{rd6/rd6} mice (Fig. 6). Interestingly, however, cone ERGs were relatively preserved at P25 and immunohistochemical studies confirmed the relative preservation of cones in *Mfrp*^{rd6/rd6} mice. In contrast, in *Vil2*^{tm1Aim} mice, cone photoreceptors were more severely affected than rods (Bonilha *et al.*, 2006). The cause of the difference in the rate of degeneration of rods and cones types is not apparent as both are normally ensheathed by RPE microvilli and are dependent upon a functional RPE for removal of shed OSs. Closer examination of the interaction of RPE microvilli with rod and cone OSs may yield insights into their different rates of degeneration of the photoreceptors.

It is interesting to note that a mutation in the C1q domain of human *C1QTNF5*, found in patients with L-ORD (Hayward *et al.*, 2003; Ayyagari *et al.*, 2005) is associated with drusen-like

deposits with subsequent retinal degeneration. In contrast to human *CIQTNF5* mutations, human *MFRP* mutations have been associated with optic nerve head drusen with severe rod-cone dystrophy (Ayala-Ramirez *et al.*, 2006) or nanophthalmos and hyperopia with no evidence of retinal degeneration (Sundin *et al.*, 2005). The apparent difference in phenotypic expression of mutations within *Mfrp* in humans and mice remains to be examined.

In summary, MFRP and C1QTNF5, two proteins that have been shown to directly interact, lead to ocular diseases in mice and humans when mutated. It appears that MFRP is necessary for the normal development of the apical microvilli of the RPE and that a mutation in *Mfrp* leads to an initially focal reduction in the number of apical microvilli in mutant mice. These RPE defects may contribute to abnormal photoreceptor OS development and/or RPE phagocytosis, ultimately leading to severe photoreceptor degeneration. Determining the role of MFRP in the development of the RPE and, potentially, in other signaling functions that it may mediate, will improve our ability to identify potential therapeutic regimens and targets for amelioration of retinal disease.

ACKNOWLEDGMENTS

We would like to thank Jennifer Torrance for expert assistance in the preparation of the figures, and Mary Robertson for technical assistance. We also thank Drs Verity Letts and Peter Fuerst for careful review of the manuscript. The *rd6* cDNA was a kind gift from Dr. Shu. We would like to thank Dr. Sylvia Finnemann for providing the retinal bleaching protocol. This study was supported by National Institutes of Health grants, EY11996 (PMN), EY15638 (NSP), a TJL institutional core grant (CA34196), VA, Foundation Fighting Blindness, and Research to Prevent Blindness.

REFERENCES

- Ayala-Ramirez R, Graue-Wiechers F, Robredo V, Amato-Almanza M, Hortadiez I, Zenteno JC. A new autosomal recessive syndrome consisting of posterior microphthalmos, retinitis pigmentosa, foveoschisis, and optic disc drusen is caused by a MFRP gene mutation. *Molecular Vision* 2006;12:1483–1489. [PubMed: 17167404]
- Ayyagari R, Mandal MN, Karoukis AJ, Chen L, McLaren NC, Lichter M, Wong DT, Hitchcock PF, Caruso RC, Moroi SE, Maumenee IH, Sieving PA. Late-onset macular degeneration and long anterior lens zonules result from a CTRP5 gene mutation. *Investigative Ophthalmology & Visual Science* 2005;46:3363–3371. [PubMed: 16123441]
- Besharse JC, Hollyfield JG, Rayborn ME. Turnover of rod photoreceptor outer segments. II. Membrane addition and loss in relationship to light. *Journal of Cell Biology* 1977;75:507–527. [PubMed: 264121]
- Bonilha VL, Rayborn ME, Saotome I, Mcclatchey AI, Hollyfield JG. Microvilli defects in retinas of ezrin knockout mice. *Experimental Eye Research* 2006;82:720–729. [PubMed: 16289046]
- Cheng T, Peachey NS, Li S, Goto Y, Cao Y, Naash MI. The effect of peripherin/rds haploinsufficiency on rod and cone photoreceptors. *Journal of Neuroscience* 1997;17:8118–8128. [PubMed: 9334387]
- Collin GB, Cyr E, Bronson R, Marshall JD, Gifford EJ, Hicks W, Murray SA, Zheng QY, Smith RS, Nishina PM, Naggert JK. Alms1-disrupted mice recapitulate human Alstrom syndrome. *Human Molecular Genetics* 2005;14:2323–2333. [PubMed: 16000322]
- Duncan JL, Lavail MM, Yasumura D, Matthes MT, Yang H, Trautmann N, Chappelov AV, Feng W, Earp HS, Matsushima GK, Vollrath D. An RCS-like retinal dystrophy phenotype in mer knockout mice. *Investigative Ophthalmology & Visual Science* 2003;44:826–838. [PubMed: 12556419]
- Farjo R, Skaggs JS, Nagel BA, Quiambao AB, Nash ZA, Fliesler SJ, Naash MI. Retention of function without normal disc morphogenesis occurs in cone but not rod photoreceptors. *Journal of Cell Biology* 2006;173:59–69. [PubMed: 16585269]
- Hawes NL, Chang B, Hageman GS, Nusinowitz S, Nishina PM, Schneider BS, Smith RS, Roderick TH, Davisson MT, Heckenlively JR. Retinal degeneration 6 (rd6): a new mouse model for human retinitis punctata albescens. *Investigative Ophthalmology & Visual Science* 2000;41:3149–3157. [PubMed: 10967077]

- Hayward C, Shu X, Cideciyan AV, Lennon A, Barran P, Zarepari S, Sawyer L, Hendry G, Dhillon B, Milam AH, Luthert PJ, Swaroop A, Hastie ND, Jacobson SG, Wright AF. Mutation in a short-chain collagen gene, CTRP5, results in extracellular deposit formation in late-onset retinal degeneration: a genetic model for age-related macular degeneration. *Human Molecular Genetics* 2003;12:2657–2667. [PubMed: 12944416]
- Kameya S, Hawes NL, Chang B, Heckenlively JR, Naggert JK, Nishina PM. Mfrp, a gene encoding a frizzled related protein, is mutated in the mouse retinal degeneration 6. *Human Molecular Genetics* 2002;11:1879–1886. [PubMed: 12140190]
- Katoh M. Molecular cloning and characterization of MFRP, a novel gene encoding a membrane-type Frizzled-related protein. *Biochemical and Biophysical Research Communications* 2001;282:116–123. [PubMed: 11263980]
- Lee Y, Kameya S, Cox GA, Hsu J, Hicks W, Maddatu TP, Smith RS, Naggert JK, Peachey NS, Nishina PM. Ocular abnormalities in Large(myd) and Large(vls) mice, spontaneous models for muscle, eye, and brain diseases. *Molecular and Cellular Neuroscience* 2005;30:160–172. [PubMed: 16111892]
- Mandal MN, Vasireddy V, Jablonski MM, Wang X, Heckenlively JR, Hughes BA, Reddy GB, Ayyagari R. Spatial and temporal expression of MFRP and its interaction with CTRP5. *Investigative Ophthalmology & Visual Science* 2006a;47:5514–5521. [PubMed: 17122143]
- Mandal MN, Vasireddy V, Reddy GB, Wang X, Moroi SE, Pattnaik BR, Hughes BA, Heckenlively JR, Hitchcock PF, Jablonski MM, Ayyagari R. CTRP5 is a membrane-associated and secretory protein in the RPE and ciliary body and the S163R mutation of CTRP5 impairs its secretion. *Investigative Ophthalmology & Visual Science* 2006b;47:5505–5513. [PubMed: 17122142]
- Molday RS. Peripherin/rds and rom-1: molecular properties and role in photoreceptor degeneration. *Progress in Retinal & Eye Research* 1994;13:271–299.
- Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC. Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking alphavbeta5 integrin. *Journal of Experimental Medicine* 2004;200:1539–1545. [PubMed: 15596525]
- Peachey NS, Goto Y, Al-Ubaidi MR, Naash MI. Properties of the mouse cone-mediated electroretinogram during light adaptation. *Neuroscience Letters* 1993;162:9–11. [PubMed: 8121644]
- Shu X, Tulloch B, Lennon A, Vlachantoni D, Zhou X, Hayward C, Wright AF. Disease mechanisms in late-onset retinal macular degeneration associated with mutation in C1QTNF5. *Human Molecular Genetics* 2006;15:1680–1689. [PubMed: 16600989]
- Strauss O. The retinal pigment epithelium in visual function. *Physiological Reviews* 2005;85:845–881. [PubMed: 15987797]
- Subrayan V, Morris B, Armbricht AM, Wright AF, Dhillon B. Long anterior lens zonules in late-onset retinal degeneration (L-ORD). *American Journal of Ophthalmology* 2005;140:1127–1129. [PubMed: 16376663]
- Sundin, OH.; Leppert, GS.; Silva, ED.; Yang, JM.; Dharmaraj, S.; Maumenee, IH.; Santos, LC.; Parsa, CF.; Traboulsi, EI.; Broman, KW.; DiBernardo, C.; Sunness, JS.; Toy, J.; Weinberg, EM. Extreme hyperopia is the result of null mutations in MFRP, which encodes a Frizzled-related protein; *Proceedings of the National Academy of Sciences of the USA*; 2005. p. 9553-9558.
- Travis GH, Brennan MB, Danielson PE, Kozak CA, Sutcliffe JG. Identification of a photoreceptor-specific mRNA encoded by the gene responsible for retinal degeneration slow (rds). *Nature* 1989;338:70–73. [PubMed: 2918924]
- Wu J, Marmorstein AD, Kofuji P, Peachey NS. Contribution of Kir4.1 to the mouse electroretinogram. *Molecular Vision* 2004a;10:650–654. [PubMed: 15359216]
- WU J, Peachey NS, Marmorstein AD. Light-evoked responses of the mouse retinal pigment epithelium. *Journal of Neurophysiology* 2004b;91:1134–1142. [PubMed: 14614107]

Fig. 1. Quantitative analysis of mRNA levels of *Mfrp* (A) and *C1qtnf5* (B). *Mfrp* and *C1qtnf5* are expressed dicistronically. Data points indicate the average level measured from control (filled circles) and *Mfrp*^{rd6/rd6} (open circles) mice at 5 ages (two pools each of 4–23 eyes per pool), ranging from e14.5 to P28. For controls, the levels of *Mfrp* and *C1qtnf5* mRNA peaked at P14 while the peak for *Mfrp*^{rd6/rd6} mice occurred at a later age.

Fig. 2. Localization of MFRP in mouse retina (A–H). Retinal sections obtained from P7 (A, B), P14 (C, D), P21 (E, F), and P28 (G, H, L) and stained by anti-hMFRP (red). Nuclear counter staining was done with DAPI (blue). Inserts show higher magnification images. Higher magnification image of control (L) was obtained using confocal microscopy to show the MFRP localization in the RPE. The arrows indicate the RPE nuclei. Western analysis using anti-mMFRP (I) and anti-hMFRP (J). Eyes for Western analysis were obtained at 2 mo. Anti-Actinin 4 was used as a loading control. (K) Western analysis of Myc-MFRP^{rd6} overexpressed in COS-7 cells. Cells were harvested 48 hours after transfection. Lysate from Myc-MFRP^{rd6} transfected cells and non-transfected control (NT) were separated on 10% SDS-PAGE gel, transferred to

nitrocellulose membrane and stained with anti-c-Myc, anti-mMFRP, and anti-hMFRP. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell layer. Bars=10 μ m.

Fig. 3. Localization of C1QTNF5 in mouse retina (A–H). Retinal sections obtained from P7 (A, B), P14 (C, D), P21 (E, F), and P28 (G, H) and stained by anti-C1QTNF5 (red). Nuclear counter staining was done with DAPI (blue). Inserts show higher magnification images. (I) Western blot analysis with anti-C1QTNF5 was performed as described in Fig 2. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell layer. Bar=10 μ m.

Fig. 4. Progressive outer segment disorganization and degeneration in *Mfrp*^{rd6/rd6} mice compared to age-matched controls (control) visualized by light microscopy. Retinal sections were obtained from P7 (A, B), P14 (C, D), P21 (E, F), P28 (G, H), and 2 months (I, J) and stained by hematoxylin and eosin (H&E). Arrows indicate aberrant cells. RPE, retinal pigment epithelium; OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell layer. Scale bars represent 50 μ m (A–H) and 10 μ m (I, J), respectively.

Fig. 5. Outer segment disorganization and apical microvilli retardation in *Mfrp^{rd6/rd6}* revealed by electron microscopy at P2 (A, B), P7 (C, D), P14 (E, F), and P21 (G, H). The arrows indicate microvilli. IS, inner segment; RPE, retinal pigment epithelium; OS, outer segment; P, pigment. Bar=500 nm.

Fig. 6. Dark-adapted ERGs obtained from control (left) and *Mfrp^{rd6/rd6}* (right) mice exposed to 5 different strobe flash intensities at P25 (A). Dashed lines indicate time of strobe flash presentation. Scale bars indicate 200 μV and 100 ms. Amplitude of the dark-adapted ERG a- and b-wave plotted as a function of flash intensity (B). Data points represent the mean ($\pm$ S.E.M) response amplitude obtained from 8 control (filled circles) and 7 *Mfrp^{rd6/rd6}* (open circles) mice. Light-adapted ERGs obtained from control (left) and *Mfrp^{rd6/rd6}* (right) mice exposed to 4 different strobe flash intensities at P25 (C). Dashed lines indicate time of strobe flash presentation. Scale bars indicate 100 μV and 100 ms. Amplitude of the cone ERG plotted as a function of flash intensity (D). Data points represent the mean ($\pm$ S.E.M) response amplitude

obtained from 8 control (filled circles) and 7 *Mfrp^{rd6/rd6}* (open circles) mice. Amplitude of cone ERG b-wave plotted as a function of rod ERG a-wave (E). Data are expressed relative to the normal mean. Each symbol represents an individual mouse. The solid diagonal line represents an equivalent decrease in the two response measures; points falling above this line indicate a relative sparing of cone responses; a relative sparing of rod responses would result in points falling below the line.

Fig. 7. Rod and cone cells in control (left) and *Mfrp*^{rd6/rd6} (right) eyes. Retinal sections obtained from P25 animals were stained with anti-rhodopsin (RHO; A, B), blue-opsin (B-OP; C, D) and red/green opsins (G/R-OP; E, F). Outer segment thinning can be seen by light microscopy at this age (G, H). ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium; INL, inner nuclear layer; GC, ganglion cell layer. Bars: A–F=10 μ m; G, H=50 μ m.

Fig. 8. dc-ERGs recorded to 7 min duration stimulus flashes from control or *Mfrp*^{rd6/rd6} mice at P25 (A). Each record indicates the grand average of 7 control or 7 *Mfrp*^{rd6/rd6} mice. Stimulus intensity was 2.2 log cd/m²; stimulus presentation is represented by the lower trace. Scale bars indicate 0.5 mV and 60 s. Amplitude of the major components of the dc-ERG (B). Bars indicate the average ($\pm$ S.E.M.) response from 7 control or 7 *Mfrp*^{rd6/rd6} mice.

Fig. 9. Immunostaining by anti- β -catenin (β -CAT; A, B) and ZO-1 (ZO-1; C, D) in *Mfrp*^{rd6/rd6} mice (right) is not significantly different compared to control (left). Retinal sections were obtained from mice at P28. Relative expression levels of *Fos* and *Tcf-1* in *Mfrp*^{rd6/rd6} and control mice (E).

Fig. 10. *Mfrp^{rd6/rd6}* have phagocytic defects (A–D). The retinal sections in panel C and D were obtained 1 h after light stimulation while the sections in panel A and B were obtained at the same time without the light stimulation from 2 mo control (A, C) and *Mfrp^{rd6/rd6}* (B, D) mice. Retinal sections were stained with anti-rhodopsin (RHO; red) to determine phagocytic uptake of rod OS in RPE and anti-ezrin (EZRIN; green) was used to distinguish the RPE-photoreceptor border. The boxed area is enlarged in the insert. In control mice, rhodopsin uptake (arrows) was increased with light stimulation (C) compared to no light stimulation (A). Quantification of rhodopsin positive material in the RPE (E). Western analysis of whole eye lysates from 2 mo control and *Mfrp^{rd6/rd6}* mice, and immunoprecipitated products of MERTK (F). Phospho-MERTK is shown by immunoblot with anti-phospho-Tyrosine (pY) antibody following immunoprecipitation with anti-MERTK (Mer). To quantify the band intensity, the membrane was reblotted with anti-MERTK. The immunoblot of 10% of lysate input was performed with

primary antibody as indicated. RPE, retinal pigment epithelium; ELM, external limiting membrane; ONL, outer nuclear layer; OS, outer segment. Bar=10 μ m.