Insights into the mechanisms of macular degeneration associated with the R172W mutation in RDS

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Mutations in the photoreceptor tetraspanin gene peripherin-2/retinal degeneration slow (PRPH2/RDS) cause both rod- and cone-dominant diseases. While rod-dominant diseases, such as autosomal dominant retinitis pigmentosa, are thought to arise due to haploinsufficiency caused by loss-of-function mutations, the mechanisms underlying PRPH2-associated cone-dominant diseases are unclear. Here we took advantage of a transgenic mouse line expressing an RDS mutant (R172W) known to cause macular degeneration (MD) in humans. To facilitate the study of cones in the heavily rod-dominant mouse retina, R172W mice were bred onto an Nrl2/2 background (in which developing rods adopt a cone-like fate). In this model the R172W protein and the key RDS-binding partner, rod outer segment (OS) membrane protein 1 (ROM-1), were properly expressed and trafficked to cone OSs. However, the expression of R172W led to dominant defects in cone structure and function with equal effects on S- and M-cones. Furthermore, the expression of R172W in cones induced subtle alterations in RDS/ROM-1 complex assembly, specifically resulting in the formation of abnormal, large molecular weight ROM-1 complexes. Fundus imaging demonstrated that R172W mice developed severe clinical signs of disease nearly identical to those seen in human MD patients, including retinal degeneration, retinal pigment epithlium (RPE) defects and loss of the choriocapillaris. Collectively, these data identify a primary disease-causing molecular defect in cone cells and suggest that RDS-associated disease in patients may be a result of this defect coupled with secondary sequellae involving RPE and choriocapillaris cell loss.

INTRODUCTION

The product of the peripherin-2/retinal degeneration slow (PRPH2/RDS) gene, hereafter referred to as RDS, is a photoreceptor-specific tetraspanin protein exclusively localized to the disc rims of both cone and rod outer segments (OSs) (1). The protein is critical for the proper formation and maintenance of OSs, and specifically for building the rim structure (2,3). To date over 80 different mutations in the RDS gene have been linked with human diseases, including autosomal dominant retinitis pigmentosa (adRP), digenic RP, pattern dystrophy, adult vitelliform macular dystrophy, central areolar chorioidal dystrophy and other forms of macular degeneration (MD) (http://www.retina-international.org/sci-news/rdsmut.htm). Yet, no curative or preventative therapies for these conditions exist.

RDS function relies on its assembly into protein complexes, which initially are either homotetramers or heterotetramers, the latter containing RDS’ non-glycosylated homologue ROM-1 (rod OS membrane protein-1); these complexes are held together via interactions between the second intradiscal (D2) loop of the two proteins (4,5). RDS and ROM-1 function together, assembling in the inner segment (cell body) of the photoreceptor into tetrameric core complexes (6). These complexes are then trafficked to the OS where they further assemble into higher order oligomeric structures, including hetero-octamers and RDS homooligomers (7). These larger complexes are held together by

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intermolecular disulfide bonds mediated by a specific cysteine residue (C150) (8,9), one of seven D2 loop cysteines (the others are all involved in the intramolecular disulfide bonding necessary for proper folding of the D2 loop). In the absence of C150, RDS and ROM-1 tetramers form, but not higher order oligomers. In transgenic mice that express C150S mutant RDS in the absence of wild-type (WT) RDS, OSs fail to form, confirming that covalently bound, higher order RDS complexes are required for proper photoreceptor OS biogenesis (9). Although RDS/ROM-1 complexes are similar in rods and cones (7), we have shown that the two cell types have differential requirements for RDS (10,11). While rods without RDS form no OSs (12), cones that lack rim structures and conventional flattened membranous lamellae, but nonetheless retain appreciable levels of retinal function (10). However, the reason why some RDS mutations result in rod-dominant retinal diseases (such as adRP), while others are not, is not yet known.

One of the most common RDS mutations is a substitution of tryptophan for arginine at position 172 (R172W), which results in autosomal dominant macular dystrophy. This mutation has been described by multiple groups and occurs in a large number of families (13–16). Key patient phenotypes include central vision loss, clinically detected macular changes (e.g. by ophthalmoscopy) and atrophy of the choriocapillaris and retinal pigment epithelium (RPE) (13,15). While full-field electroretinograms (ERGs) can be normal, the multi-focal ERG is almost always decreased. Affected individuals typically present with faltering visual acuity in the third to fourth decade of life, although macular changes can be detected in asymptomatic children and young adults carrying the mutation (15). Although it is unclear why the R172W mutation causes this distinct cone-associated phenotype, having arginine at position 172 in RDS is critical for cone structure and function. Each of the known substitutions at position 172 (R172W, R172Q) in RDS causes a cone- or a fovea-dominant defect in patients (15,17) in contrast to some other residues in which mutations at the same site can cause both rod- and cone-dominant phenotypes (such as K153Δ) (18) or (N244H/K) (19,20).

Our previous studies have suggested that the underlying disease mechanism for RDS mutations that cause rod-dominant disease (such as C214S) may be haploinsufficiency (21,22). However, the mechanisms underlying RDS-associated cone-dominant or macular disease are more complex. To study these disease mechanisms we generated and characterized transgenic mice carrying the R172W mutation on multiple RDS genetic backgrounds (23,24). We showed that expression of R172W caused a severe dominant-negative defect in cone function, consistent with patient phenotypes, while rod function was unaffected (24). This dominant effect is not due to excessive levels of RDS, since we have previously shown that overexpression of WT RDS does not have any structural or functional consequences to the retina (25). Biochemical studies showed that the R172W RDS protein was slightly more susceptible to tryptic digestion than WT RDS, but retained the ability to bind ROM-1 and form proper RDS complexes in rods (24). These observations are consistent with improved, rather than defective, rod structure and function in the presence of R172W protein. However, the small number of cones in the WT mouse retina, which account for only ~3% of the total photoreceptor population, precluded studies on the function of R172W protein in cones.

Hence, we undertook additional studies to extend our understanding of the mechanism of cone-dominant disease in the presence of the R172W mutation in RDS. To facilitate characterization of cones, we took advantage of the Nrl+/− (neural retinal leucine zipper) knockout mouse, in which developing rods are converted to cone-like cells (26). Herein, we show that R172W mice on the Nrl+/− background also exhibit defects in cone vision, consistent with their counterparts on the WT background. We observed that the formation of RDS/ROM-1 complexes in cones of the R172W mice was altered, suggesting that this molecular defect may underlie the cell-type specific disease phenotype. In addition, we show that the R172W mice exhibited clinical signs of disease consistent with those seen in patients, suggesting that this model has potential utility for investigating the relationship between molecular defects and the development of this type of retinal degeneration.

RESULTS

Expression and localization of R172W in the Nrl−/− retina

To facilitate our studies on the mechanism of disease in the case of the R172W mutation, we crossed a WT R172W transgene on different rds backgrounds onto the Nrl−/− genetic background (herein also referred to as the ‘cone-dominant background’). Throughout this study, we compared the characteristics of the R172W protein expressed on the rod-dominant background (i.e. WT, rds+/− or rds−/−) with that on the cone-dominant background (i.e. Nrl−/−, rds+/−/Nrl−/− or rds−/−/Nrl−/−).

Western blot (WB) analysis of extracts from retinas containing only WT RDS (WT/Nrl−/−) or mutant RDS (R172W/rds+/− or R172W/rds−/−/Nrl−/−) under reducing conditions demonstrated that the R172W protein had the correct size in both the rod- and cone-dominant background (Fig. 1A). Our previous studies showed that animals homozygous for the R172W transgene had R172W protein levels that were ~75% of WT levels (24). Here, we observed that R172W levels in R172W homozygous mice on the rds+/−/Nrl−/− genetic background were ~50% of RDS levels in Nrl+/−/rds−/− retinas compared with WT and R172W/rds−/−/Nrl−/− versus Nrl+/−/rds−/− retinas (Fig. 1B, bottom), although the difference in ROM-1 levels was of smaller magnitude than the difference in RDS levels. Interestingly, these results also confirm an observation that we made previously, namely that the ratio of RDS:ROM-1 is different in rods (WT background) than in cones (Nrl−/− background) (27). RDS levels in the Nrl−/− were decreased by ~30% compared with WT levels, while ROM-1 levels in the Nrl−/− line were decreased by ~70% compared with WT levels (Fig. 1B).

We next assessed the localization of the R172W protein (hereafter referred to as just R172W). Frozen retinal sections were collected from R172W/Nrl+/− or control Nrl+/− eyes harvested at P30 and immunolabeled with antibodies which are either specific to transgenic R172W (mAB 3B6, green Fig. 1C) or
which recognize both endogenous and transgenic RDS isoforms (RDS-CT, red, Fig. 1C). Consistent with the specificity of the IRBP promoter, the expression of R172W was restricted to photoreceptor cells and the mutant protein co-localized with WT RDS. To determine whether the transgene was expressed in either or both S- and M-cones, immuno-florescence (IF) labeling was performed on tissue sections from Nrl<sup>2−/2</sup>, R172W/<sup>+/−</sup>, Nrl<sup>−/−</sup>, R172W/<sup>+/−</sup>, Nrl<sup>−/−</sup>, R172W/<sup>+/−</sup>, Nrl<sup>−/−</sup>, and R172W/<sup>+/−</sup>, Nrl<sup>−/−</sup> eyes using mAB 3B6 (R172W-specific; green, Fig. 1D) and either S- or M-cone opsin-specific antibodies (red, Fig. 1D). Co-localization with mAB 3B6 and S-/M-opsin antibodies confirmed that R172W was expressed in both cone cell types. While the images shown were taken of photoreceptors whose OSs were inside the photoreceptor rosettes typical of the Nrl<sup>−/−</sup> retina [10],
similar results were observed in photoreceptors whose OSs faced
the RPE (Supplementary Material, Fig. S1). RDS functions as a
complex with ROM-1; hence, to confirm that ROM-1 localization
was not altered in the presence of R172W, retinal sections
from Nrl−/− and R172W/rds−/−/Nrl−/− were probed by IF
using both RDS-CT and ROM1-2H5 antibodies (Fig. 2A). We
observe co-immunolocalization of the two proteins and no differ-
cences in their distribution between the two genotypes.
Finally, to confirm that RDS and ROM-1 were trafficking to
the OS and not accumulating abnormally in the inner segments
of photoreceptors, tissue sections were probed with antibodies
to an inner segment marker (Na+K+-ATPase, green, Fig. 2B)
and either RDS-CT or ROM1-CT (red, Fig. 2B). We observed
that the green IF labeling was in a distinct layer (corresponding
to photoreceptor inner segments) from the red IF labeling
obtained with the anti-RDS/ROM-1 antibodies (confined to
OSs) in both the Nrl−/− and R172W/rds−/−/Nrl−/− mouse
lines. Hence, both R172W and ROM-1 properly trafficked to
the OS on the given genetic backgrounds. These data are in con-
trast to prior observations regarding some other cone-dominant
RDS mutations, which cause mislocalization of mutant RDS
protein and cone opsins throughout the outer nuclear and synap-
tic (outer plexiform) layers (9,28). In the present study, all four
proteins (R172W, ROM-1, S-opsin and M-opsin) were exclu-
sively localized to the OS (Fig. 1D, Fig. 2B).

S- and M-cone functions is similarly affected
by the R172W mutation

Previously, we observed that R172W caused a severe dominant
defect in cone ERG amplitudes, even when the transgene was
expressed in the presence of WT level of RDS (24). In contrast,
rod function was only slightly decreased (in R172W/WT mice
versus WT) or was improved (in R172W/rds+/− versus rds+/−,
as shown in (24) and Supplementary Material, Figure S2). Here
we asked whether this dominant cone defect was specific to a par-
ticular cone subtype. Full-field spectral photopic ERG was con-
ducted at P30 on R172W or non-transgenic (Non-T) mice on
either the rod-dominant (Fig. 3A) or cone-dominant (Fig. 3B)
background. S- (Photopic B-UV) and M- (Photopic B-Green)
cone function, as measured by maximum photopic b-wave
amplitudes, were significantly reduced in R172W/WT and R172W/
Nrl−/− compared with their non-transgenic counterparts (WT/
Nrl−/−, respectively, Fig. 3A and B), confirming that the cone-
dominant defect associated with the R172W mutation is recapitu-
lated in the Nrl−/− model. In the rod-dominant background,
S- and M-cones were similarly affected: green photopic b-wave
amplitudes were reduced by 59%, while UV-photopic b-wave
amplitudes were reduced by 57% (R172W/WT versus WT,
Fig. 3A). In the Nrl−/− cone-dominant background, M-cone pho-
topic b-wave amplitudes were slightly more affected than S-cone
b-wave amplitudes (65 and 47% decrease between Nrl−/− and
R172W/Nrl−/−, respectively; Fig. 3B).

Interestingly, when the R172W transgene was expressed in
the presence of only one allele of WT RDS (i.e. the rds+/− back-
ground), there was a dramatic decrease in S- and M- cone b-wave
amplitudes on the rod-dominant background (R172W/rds+/−
versus rds+/−, Fig. 3A), while amplitudes were unchanged on
the cone-dominant background (R172W/rds+/−/Nrl−/−
versus rds+/−/Nrl−/−, Fig. 3B). This observation highlights
the fact that cones in the retina of the Nrl−/− mouse line
(which is devoid of rods) may not behave in precisely the same
way as WT mouse cones (which are surrounded by rods),
although they are quite similar in structure and function.
Finally, we examined cone function in cone-dominant mice
lacking any WT RDS (R172W/rds−/−/Nrl−/−). While
M-cone b-wave amplitudes were not improved compared with
non-transgenic controls (rds−/−/Nrl−/−), mean S-cone b-wave
amplitudes tended to be higher than in WT controls (although
the difference was not statistically significant, Fig. 3B). This

Figure 2. R172W and ROM-1 properly traffic to the OS. (A) Frozen retinal sec-
tions from transgenic and non-transgenic animals were immunolabeled with
ROM1-2H5 (green) and RDS-CT (red) antibodies. (B) Sections were labeled
with the inner segment marker Na+K+-ATPase (ATPase, green) and either
RDS-CT or ROM1-CT (red). N = 3 eyes per genotype. Abbreviations as in
Figure 1. Scale bar: 10 μm.
small improvement in S-cone b-wave amplitudes is consistent with our previously reported small improvement in non-spectral (i.e. white light-stimulated) photopic b-wave amplitudes in R172W mice lacking endogenous RDS (24), and again supports the utility of the Nrl2/2 line as a cone-dominant model. This observation also suggests that while R172W is not capable of supporting normal cone photoreceptor function, it apparently is better than no RDS at all.

Cone OS ultrastructure reflects cone function in R172W mice

Due to the low number of cones in the rod-dominant WT mouse retina, we previously had not been able to assess the effects of the R172W mutation on cone OS ultrastructure. Therefore, in the present study, we conducted histological analyses at the light and EM levels on 1-month old R172W mice on the Nrl2/2 background. No gross changes in retinal morphology were observed at the light level in R172W mice compared with non-transgenic controls (Fig. 4A), although consistent with our previous observation (10), we did observe fewer rosettes in the rds−/−/Nrl−/− retina (and in the R172W/rds−/−/Nrl−/−) than in the Nrl−/− retina (Fig. 4A).

At the ultrastructural level, however, defects became more readily apparent. Cone OSs in the Nrl−/− line exhibit nicely stacked lamellae; however, while some similar stacks of lamellae appeared in retinas in the R172W/Nrl−/− line, many OSs contained open, unflattened membranous structures (black arrow, Fig. 4B; see also additional representative images in Supplementary Material, Fig. S3). Consistent with the lack of detectable functional (ERG) difference, the ultrastructure of OSs of R172W/rds+/−/Nrl−/− mice were indistinguishable from their non-transgenic rds+/−/Nrl−/− counterparts, both exhibiting large swirls of membranous lamellae (11) that were not properly oriented. Finally, as reported previously (10), we observe that the OSs of rds−/−/Nrl−/− retinas completely lack disc rims and lamellae, and instead were just open, somewhat amorphous membranous structures. However, consistent with the small improvement we observed in the ERG function, we also detected some improvements in OS ultrastructure in the R172W/rds+/−/Nrl−/− mouse retina, including nascent lamellae formation and membrane stacking (white arrow, Fig. 4B). EM immunogold (IG) labeling with S- and M-opsin antibodies (Supplementary Material, Fig. S4A and B) did not identify any ultrastructural differences in S- versus M-cones in transgenic versus non-transgenic animals. Finally, EM IG labeling with RDS-CT antibodies confirmed that RDS (both mutant and WT isoforms) was located in these abnormal OS structures (Supplementary Material, Fig. 4C).

R172W causes defects in ROM-1 complex formation

One of the most useful aspects of Nrl−/− mice is the ability to study RDS biochemistry in cones in this model. Our previous data from the oligomerization-incompetent C150S RDS mutant suggest that RDS/ROM-1 complexes may assemble differently in rods versus cones (9,28). Therefore, we conducted
and mice. Consistent with our prior observations (28), retinas from ROM-1 distribution between transgenic and non-transgenic differences were observed with respect to either RDS or ROM-1 (blots and graphs plotting the percent of total RDS (Figure 5A and B) shows representative RDS/ROM-1 immuno-

-5) and higher order RDS homo-oligomers (fractions 1–3). Figure 5A and B shows representative RDS/ROM-1 immuno-blots and graphs plotting the percent of total RDS (solid line) or ROM-1 (dashed line) found in each fraction; no significant differences were observed with respect to either RDS or ROM-1 distribution between transgenic and non-transgenic mice. Consistent with our prior observations (28), retinas from cone-dominant animals (Nrl+/− and R172W/rds+/−/Nrl−/−, Fig. 5B) had a greater percentage of their total RDS in higher order oligomeric complexes (fractions 1–3) than did retinas from rod-dominant animals.

RDS complexes are formed via both covalent (disulfide) and non-covalent bonds, and intermolecular disulfide bonding is responsible for the assembly of octameric and higher order oligomeric complexes. The relative contribution of covalently bound versus non-covalently bound forms of RDS/ROM-1 to the total pool of RDS/ROM-1 complexes can be assessed by examining fractions obtained from non-reducing sucrose gradients that subsequently are analyzed by non-reducing SDS-PAGE/WBs. Shown in Figure 6A are representative non-reducing RDS WBs and companion quantification graphs (as in Fig. 5) plotting the percent of monomer (dashed line, non-covalently bound form) and dimer (solid line, covalently bound form). Most of the RDS was found in the dimeric form, but there were no significant differences between genotypes. In contrast, when these non-reducing blots were probed with anti-ROM-1 antibodies, an anomalous third band was detected in addition to the monomeric and dimeric forms (Fig. 6B). These abnormal, large molecular weight complexes (dotted line, Fig. 6B) were found primarily in the octameric fractions, and were highly enriched in R172W/rds+/−/Nrl−/− retinal extracts. These complexes also were observed when R172W was expressed in conjunction with WT RDS in the cone-dominant background (R172W/rds+/−/Nrl−/−, Supplementary Material, Fig. 5A). Importantly, these data indicate that while RDS complexes assemble properly in the presence of R172W, the ability of ROM-1 to complex normally in cones expressing R172W is impaired.

As a result of the appearance of this aberrant third type of ROM-1 complex, we wanted to confirm that ROM-1 still retained the ability to interact with RDS in the presence of R172W transgenic protein. Therefore, we conducted reciprocal co-immunoprecipitation (IP) with antibodies against RDS and ROM-1 (bound fractions are shown in Fig. 7A; input and unbound fractions are in Supplementary Material, Fig. 5B). Although RDS and ROM-1 protein levels are lower in R172W/rds+/−/Nrl−/− and R172W/rds−/− retinas than in controls, the mutant RDS was able to pull down ROM-1 (and vice versa). We next asked whether the abnormal large molecular weight ROM-1 complexes identified in the velocity sedimentation studies retained the ability to bind R172W. Using non-reducing SDS-PAGE/WB analysis following IP of retinal extracts with anti-RDS antibodies, we confirmed that this aberrant large complex does bind to RDS (Fig. 7B).

R172W mice exhibit RPE defects and degeneration of the choriocapillaris consistent with patient phenotypes

Although vision defects in patients carrying the R172W RDS mutation are primarily attributed to defective cones, the maculopathies seen clinically suggest that such defects may be a combination of direct effects on cones coupled with secondary sequelae, such as toxicity to the RPE, which firsts affects the macula due to the higher demands on the RPE in that region
Figure 5. R172W does not alter the distribution of RDS complexes. Retinal extracts collected at P30 from transgenic or non-transgenic controls in the rod-dominant (A) or cone-dominant (Nrl<sup>−/−</sup>, B) background were fractionated on continuous 5–20% non-reducing sucrose gradients. Fractions were separated on reducing SDS-PAGE and blots were probed for RDS or ROM-1. Graphs show the amount of RDS (solid line) or ROM-1 (dashed line) in each fraction as a percent of total immunoreactivity. Plotted are means ± SEM. Peak fractions for molecular weight markers in the gradient are shown with arrows: apoferritin (443 kD), beta-amylase (200 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD) and carbonic anhydrase (29 kD). Two retinas were pooled for each extraction/fractionation and three to six independent experiments were performed per genotype.

Figure 6. R172W leads to the formation of abnormal ROM-1 complexes in the Nrl<sup>−/−</sup> background. Retinal extracts from the non-reducing gradients shown in Figure 5 were separated on non-reducing SDS-PAGE. Blots were probed with RDS (A) or ROM-1 (B). Graphs show the amount of RDS/ROM-1 found as monomer (dashed line, A and B), dimer (solid line, A and B) or in the case of ROM-1, an abnormal large MW complex (dotted line, B) as a percent of total immunoreactivity. Plotted are means ± SEM. Peak fractions for molecular weight markers in the gradient are shown with arrows as in Figure 5. Two retinas were pooled for each extraction/fractionation and three to six independent experiments were performed per genotype.
compared with other retinal regions. Clinically, this manifests as peripapillary atrophy, RPE atrophy and loss of the choriocapillaris, in addition to other pronounced phenotypic changes in the macula (RPE granularity, bull’s eye maculopathy, etc.) (13,15), all of which are visible by fundus imaging and fluorescein angiography (FA). Because mice lack a macula, they are not a good model for such characteristic central retinal defects. However, to determine whether R172W transgenic mice exhibit any of the other clinical signs, we conducted fundus imaging and FA at 2 months of age. The Nrl−/− model is known to degenerate over time (10) and the rosettes commonly seen in this model appear on fundus images, potentially clouding interpretation of the phenotype, and so these studies were restricted to mice that had the rod-dominant background.

Fundus images from R172W/rds−/− were often grossly normal, although some exhibited an abnormal ring around the optic nerve (Fig. 8A, left column, yellow arrows), as well as a lack of the normal star pattern of retinal vasculature. In contrast, the phenotype was quite striking upon FA; transgenic mice exhibited extensive loss of the choriocapillaris (Fig. 8A, right column, only choroidal arterioles are visible, capillary beds are gone). This phenotype is nearly identical to that seen in RDS-associated maculopathy patients (13,15). In the R172W/rds−/− eyes, retinal vasculature was difficult to see, but appeared to be severely attenuated, and also was found in a very different imaging plane (observe faint out-of-focus retinal vessels in some specimens, red arrows Fig. 8A) from the newly exposed, abnormal choroidal vasculature. Interestingly, in mice (as in patients), this FA phenotype exhibited incomplete penetrance: half (4 of 8 eyes examined) exhibited the severe phenotype shown in the top two rows of Figure 8A, while the other eyes (4 of 8) showed no or only minor changes (Fig. 8A, bottom row). In addition, the phenotype was not uniformly bilateral: some mice exhibited this choroidal atrophy in both eyes, while in others only one eye appeared to be affected. None of the age-matched WT animals examined (Fig. 8B) showed any fundus or FA abnormalities. In addition, these atrophic changes were specific to the R172W mutation and not merely due to RDS deficiency as they were observed in age-matched non-transgenic rds−/− (Fig. 8C) or rds−/− eyes (Supplementary Material, Fig. 6).

A subset of R172W/rds−/− animals were aged out to observe the FA phenotype at later time points (5 months of age). FA and histological sections from R172W/rds−/− eyes shown in Figure 8D are the same eyes imaged in Figure 8A (control images in Fig. 8E and F are age matched but not the same animals as in Fig. 8B and C). Vascular phenotypes in eyes exhibiting abnormalities at 2 months of age persisted or worsened at 5 months of age: for example, the eye shown in the top row of Figure 8D showed further degeneration of choroidal vessels from 2 to 5 months of age. In contrast, the eye which showed no FA abnormalities at 2 months of age continued to exhibit normal vasculature at 5 months of age (Fig. 8D, bottom row). As before, WT and rds+/− controls exhibited no FA abnormalities at 5 months of age. These severe choroidal abnormalities were also observed in histological sections. In the WT and rds+/− eyes, the choroid consists of a continuous plexus with the small vessels of the choriocapillaris readily visible (Fig. 8E and F, white arrows in right panels). In contrast, in R172W/rds−/− eyes, it was often impossible even to detect the choriocapillaris and the remaining choroidal tissue itself was largely degenerated with large open spaces (Fig. 8D, right panels). This phenotype correlated with the FAs; eyes with severe FA abnormalities exhibited the worst choroidal structure upon histological examination of tissue sections.

Given these observed choroidal abnormalities, we next asked whether the neural retina exhibited degeneration at 5 months of age, especially in view of the photoreceptor-specific expression of the mutant protein. We conducted morphometric assessment of the thickness of the outer nuclear layer (ONL, a measure of photoreceptor degeneration) and found that the R172W/rds−/− eyes exhibited dramatic thinning of the ONL compared with both the WT and rds+/− (Fig. 9A and B). The mean ONL thickness in R172W/rds−/− mice was reduced to ~45% of WT thickness with similar magnitude losses across the retina (Fig. 9B).

Again, this deterioration is not due to deficiency of the RDS protein, but rather is a specific gain-of-function of the R172W mutation, since we have previously shown that mice homozygous for the transgene express ~75% of WT levels of RDS protein (in comparison to ~50% in the rds−/−). Interestingly, we did not observe the same variability in ONL thickness that we observed in FA. All R172W/rds−/− animals studied showed thinning of the ONL throughout the retina. Patients typically exhibit fundus signs of RPE atrophy and so we next examined the RPE structure (Fig. 9C). R172W/rds−/− exhibited large vacuoles in the RPE (Fig. 9C, black arrows and 8D, white arrowheads). These were only observed in the central retina and were mostly pronounced.
in the peripapillary region near the optic nerve. Interestingly, in contrast to the ONL thinning, this RPE defect was only seen in animals that exhibited FA abnormalities.

DISCUSSION

Here we present exciting new evidence that sheds significant light on the mechanisms of RDS-associated cone-dominant disease. We showed that the cone-dominant functional effects of the R172W are recapitulated in the Nrl2/2 background, supporting its use for analysis of the effects of R172W on cone photoreceptors. The effects of R172W impact both S- and M-cones, i.e. they are not cone subtype specific; consistent with the fact that most murine cones express both M- and S-opsin. Importantly, we found that the expression of R172W in cones leads to abnormal RDS/ROM-1 complex formation, specifically changes in the biophysical behavior of ROM-1. This abnormality also was detected when R172W was co-expressed with WT RDS, consistent with the dominant nature of the defect in maculopathy patients. Furthermore, we observed striking clinical signs of RPE defects and degeneration of the choriocapillaris, consistent with comparable findings in human RDS-associated disease.

**Figure 8.** R172W causes RPE atrophy and degeneration of the choriocapillaris. R172W mice (A) or age-matched non-transgenic controls (WT; B, rds+/−; C) underwent fundus imaging (left column) and FA (right column) at 2 months of age. (A) Images from three different R172W/rds−/− eyes to illustrate the varied phenotype in this background. Images in the left column are from the same eye as those in the right column. Yellow arrows in (A) show fundus abnormalities and red arrows show shadows of retinal vasculature in a different imaging plane. (D–F) Shown are fluorescein angiograms (right) and histological sections (left) from the indicated genotypes at 5 months of age. White arrows point to small vessels of the choriocapillaris and white arrowheads show RPE vacuoles. N = 6/8 eyes per genotype. Scale bar, 10 μm. Sc, sclera; Ch, choroid; RPE, retinal pigment epithelium; ChC, choriocapillaris.
Defects in RDS complex formation may contribute directly or indirectly to many different RDS-associated retinal disease phenotypes, a fact which undoubtedly accounts for the observation that the majority of RDS disease-causing mutations, including R172W, reside in the D2 loop (the region of RDS/RDS and RDS/ROM-1 interactions) (4,5). The defect in complex formation we observed here was relatively minor. The distribution of RDS complexes is completely normal in R172W retinas, both in the rod- and cone-dominant background. The only change we observed was the appearance of abnormal, large molecular weight ROM-1 complexes in the cones of R172W transgenic retina. These complexes are not likely to be misfolded aggregates awaiting degradation in the inner segment (where protein is synthesized), for three reasons. First, we did not detect any ROM-1 in the inner segment, suggesting that the protein is trafficked out of the ER/Golgi to the OS. Secondly, the abnormal complexes were reduced to monomers in the presence of reducing agent. In the case of RDS mutants that aggregate and are degraded, such as C214S (21,29), we have observed that the large aggregate band seen on immunoblots persisted even in the presence of reducing agent (29). Thirdly, the abnormal large-molecular-weight ROM-1 complexes were reduced to monomers in the presence of reducing agent (29). The reason for this differential photoreceptor subtype sensitivity is unknown, but may be tied to the different structural organization of cone OSs (which have lamellae/rims exposed to the extracellular space) and rods (in which discs are fully enclosed by the OS plasma membrane). It also may be tied to differences in complex assembly in the two cell types. Our data from mice carrying the C150S RDS mutation suggest that RDS complexes may assemble differently in the inner segments of rods versus cones (although the nature of these differences is not known) (9,28). In any case, when mutant R172W is expressed in cone OSs, the RDS complexes become just sufficiently abnormal to cause alterations in ROM-1.

However, the reason that R172W (and possibly other RDS mutations) causes MD is likely more complex than a simple...
molecular defect in cones. A key phenotype of R172W-associated disease in patients, often observable before overt vision loss or ERG changes are detectable, is maculopathy and the development of atrophic patches in the RPE, which are visible by fundus examination (13). This phenotype is not uniformly associated with defects in cones. For example, mutations in the cone phototransduction ion channel protein CNGA3 cause a complete lack of cone function and color vision from birth (30), but except in rare cases in older individuals, do not cause alterations in fundus appearance or RPE (31). As a result, we hypothesize that the severe MD coupled with choroidal/RPE atrophy seen in patients carrying the R172W mutation is due to a combination of primary cone photoreceptor molecular defects coupled with secondary sequelae that impact RPE as well as choriocapillaris viability and structural integrity. Precedence for this hypothesis exists. For example, mutations in the photoreceptor gene ABCA4 lead to accumulation of abnormal retinoids (derived from the visual cycle), which are toxic to the RPE, resulting in Stargardt’s MD (32,33). Importantly, we showed in the present study that the transgenic R172W RDS mouse recapitulates the RPE/choriocapillaris defects so prevalent in maculopathy patients, even though the mouse lacks a macula. The fact that this phenotype is not observed in rds−/− mice indicates that it is specific to the R172W mutation, and also is not merely caused by ongoing degeneration, OS loss or RDS-associated defects in vision. Interestingly, in common with the case in patients, we see striking animal-to-animal variability in fluorescein angiogram and RPE phenotypes in R172W/rds−/−, while degeneration of photoreceptors is fairly consistent among all animals. This suggests that the clinical disease severity is determined less by the primary defect in photoreceptors and more by the varying ability of neighboring tissues (RPE and choroid) to cope with such a defect. Furthermore, we observe significant pan-retinal degeneration of the choriocapillaris in animals who exhibit only modest, centrally localized defects in RPE cells and no sign of overt loss of RPE cells. That death of the RPE is not a prerequisite for degeneration of the neighboring choroid suggests that more subtle toxic changes in the RPE may influence the adjacent vasculature, and in-depth assessment of the RPE will be part of future work.

In conclusion, we have provided new findings that may be informative with regard to the mechanisms underlying MD in the case of the R172W mutation in RDS. We hope to capitalize on these new insights to develop rationally designed, mechanism-based therapeutics that will target disease processes ongoing both in photoreceptors as well as other affected cell types. This excellent mouse model will facilitate future work to more closely evaluate the interplay between photoreceptor defects and resulting loss in RPE structural and functional integrity. Of particular importance will be an assessment of the role of the abnormal RDS/ROM-1 complexes we have described here in the development of the associated RPE and choriocapillaris defects.

MATERIALS AND METHODS

Animal care and use

R172W transgenic mice were generated and characterized as described previously (24). Transgene expression is driven by the photoreceptor-specific interphotoreceptor retinoid-binding protein promoter, and the murine RDS cDNA carries the pathogenic R172W point mutation. It also carries a P341Q modification to facilitate antibody recognition (see below), which we have previously shown has no effect on protein function or gross biochemical properties (25). R172W mice were bred onto either an Nrl−/− background (generously shared by Dr Anand Swaroop, National Eye Institute), or a rds−/−/Nrl−/− background, as described and characterized previously (10). Animals were maintained under dim cyclic light (12 h/12 h L:D cycle; ∼30 lux). All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center, and conformed to the Statement for the Use of Animals in Ophthalmic and Vision Research as set forth by the Association for Research in Vision and Ophthalmology and the NIH Guide for the Care and Use of Laboratory Animals.

Antibodies

RDS-CT and ROM1-CT rabbit polyclonal antibodies against the C-terminus of RDS were generated in-house and characterized as previously described (9,10,24). RDS-CT recognizes both endogenous RDS and the R172W mutant RDS isoform, and was used at a dilution of 1:1000 for WB and immuno-fluorescence (IF) microscopy or at 1:10 for EM IG microscopy. ROM1-CT was used at 1:1000 dilution (WB and IF). ROM-1 monoclonal antibody (mAB) 2H5 also was generated in-house and used at 1:10 dilution for WB. mAB 3B6 (generously provided by Dr Robert Molday, University of British Columbia) specifically recognizes the transgenic (R172W) RDS isoform, but not WT murine RDS, due to the P341Q silent mutation and was used at 1:100 dilution for IF. Rabbit polyclonal antibodies against short (S-) and medium (M-) wavelength cone opsins (generously shared by Dr Cheryl Craft, University of Southern California) were used at 1:10 dilution for IG or at 1:30 000 dilution for IF. HRP-conjugated anti-actin polyclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used at 1:50 000 dilution. The following secondary antibodies were employed: anti-rabbit/anti-mouse HRP-conjugated IgG (KPL, Gaithersburg, MD, USA, at 1:50 000 dilution for WB); Alexafluor 488-, 555- or 647-conjugated anti-mouse/anti-rabbit IgG (Life Technologies, Grand Island, NY, USA, at 1:1000 dilution for IF); and AuroProbe™ 10 nm colloidal gold-conjugated anti-rabbit IgG (GE Healthcare, Pittsburgh, PA, USA, at 1:10 dilution for EMIG).

IF microscopy

Eyes were enucleated, fixed and dissected as described previously (21,28). After cryoprotection in serial sucrose dilutions (28), frozen retinal tissue sections (10-mm thickness) were collected onto glass microscope slides and IF was performed as described in detail previously (28). In brief, sections were incubated in 1% NaNBH4, washed in PBS, blocked in PBS containing 5% (w/v) BSA (Sigma-Aldrich), 1% (w/v) fish gelatin (Sigma-Aldrich), 20% (v/v) donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 1% (v/v) Triton X-100 (VWR). Sections were then incubated overnight with primary antibodies diluted in blocking buffer, then rinsed, incubated with secondary antibodies, rinsed again and mounted using ProlongGold™.
mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Images were captured on a BX-62 spinning disk confocal microscope (Olympus, Japan) and analyzed with Slidebook™ 4.2 software (Intelligent Imaging Innovations, Denver, CO, USA). All images are presented as single planes from a confocal stack unless otherwise specified. Exposure times and display settings for all images were normalized to a control section where primary antibody was omitted during processing.

**Fundus imaging**

Fundus imaging was performed on anesthetized, dilated mice using a Micron III system (Phoenix Research Laboratories, Pleasanton, CA, USA) as described previously (34,35). After collecting bright-field fundus images, animals were injected intraperitoneally with 100 µl of 1% (w/v) fluorescein sodium (Sigma-Aldrich) and FA images were captured using an excitation filter of 486 nm and an emission filter of 436 nm. All images were captured using StreamPix® software (Phoenix Research Labs).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflicts of Interest statement.** None of the authors has any conflict of interest to declare.

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