The Cys²¹⁴ → Ser mutation in peripherin/*rds* causes a loss-of-function phenotype in transgenic mice

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P/rds (peripherin/retinal degeneration slow) is a photoreceptor-specific membrane glycoprotein necessary for outer segment disc morphogenesis. Mutations in *P/rds* are associated with different blinding diseases. A C214S (Cys²¹⁴ → Ser) missense mutation has been shown to be the cause for a late-onset form of ADRP (autosomal dominant retinitis pigmentosa) in humans. In the present study, we generated transgenic mice expressing *P/rds* with the C214S mutation and crossed them into *rds* mutant mice to elucidate the mechanism underlying the pathology of ADRP. Although an ample amount of transgene message was formed in C214S retinas from all transgenic lines, only a trace amount of the mutant protein was detected by Western blotting and immunoprecipitation. C214S mice on the wild-type or *rds*^{+/-} backgrounds exhibited no signs of negative effects of the mutation on retinal

structure or function, suggesting a loss-of-function phenotype. This phenotype is further supported by the absence of outer segment formation in the C214S mice on the *rds*^{-/-} background. In contrast, expression of C214S protein in the inner retinal cells of transgenic mice or in COS cells resulted in the formation of a substantial amount of mutant protein, signifying a possible photoreceptor-specific regulation of *P/rds*. These results provide evidence that the loss-of-function phenotype seen in C214S transgenic mice shows a disease progression that correlates with ADRP patients carrying the same mutation, indicating that the C214S mutation on one allele of *P/rds* results in haploinsufficiency.

Key words: C214S, loss-of-function phenotype, peripherin/*rds*, retinal degeneration, retinitis pigmentosa, transgenic mice.

INTRODUCTION

P/rds (peripherin/retinal degeneration slow), a tetraspanning membrane protein, is involved in photoreceptor OS (outer segment) disc morphogenesis and maintenance [1–4]. *P/rds* is named after the *rds* mouse [5], which has a 9.2 kb insertion of foreign DNA in exon II of the *rds* gene, making it untranslatable [6]. While *rds*^{-/-} mice fail to develop OSs, *rds*^{+/-} mice form disorganized OS structures, signifying that one correct copy of the gene is not sufficient for normal function [7,8].

Rom-1 is homologous with *P/rds*, having similar sequence, structure and localization [9,10]. Rom-1 plays a lesser role in disc formation, since Rom-1^{-/-} mice develop OS structures [11]. Furthermore, Rom-1 is absent from lower vertebrates, suggesting that it may have evolved from *P/rds* by gene duplication [12–14]. *In vivo* and *in vitro* studies have shown that the non-covalent interactions between *P/rds* and Rom-1 act to form homomeric and heteromeric core complexes that are required for *P/rds* targeting and incorporation into disc membranes [15–19]. These complexes are linked together through intermolecular disulphide bonds to form higher-order oligomers that are crucial for disc-rim formation [17].

Several pathogenic mutations in *P/rds* are associated with ADRP (autosomal dominant retinitis pigmentosa) and macular dystrophy (see <http://www.sph.uth.tmc.edu/RetNet> and <http://www.retina-international.org/sci-news/rdsmt.htm>). Most of these mutations are found in the large intradiscal loop that contains seven conserved cysteine residues. Replacement of any of these cysteine residues in COS cells alters *P/rds* interactions, particularly intra-disulphide bond formation [19].

Humans with the mutation C214S (Cys²¹⁴ → Ser) of *P/rds* acquire ADRP, a disease that causes progressive rod degeneration

followed by cone degeneration [20]. In COS cells, C214S protein loses its ability to form higher-order complexes, but is capable of dimerization [19]. In addition, C214S protein cannot associate with Rom-1 [19]. Similar results were seen in *Xenopus laevis* retinas expressing *Xenopus* C214S fused to GFP (green fluorescent protein) [18]. This study further demonstrated that the C214S fusion protein is retained in the inner segments of *Xenopus* rods. Although the COS and *Xenopus* studies provided important information regarding *P/rds* binding and trafficking, neither is sufficient for evaluating the abnormalities observed in humans carrying mutations in *P/rds*. Therefore to confirm the causative nature of the defect associated with C214S, we studied transgenic mice expressing the mutant protein in rods and cones. Although C214S transgenic message was abundant, the level of the mutant protein was scarcely detectable, suggesting a loss-of-function mutation. The phenotype of C214S mice implies that there is a haploinsufficiency defect in patients carrying the C214S mutation in which the protein level from one wild-type allele is not enough for maintaining the OS structure. Expression of C214S in mice provides compelling biochemical, structural and functional evidence that the ADRP phenotype associated with the C214S mutation is a consequence of haploinsufficiency.

MATERIALS AND METHODS

Generation of C214S transgenic mice

The C214S mutation in *rds* cDNA was introduced by PCR-mediated mutagenesis using two sets of primers as described previously [21] to generate two overlapping fragments containing the TGC → TCC modification. The NMP (normal mouse *P/rds*)

Abbreviations used: ADRP, autosomal dominant retinitis pigmentosa; CT, C-terminal; DAPI, 4,6-diamidino-2-phenylindole; ERG, electroretinography; hIRBP, human interstitial retinal binding protein; *rds*, retinal degeneration slow; NMP, normal mouse *P/rds*; OS, outer segment; *P/rds*, peripherin/*rds*.

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Figure 1 Generation and expression of the C214S transgene

(A) Diagram of the mutation and transgene construct. The hIRBP promoter directs expression to rods and cones; *rds* cDNA contains the C214S mutation and the P341Q modification; SV40 poly A signal allows for recognition of transgene expression. (B) Northern-blot analysis of whole retinal RNA extracts (20 μ g/sample) taken from 20 days old mice of indicated genetic backgrounds of non-transgenic controls (lanes 1–3) and transgenic littermates (lanes 4–6). Two different probes were hybridized to the membrane: a cDNA probe that recognizes both endogenous and transgenic message, and an SV40 poly A probe that only recognizes the transgenic message. The 28 S band was included as a control for loading. This blot is representative for three independent experiments.

transgene, an *rds* cDNA clone containing the P341Q C-terminal modification, was used as a template [22]. Extension of the two amplified fragments in a second PCR produced the full-length *rds* sequence with the C214S mutation and P341Q modification. The primers used to generate the first fragment is the forward primer (5'-TAGCTCCGGCTACCGTTACT-3'), which starts at position 30 in the *rds* cDNA sequence, and the reverse primer (5'-CCGCGGTGA^{ACT}CGGATTGGAGCA-3'), which starts at position 872 and contains the C214S nucleotide change (shown in boldface). The set of primers used to generate the second fragment is the forward primer (5'-GCTGCTCCAATCCGAGTTCACCGC-3'), which starts at position 847 and contains the C214S nucleotide change (shown in boldface), and the reverse primer (5'-TGCACCTTCAGCCTCCACCTG-3'), which starts at position 1223. The underlined nucleotides are the changes introduced in the *rds* sequence. All changes other than the C214S point mutation did not change the amino acid sequence and were introduced for specific recognition of the transgene transcript. The transgene was created as described before [23], where the C214S cDNA clone was digested with BamHI–EcoRI and subcloned into the NMP transgene that consists of 1.3 kb of the hIRBP (human interstitial retinal binding protein) promoter, 1.5 kb of the mouse *rds* full-length cDNA (60–1531) with the P341Q modification and 0.9 kb of the SV40 poly A signal (Figure 1A). The P341Q modification was included to facilitate detection of the transgene product in the presence of wild-type *rds*, using the monoclonal antibody 3B6 (a gift from Dr R. S. Molday, Department of Biochemistry and Molecular Biology, University of British Columbia,

Vancouver, BC, Canada). The transgene sequence was confirmed from both strands before embryo injection.

The effects of the P341Q modification on *P/rds* function on the NMP retina have been well characterized [22]. Structural, functional and biochemical analyses of the NMP retina were indistinguishable from the wild-type retina. A total of 13 C214S founders were generated and confirmed to contain the transgene by Southern-blot analysis and PCR (results not shown). All founders passed the transgene to their offspring in Mendelian fashion, and a separate transgenic mouse line was generated for each one. After eliminating the *rd* mutation by successive backcrossing into the C57BL background, transgenic mice were mated to both wild-type and *rds* mice to generate C214S mice on all backgrounds (wild-type, *rds*^{+/-} and *rds*^{-/-}). As a result of primary analysis, one line was chosen for further characterization, designated line 204, based on the comparable level of the transgene message to that of the wild-type. All experiments and animal maintenance were approved by the local Institutional Animal Care and Use Committee (Oklahoma City, OK, U.S.A.) and conformed to the guidelines on the care and use of animals adopted by the Society for Neuroscience and the Association for Research in Vision and Ophthalmology (Rockville, MD, U.S.A.).

Northern-blot analysis

Total RNA was extracted from mouse retinas of indicated genotypes using TRIzol[®] reagent (Invitrogen, Carlsbad, CA, U.S.A.), and 20 μ g of total RNA was electrophoretically separated on a 1 % agarose gel containing 18 % (v/v) formaldehyde. The number of retinas used to meet this criterion ranged from 2 to 4. Images of the 18 and 28 S ribosomal bands were captured before transfer to determine the integrity of the RNA in the samples. RNA was then transferred to an S&S NYTRAN[®] SuPerCharge nylon membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and hybridized sequentially with a ³²P-labelled SV40 polyadenylated DNA fragment to detect the transgene transcript and an *rds* cDNA fragment to detect both the transgene and endogenous transcripts. Hybridization was performed in 50 % (v/v) formamide, 5 $\times$ SSC (saline sodium citrate; 1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate) buffer, 1 $\times$ Denhardt's buffer (0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 0.5 % SDS and 0.5 mg/ml salmon sperm DNA. The membranes were washed for 20 min each with 2 $\times$ SSC buffer/0.5 % SDS, 0.5 $\times$ SSC/0.5 % SDS and 0.1 $\times$ SSC/0.5 % SDS at 55 °C. The blots were then exposed overnight to Kodak Biomax MR film at –80 °C with an intensifying screen.

Immunoprecipitation and Western-blot analysis

Polyclonal antibodies against the anti-*P/rds*-CT (*P/rds* C-terminal) region and the anti-Rom-1-CT (Rom-1 C-terminal) region were used for the detection of *P/rds* and Rom-1 respectively [24]. *P/rds* D2 loop (D2) antibody was also used for the detection of *P/rds* [14,22,24]. Anti-actin polyclonal antibody from the IgG fraction of antiserum was purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Retinas and COS-1 cells were homogenized on ice in solubilization buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1 % (v/v) Triton X-100, 0.05 % SDS, 2.5 % (v/v) glycerol and 1.0 mM PMSF. After solubilization at 4 °C for 1 h, the homogenates were centrifuged at 100 000 *g* for 25 min, and supernatants were collected for analysis of protein concentration using a Bio-Rad Protein Assay[™] kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For Western blotting, retinal extracts or COS-1 cell extracts were subjected to SDS/PAGE and transferred on to a PVDF membrane. Blots were incubated with the primary antibody at a dilution ratio of 1:250

(anti-actin) or 1:1000 (anti-*P/rds*-CT, D2 or anti-Rom-1) for 2 h at room temperature (25°C). After 3×10 min washings with $1 \times$ TBST (Tris-buffered saline with Tween 20), the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Kirkegaard & Perry, Gaithersburg, MD, U.S.A.) at a dilution ratio of 1:12 500 (anti-actin) or 1:25 000 (anti-*P/rds*-CT, D2 and anti-Rom-1) for 1 h at room temperature. After rinsing several times with $1 \times$ TBST, SuperSignal® chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) was applied to detect binding of the primary antibodies to their cognate antigens.

For immunoprecipitation, retinas were incubated with either anti-*P/rds*-CT or anti-Rom-1-CT antibody (dilution ratio of 1:100) for at least 4 h at 4°C. Protein A-Sepharose beads (Sigma-Aldrich) were then added for incubation at 4°C overnight in solubilization buffer. Beads without the primary antibodies were used as negative controls. After adsorption, the beads were washed several times, and bound proteins were eluted with Laemmli sample buffer [25] for immunoblotting analysis using the anti-*P/rds*-CT, D2 or anti-Rom-1-CT antibodies.

COS cell transfection

A pcDNA3.1 (Invitrogen) construct containing the wild-type *rds* cDNA sequence corresponding to nt 1–1820 was used as a template for site-directed mutagenesis using the Transformer™ Site-Directed Mutagenesis kit (BD Biosciences, Palo Alto, CA, U.S.A.). The primer sequence (5'-CAGCTGCTCCAACCC-AGCTCCCCG-3') was used to introduce the TGC → TCC mutation (shown in boldface) to create the C214S mutation in the *rds* sequence. The presence of the introduced mutation and the absence of any mismatch during PCR amplification were confirmed by sequence analysis from both strands. COS-1 cells (a gift from Dr M. Al-Ubaidi, University of Oklahoma Health Sciences Center) were grown on 100 mm dishes to 80 % confluency before transfection with 10 µg of either the wild-type (pcDNA3.1-Wt) or C214S construct (pcDNA3.1-C214S). The transfection procedure was performed using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection; one portion of the cells was seeded on to coverslips and the other portion was used for protein extraction.

Histology and immunohistochemistry

For light microscopy and immunostaining of retinas, animals were killed, and eyes were fixed in 90 ml of Davidson's fixative [30 ml of 95 % (v/v) ethanol, 20 ml of 10 % neutral buffered formalin, 10 ml of acetic acid and 30 ml of distilled water] overnight at 4°C. The eyes were then washed three times with PBS and stored in 70 % ethanol before serial dehydration and embedment in paraffin. Eyes were sectioned (6–8 µm thickness) on a standard microtome and either stained with haematoxylin/eosin or subjected to immunostaining.

For immunostaining, retinal sections were blocked for 1 h using 5 % (w/v) BSA in PBS and then incubated overnight at 4°C with the primary antibodies diluted with 1 % BSA in PBS. Anti-*P/rds*-CT polyclonal antibody and 1D4 (a monoclonal antibody to opsin; a gift from Dr R. S. Molday) were applied at a 1:100 dilution. The monoclonal antibody 3B6 was applied at a dilution of 1:10, and the polyclonal antibody to opsin [26] was applied at a dilution of 1:1000. Three retinal sections per slide were incubated with 1 % BSA in PBS without the primary antibody as a negative control. Sections were then rinsed for 3×10 min in PBS, then incubated with a mixture of goat anti-mouse FITC and goat anti-rabbit Cy3 (each diluted 1:100) for 1 h at room temperature in the dark. The sections were washed for 3×10 min with PBS, and coverslips were mounted on to the slides with Vectashield™

containing DAPI (4,6-diamidino-2-phenylindole) stain (Vector Laboratory, Burlingame, CA, U.S.A.) and photographed with a Zeiss universal microscope equipped for epifluorescence. Digitally captured images were saved as TIFF files on a computer and imported into Adobe PHOTOSHOP™ 7.0, where colour pictures were generated.

COS-1 cells were grown on coverslips in DMEM containing 10 % (v/v) fetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A.). The cells were washed for 2×5 min with PBS, fixed with 4 % (w/v) paraformaldehyde for 20 min at room temperature, washed again for 2×5 min with PBS, permeabilized with 0.3 % (v/v) Tween 20 (Bio-Rad Laboratories) in PBS for 15 min at room temperature, and then blocked for 1 h at room temperature in 5 % BSA in PBS. Anti-*P/rds*-CT antibody in a 1:500 dilution in PBS with 1 % BSA was added to the cells and incubated overnight at 4°C. The primary antibody was washed for 3×10 min with PBS. Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) in PBS with 1 % BSA was then added to the cells for 1 h at room temperature and then washed for 3×10 min with PBS. Vectashield™ containing DAPI stain (Vector Laboratories) was used to mount the coverslips on to the slides.

Electron microscopy

Immediately after killing, eyes were orientated by marking the superior hemisphere along the vertical meridian at the limbus with a hot needle, before starting the dissection. A slit was made in the superior cornea and the eyes were fixed for 2 h at 4°C in fresh 0.1 M sodium phosphate buffer (pH 7.4), containing 2.5 % (v/v) glutaraldehyde, 2.0 % formaldehyde and 0.025 % CaCl_2 . After the 2 h primary fixation, the superior cornea was cut away, the lens was removed and fixation was continued overnight. The fixed eyes were washed with 0.1 M sodium cacodylate buffer (pH 7.4), containing 0.025 % CaCl_2 , and then post-fixed for 1 h in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. After post-fixation, the eyes were rinsed twice in 0.1 M sodium cacodylate buffer and once in distilled water. Eyes were dehydrated in a graded ethanol series followed by propylene oxide and infiltrated overnight in Spurr's resin. The eyes were then embedded in resin-filled BEEM® capsules (Polysciences, Warrington, PA, U.S.A.) and allowed to polymerize in a 70°C oven for 48 h. Tissue sections were obtained with a Reichert–Jung Ultracut E microtome using glass or diamond knives. Thin (600–800 Å thickness; 1 Å = 0.1 nm) sections were collected on copper 75/300 mesh grids and stained with 2 % (v/v) uranyl acetate and Reynolds' lead citrate. Sections were viewed with a JEOL 100CX electron microscope at an accelerating voltage of 60 keV. For light microscopy, thick (0.75–1 µm) sections were viewed and photographed with an Olympus BH-2 photomicroscope in the auto-expose mode, using $\times 20$ or $\times 60$ DPlanApo objective and images were collected with a Nikon digital camera system.

ERG (electroretinography)

ERGs were recorded from the corneal surface of mice, as described previously [8]. For the assessment of rod photoreceptor function, a flash stimulus was presented to the dark-adapted (4 h), dilated eyes in a Nicolet Ganzfeld (GS-2000) with a 137 cd (s/m^2) flash intensity. The amplitude of the rod *a*-wave was measured from the prestimulus baseline to the *a*-wave trough. The amplitude of the rod *b*-wave was measured from the trough of the *a*-wave to the peak of the *b*-wave. To evaluate the light response of cones, a strobe flash stimulus was presented to the light-adapted (5 min), dilated eyes in the same apparatus with a 77 cd (s/m^2) flash intensity. The amplitude of the cone *b*-wave was measured from the prestimulus baseline to the peak of the *b*-wave.

RESULTS

C214S transgene expression

To test the hypothesis that the C214S mutation in *P/rds* causes ADRP through haploinsufficiency, transgenic mice carrying this point mutation were generated (see the Materials and methods section). Expression of the C214S transgene was specifically targeted to rods and cones using the 1.3 kb hIRBP promoter (Figure 1A). All founders were bred on to the wild-type, *rds*^{+/-} and *rds*^{-/-} backgrounds to characterize the mutant protein alone and in the presence of wild-type *P/rds*. A transgenic line was generated for each of the 13 founders identified, and their phenotypes were characterized structurally and functionally. The lines were mated to achieve homozygosity of the transgene on all *rds* genetic backgrounds. To determine levels and integrity of the C214S transcript, Northern-blot analysis was performed on C214S retinas on different *rds* genetic backgrounds. Using an *rds* cDNA probe, two wild-type transcripts (1.6 and 2.7 kb) were detected in all retinal extracts containing the wild-type allele as reported previously [8], whereas a 2.4 kb C214S transcript was detected only in retinal extracts from transgenic mice (Figure 1B, lanes 4 and 5). This result was observed in all transgenic lines. The large band migrating above 9 kb represents the *rds* insertion [6,8]. Retinas from line 204 contain the C214S message comparable in level with the 2.7 kb wild-type transcript seen with the *rds* cDNA probe (Figure 1B, lanes 3 and 4). The SV40 poly A probe detects only the C214S transcript in transgenic retinas on all *rds* genetic backgrounds. With this probe, no bands were detected in RNA extracts from non-transgenic retinas (Figure 1B, lanes 1–3). Homozygous animals from the 204 line were used for further analysis of retinal phenotype due to a transgenic expression level that is comparable with the wild-type.

C214S protein in transgenic retinas

To evaluate the endogenous *P/rds*, C214S *P/rds* and Rom-1 protein formed in the mouse retinas, retinal homogenates from transgenic homozygous mice and their non-transgenic littermates on all *rds* genetic backgrounds were subjected to SDS/PAGE and Western-blot analysis (Figure 2A). Blots were probed with anti-*P/rds*-CT, anti-Rom-1-CT and polyclonal anti-actin antibody respectively. The *P/rds* monoclonal antibody, 3B6, failed to detect the C-terminal modification on Western blots (results not shown) as reported previously [22,23]. In retinas containing wild-type *P/rds*, two bands were detected approximately coincident with the 37 kDa protein standard, a minor, thin upper band and a major, thicker lower band. The band below the 99 kDa standard probably represents the *P/rds* inter-disulphide homodimer (~72 kDa), whereas the highest band is consistent with oligomers created through disulphide bonding [9,15]. In the C214S transgenic retinas on the *rds*^{-/-} background, a trace amount of C214S protein was detected (Figure 2A, lane 6). This faint amount of mutant protein migrated exclusively with the upper (minor) band seen in the wild-type retinal extracts. Similar results were obtained with the other transgenic lines. Rom-1 was barely visible in the Western blots of solubilized *rds*^{-/-} retinas (Figure 2A, lane 5). Although Rom-1 was not seen in the C214S retinas on the *rds*^{-/-} background of the same blot (Figure 2A, lane 6), a longer exposure of the blot revealed a faint band (results not shown).

C214S protein association with Rom-1

The C214S mutant protein was tested for its ability to interact with Rom-1 (Figure 2B). When retinal extracts from C214S/*rds*^{-/-} mice were immunoprecipitated with anti-*P/rds*-CT antibody in a

Figure 2 C214S protein expression, localization and interaction with Rom-1

(A) Western-blot analysis of retinal extracts (20 µg/sample) from 20–30 days old mice that were subjected to a reducing SDS/PAGE (10% polyacrylamide). Lanes 1–6 show a side-by-side comparison of transgenic retinas on the wild-type and *rds* backgrounds relative to non-transgenic retinas on the same backgrounds. The blot was probed with anti-*P/rds*-CT, anti-Rom-1-CT and anti-actin antibodies. (B) Western blot of *P/rds* and Rom-1 immunoprecipitates of retinal extracts (20 µl of sample from the 50 µl eluate/lane). In lanes 1–3, 200 µg of whole retinal protein extract was loaded into the reaction mixture. Lane 4 contained 300 µg of protein, while lanes 5 and 6 contained 400 and 500 µg of protein respectively. The blot was probed with the same anti-*P/rds*-CT and anti-Rom-1-CT antibodies that were used for immunoprecipitation. (C, D) Immunohistochemical analysis of non-transgenic and C214S retinas on wild-type, *rds*^{+/-} and *rds*^{-/-} backgrounds using anti-*P/rds*-CT and 1D4 (C) and the monoclonal antibody (3B6) and the polyclonal anti-opsin (D). NMP retina in (D) was used as a positive control for 3B6. Two different anti-opsin antibodies, monoclonal 1D4 (C) and a polyclonal IgG (D), were used as correlative, photoreceptor-specific reagents to label the outer and inner segments of the rod.

satürating amount, Rom-1 could not be detected by Western-blot analysis (Figure 2B, upper panel, lanes 3–6). Conversely, C214S *P/rds* could not be detected when immunoprecipitating with anti-Rom-1-CT in a saturating amount (Figure 2B, lower panel, lanes 3–6), whereas Rom-1 was clearly detected in the same samples using the anti-Rom-1-CT antibody. Since *P/rds* from wild-type retinal extracts was capable of co-immunoprecipitating with Rom-1 (lane 1), only the C214S mutant protein seems to have lost its association with Rom-1. Because there is very little C214S protein present in this assay, the interactions may exist at a level too low to be detected. However, this is probably not the case in our work, since previous COS cell data showed a distinct loss of interaction between C214S and Rom-1 when their protein expression levels were high [19]. Increasing the amount of retinal extract in the reactions yielded a proportional increase in the amount of C214S *P/rds* when the beads were eluted (Figure 2B, lanes 3–6). This effect was seen with both the anti-*P/rds*-CT

antibody and the anti-P/*rds*-D2 antibody. Similar results were seen with Rom-1 when an increasing amount of retinal extract was used in the immunoprecipitation. Although Rom-1 is present, it does not bring down C214S P/*rds* (Figure 2B, lanes 3–6). It is also important to note that immunoprecipitation of C214S P/*rds* only yielded the upper (minor) band, verifying the results seen in Figure 2(A).

C214S protein localization

Next, we investigated whether the trace amount of C214S protein visualized in the Western blot can be localized by immunohistochemistry. Anti-P/*rds*-CT and 3B6 antibodies were used for the present study. Two different monospecific antibodies (a rabbit polyclonal and a mouse monoclonal) against rod opsin were used to label rod OSs (Figures 2C and 2D). Although retinal sections from transgenic and non-transgenic animals on the wild-type background showed background showed labelling in the OS with anti-P/*rds*-CT antibody, no labelling was detected with transgenic and non-transgenic retinas on the *rds*^{-/-} background. This indicates that the small amount of C214S protein on the *rds*^{-/-} background observed in the Western blot (Figure 2A, lane 6) is below the sensitivity of detection with immunohistochemistry, using either anti-P/*rds*-CT (Figure 2C) or 3B6 (Figure 2D). However, these same antibodies prominently label OSs in wild-type (Figure 2C) and NMP transgenic retinas respectively (Figure 2D). Retinal sections from transgenic and non-transgenic animals on the wild-type background showed opsin labelling exclusively in the OS, whereas both retinas on the *rds*^{-/-} background showed labelling of the inner segments with the 1D4 opsin monoclonal and labelling of the inner segments and around the nucleus with the polyclonal anti-opsin antibody, indicating that expression of the C214S transgene does not affect rod opsin localization.

C214S retinal histology

Retinal phenotype and progression of disease were evaluated in all lines and compared with their non-transgenic littermates on different *rds* genetic backgrounds. In the present study, we provide the structural analysis at 1 month (Figure 3A) and 6 months (Figure 3B) of age on the wild-type, heterozygous (*rds*^{+/-}) and null (*rds*^{-/-}) backgrounds. C214S retinas on the wild-type background were morphologically indistinguishable from non-transgenic littermates at ages up to 6 months, as indicated by the number of photoreceptor nuclei and OS length (Figure 3). Histological analysis of the same genotypes given above was performed at 1 year of age with the same results (results not shown). This indicates the absence of a dominant negative effect of the transgene on the wild-type background. It also suggests that the C214S protein does not affect the endogenous P/*rds* in its ability to make OSs, even at 6 months of age (Figure 3B). However, on the *rds*^{+/-} and *rds*^{-/-} backgrounds, expression of the C214S transgene was insufficient to rescue the *rds*^{+/-} phenotype or to support the OS formation in the *rds*^{-/-} retina, which suggests a loss-of-function defect. If such a structural rescue were to occur, it would most probably be observed at 1 month of age (Figure 3A), with a noticeable improvement on OS structure in the *rds*^{+/-} retinas and at least partial formation of OS material in the *rds*^{-/-} retinas.

Electron microscopy was used to compare OS ultrastructure in C214S retinas on the wild-type and *rds*^{+/-} backgrounds in relation to the non-transgenic controls (Figure 3C). On the wild-type background, OSs in C214S retinas contained uniformly stacked discs, whereas on the *rds*^{+/-} background they exhibited disorganized and whorled discs similar to those in non-transgenic *rds*^{+/-} retinas. These observations establish the fact that even at an ultrastruc-

Figure 3 Morphology of C214S retinas is indistinguishable from controls on the same *rds* genetic backgrounds

Retinas taken from (A) 1 month old mice and (B) 6 months old mice. Retinal sections are representative of retinas from four different animals. The left panels of both (A) and (B) represent non-transgenic retinas, whereas the right panels represent C214S animals bred into the corresponding backgrounds (+/+, *rds*^{+/-} or *rds*^{-/-}). (C) Electron microscopy of rod OSs from non-transgenic retinas (left panel) and C214S retinas in the indicated genetic backgrounds (right panel). Scale bar, 4 μm.

tural level, the OS structure in C214S transgenic mice is indistinguishable from the *rds*^{+/-} background. Again, due to the loss-of-function mutation, the amount of mutant protein is too little to positively or negatively impact the OS appearance in the *rds*^{+/-} and *rds*^{-/-} mice.

C214S mice and retinal function

To correlate retinal structure and function, dark- and light-adapted ERGs were obtained from C214S mice and corresponding non-transgenic littermates on all *rds* genetic backgrounds to assess rod and cone function respectively. At 1 month of age, both the scotopic and photopic ERGs from the C214S retinas on the wild-type background were comparable with that from non-transgenic controls, suggesting that the mutant protein does not exert a dominant negative effect on the function of rods and cones. Similarly, the C214S/*rds*^{+/-} and *rds*^{+/-} mice showed a comparable reduction in rod *a*-wave amplitude (~40 % of wild-type) and a similar level of decline in cone *b*-wave response (~90 % of wild-type) at 1 month of age (Figure 4A). Furthermore, the C214S/*rds*^{-/-} and *rds*^{-/-} mice had no signs of rod or cone responses to the light stimulus, confirming our earlier observation that the small amount of mutant protein could not alleviate the *rds*^{-/-} phenotype (Figure 4A).

Figure 4 The C214S mutation causes a loss-of-function phenotype

Dark-adapted (left panels in **A** and **B**) and light-adapted (right panels in **A** and **B**) ERG amplitudes from 1 month old (**A**) and 6 months old (**B**) C214S and non-transgenic mice on different *rds* backgrounds. ERG values are an average of 8–10 eyes for each genotype.

The trend between C214S and non-transgenic mice seen at 1 month of age was also noticed at 6 months of age (Figure 4B). At this age, the C214S/*rds*^{+/-} and *rds*^{+/-} mice exhibited a further loss of scotopic *a*-wave amplitudes, to approx. 20% of the wild-type level (Figure 4B, left panel), whereas photopic *b*-wave amplitudes were further decreased to approx. 60% of the wild-type level (Figure 4B, right panel). Again, at 6 months of age, the C214S/*rds*^{-/-} and *rds*^{-/-} mice showed absolutely no response to light (Figure 4B). Both transgenic and non-transgenic littermates showed less robust rod and cone amplitudes at 6 months of age when compared with the 1 month old animals. This further confirms our earlier observation of a progressive, age-related retinal degeneration as the natural course in wild-type mice [27]. Overall, the ERG data demonstrate that the C214S mutation causes a loss-of-function defect similar to the haploinsufficiency phenotype seen with the *rds* mutation.

C214S protein expression in non-photoreceptor cells

Characterization of *P/rds* with the C214S mutation in 13 different transgenic lines demonstrated that the mutant protein level is significantly low, despite the presence of a substantial amount of transgene message. Additionally, results obtained from a transgenic line (line 550), which initially was not considered for inclusion in the present study, showed ectopic expression of C214S *P/rds* in the inner retinal cells, most probably because of a site-of-integration effect (Figure 5). This line was further characterized to confirm this observation and to assess its structural and functional phenotype. Northern-blot analysis of retinal RNA from this line showed a lower expression level of the C214S transgene when compared with the wild-type using the *rds* cDNA probe (Figure 5A, lanes 1 and 6). When evaluating the immunolocalization of the C214S protein in retinas from the 550 line on the *rds*^{-/-} background, we detected specific labelling in the inner nuclear layer (Figure 5B, lower right panel); in contrast, wild-type retinas exhibited immunolabelling exclusively in the OSs (Figure 5B, upper right panel). This phenomenon was seen in several different retinal sections from the 550 line on various *rds* backgrounds (results not shown). Furthermore, this line was capable of expressing

a low amount of C214S protein (both the upper and lower bands) with the protein levels accumulating with age (Figure 5C, lanes 2–5). As expected, immunoprecipitation of this small amount of C214S protein did not pull-down Rom-1 (Figure 5D, lane 6), because of the absence of Rom-1 in these non-photoreceptor cells. To investigate further the possibility of cell-specific behaviour, C214S and wild-type *P/rds* proteins were transiently expressed in COS cells. Figure 6(A) shows the Western-blot analysis of COS cell extracts (30 µg each) compared with wild-type retinal extracts (5 µg). The amount of loaded wild-type retinal sample was decreased to obtain better comparison with the weaker signal from COS cell extracts. Recombinant wild-type *P/rds* expressed in COS cells (Figure 6A, lane 3) is comparable with wild-type retinal *P/rds* (Figure 6A, lane 1) with monomeric, dimeric and oligomeric forms of the protein. However, the recombinant C214S protein (Figure 6A, lane 4) shows signs of aggregation, with a clear decrease in the monomeric form of the protein. The subcellular distribution of wild-type and mutant *P/rds* was examined by immunocytochemistry (Figure 6B). As seen in the top panel of Figure 6(B), wild-type *P/rds* is uniformly localized to internal membranes throughout the cell, with no apparent localization at the cell surface. Previous studies have also documented this phenomenon and have proposed that a targeting signal in *P/rds* prevents its translocation to the plasma membrane [15]. Since COS-1 cells do not possess analogous disc structures, it is not surprising to observe the insertion of *P/rds* in the internal membranes of COS cells. Although internal localization of recombinant proteins in eukaryotic cells is generally believed to indicate improper folding of the protein, *P/rds* expressed in COS cells has consistently exhibited biochemical behaviour similar to retinal *P/rds* [15–19]. Furthermore, *P/rds* is capable of forming complexes with Rom-1 when co-expressed in COS cells, indicating that the recombinant protein is likely to be functional [15]. In contrast, recombinant C214S protein forms aggregates that are typical of misfolded proteins localized in compartments consistent with the endoplasmic reticulum (Figure 6B, bottom panel), which is an observation previously made by others [28].

DISCUSSION

The present study is the first to examine the disease pathogenesis of the C214S mutation in a transgenic mouse model. The generation of transgenic mice expressing C214S *P/rds* in rods and cones affords a unique opportunity to assess the short- and long-term effects of the C214S mutation *in vivo*. Using this animal model, we have demonstrated that the retinal phenotype associated with this mutation is a loss-of-function defect that causes a late onset autosomal dominant rod dystrophy. This mouse phenotype resembles those seen in patients carrying the C214S mutation in *P/rds*. We propose that the phenotype associated with the C214S mutation is a consequence of haploinsufficiency of *P/rds* similar to the phenotype seen in the *rds*^{+/-} retina.

Four lines of evidence implicate the C214S-mediated haploinsufficiency defect as the basis for ADRP seen in mice and patients carrying this mutation. First, a small amount of C214S protein was recovered from 13 different transgenic lines, despite the presence of an ample amount of transgene message, which suggests that either the mutation hampered the production of the mutant protein or the mutant protein is unstable. Secondly, the loss of cysteine residue at this position hindered associations of the mutant protein with Rom-1. Thirdly, expression of the C214S transgene on the background of two wild-type alleles caused no structural or functional defects in the transgenic retinas, which rules out the possibility of a gain-of-function defect associated with this mutation.

Figure 5 Expression of C214S protein in non-photoreceptor cells

(A) Northern-blot analysis of the 550 mouse line with the C214S transgene. Lanes 1–6 show the comparison of wild-type and *rds* RNA transcripts with the C214S RNA transcripts from the 550 line in the corresponding genetic backgrounds. (B) Immunohistochemical analysis of wild-type and C214S retinas from line 550 on the *rds*^{-/-} background at 1 month of age. The OS, inner segment (IS), outer nuclear layer (ONL) and inner nuclear layer (INL) are indicated. The C214S retina does not have any OSs, due to the *rds*^{-/-} background. Sections are representative of four different retinas per genotype. (C) Western blot of retinal extracts (20 µg/lane) from wild-type (lane 1) and C214S animals in the *rds*^{-/-} background at different ages (lanes 2–5; P, post-natal. Ages in days.) that were run on a 10% reducing gel. The blot was probed with anti-P/rds-CT and anti-actin antibody. (D) Western blot of *P/rds* immunoprecipitates (IP) of retinal extracts (20 µl of sample from the 50 µl eluate). Whole retinal extract (200 µg) was added to each reaction mixture (lanes 1–6). The blot was probed with anti-P/rds-CT and anti-Rom-1-CT antibody. The IgG was included as a control for loading.

Fourth, expression of the C214S transgene on the *rds*^{+/+} or *rds*^{-/-} backgrounds neither rescued the haploinsufficiency defect of the *rds*^{+/+} retina nor supported the formation of OSs in the *rds*^{-/-} retinas.

Another mutation at position 214 in *P/rds* has been reported in a Spanish family [29], where the cysteine residue is replaced by tyrosine residue. Interestingly, these patients develop autosomal dominant macular dystrophy, a disease that primarily affects the cones. The drastic difference in the pathogenic defects caused by the C214Y and the C214S mutations is probably due to the diverse properties of these two residues on the function of the protein in rods versus cones. This difference can be explained by the fact that cysteine and tyrosine are both hydrophobic, whereas serine is hydrophilic. Furthermore, the change from cysteine to either serine or tyrosine may affect the protein in various ways. For example, an interaction with another protein may be disrupted or the overall structure of the protein may be altered. For C214S, the hydrophilic serine probably causes a major change in the structure of the protein in addition to the disruption of the disulphide bond formation, which may lead to a global misfolding. In C214Y, although the disulphide bond is also interrupted, the hydrophobic tyrosine may behave more like cysteine regarding its position inside the protein, leading only to a minor alteration in the overall protein structure. It is possible that C214Y interferes

specifically with the association of *P/rds* with cone-specific proteins that are yet to be discovered while maintaining its interaction with Rom-1. This is analogous to the pathogenic defect caused by the replacement of arginine with tryptophan at position 172, which causes a cone-rod dystrophy similar to C214Y [23,29,30]. Studies in transgenic mice demonstrate that this mutant protein is capable of interacting with Rom-1 in the mouse retina [23], which is indicative of proper folding. Although specific alteration of amino acid properties may yield insight into disease-causing mutations, it is feasible that the differential association of proteins in cones versus rods also plays a substantial role in disease pathogenesis.

Since *P/rds* and Rom-1 normally interact non-covalently to build functional oligomeric complexes, the lack of such interactions caused by the C214S mutation is indicative of abnormalities in the conformation of the mutant protein. This phenomenon was supported by the abnormal profile observed by velocity-sedimentation analysis of C214S expressed in COS cells [19]. When C214S is expressed in photoreceptor cells *in vivo* on all *rds* backgrounds, only a small amount of the C214S mutant protein was detected, having characteristics consistent with those of a newly synthesized product that has not completed its transit through the endoplasmic reticulum/Golgi. That is, it appears that the protein neither fully completed its maturation through

Figure 6 Protein analysis of C214S and wild-type *P/rds* expressed in COS cells

(A) Western blot of *P/rds* from wild-type retinal extract (5 μ g in lane 1) and COS cell extracts (30 μ g in lanes 2–4). The control is extracted from COS cells transfected with the pcDNA3.1 vector alone. (B) Immunocytochemistry of COS cells transiently transfected with either wild-type *rds* cDNA (pcDNA3.1-WT) or C214S *rds* cDNA (pcDNA3.1-C214S). The inset is a picture of a single cell without the DAPI staining. Pictures are representative of five different fields for three separate experiments.

post-translational processing (e.g. trimming of its glycan chains) nor did it encounter the cellular protein degradation machinery in the cytosol. This faint amount of mutant protein exhibited neither homotypic nor heterotypic binding capacity. The mutant protein also could not form tetramers, a complex that seems to be necessary for stability and trafficking of *P/rds* complexes to the photoreceptor OSs [18,19,31]. In transgenic *Xenopus* retinas, although the *Xenopus* C214S–GFP fusion protein was capable of forming homodimers, it failed to form tetramers (even with wild-type *P/rds*) and was retained in the cell body and inner segment of the rod cells [18]. Additionally, C214S protein appeared to be held at a checkpoint near the base of the *Xenopus* inner segment. In the present mouse model, the loss-of-function mutation causes little protein to remain in the cell because the mutant protein is either not synthesized properly or degraded quickly. The processing and/or degradation of the mutant protein are important steps that precede the inner segment checkpoint. Perhaps the checkpoint(s) and protein processing of *P/rds* have diverged throughout the evolution of vertebrate organisms. Certainly, *P/rds* has undergone several modifications throughout evolution [32,33]. Modification of protein processing may have evolved concomitantly with the protein structure modifications. Notably, *Xenopus* contains three endogenous homologues of the *rds* gene and lacks Rom-1 [13], making *Xenopus* a complicated model for studying the pathophysiology of *P/rds*-associated human retinal dystrophies and the subsequent development of therapeutic strategies. The complexity of *P/rds* processing may explain the diversity of data obtained from the *Xenopus* and the mouse model. Transgenic mice are acceptable animal models for studying the pathogenesis of retinal disease due to the numerous studies in mice that correlate well with human retinal physiology and disease processes.

A variant transgenic C214S *P/rds* line (550) revealed an age-dependent accumulation of the mutant protein in inner retinal cells, rather than in photoreceptor cells. Also, unlike the mutant protein from the 204 line, C214S protein from the 550 line mi-

grated as two distinct bands. The results obtained from this line, compared with those obtained with the other transgenic lines (on all *rds* backgrounds) and with transiently transfected COS cells, clearly demonstrate that the stability of the C214S protein is a function of the cell type in which it is expressed. Because photoreceptor cells are highly specialized, their protein processing machinery may have evolved accordingly to sustain their distinctiveness. In particular, since *P/rds* resides only in photoreceptors, it may interact with chaperones that are also photoreceptor-specific.

Retinal degenerative diseases represent a qualitatively and quantitatively significant healthcare issue worldwide, profoundly and negatively impacting quality of life and human productivity. Understanding the mechanisms behind these diseases is imperative for the development of rational therapeutic prevention and intervention regimes. Analysis of the C214S mutation in transgenic mice provides an informative basis for the further understanding of ADRP associated with this mutant, and may be extended to the understanding of other mutations in *P/rds*, perhaps leading to global therapeutic approaches. Recent experiments in our laboratory, employing overexpression of wild-type *P/rds* gene product as a means of counteracting the C214S mutant protein in transgenic animals on the *rds*^{+/-} background, suggest a potentially viable method of blocking the retinal degeneration caused by this mutation (M. Nour, H. M. Stricker, S. J. Fliesler and M. I. Naash, unpublished work). Results of the present study confirm that the C214S mutation causes a loss-of-function phenotype, similar to the haploinsufficiency phenotype observed in the *rds* mice, and that the C214S mouse model is suitable for studying the pathogenesis of ADRP in relation to humans who carry the same mutation.

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