Differential Loss of Prolyl Isomerase or Chaperone Activity of Ran-binding Protein 2 (Ranbp2) Unveils Distinct Physiological Roles of Its Cyclophilin Domain in Proteostasis*

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Kyoung-in Cho‡1, Hemangti Patil†1, Eugene Senda‡2, Jessica Wang‡2, Haqing Yi‡3, Sunny Qiu1, Dosuk Yoon1, Minzhong Yu1, Andrew Orry1, Neal S. Peachey§**, and Paulo A. Ferreira†‡4

From the ‡Departments of Ophthalmology and Pathology, Duke University Medical Center, Durham, North Carolina 27710, the §Department of Ophthalmic Research, Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, †MolSoft LLC, San Diego, California 92121, the †‡Research Service, Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106, and the **Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44195

The immunophilins, cyclophilins, catalyze peptidyl cis-trans prolyl-isomerization (PPIase), a rate-limiting step in protein folding and a conformational switch in protein function. Cyclophilins are also chaperones. Noncatalytic mutations affecting the only cyclophilins with known but distinct physiological subdomains, the Drosophila NinaA and its mammalian homolog, cyclophilin-B, impair opsin biogenesis and cause osteogenesis imperfecta, respectively. However, the physiological roles and substrates of most cyclophilins remain unknown. It is also unclear if PPIase and chaperone activities reflect distinct cyclophilin properties. To elucidate the physiological idiosyncrasy stemming from potential cyclophilin functions, we generated mice lacking endogenous Ran-binding protein 2 (Ranbp2) and expressing bacterial artificial chromosomes of Ranbp2 with impaired C-terminal chaperone and with (Tg-Ranbp2WT-HA) or without PPIase activities (Tg-Ranbp22944A-HA). The transgenic lines exhibit unique effects in proteostasis. Either line presents selective deficits in M-opsin biogenesis with its accumulation and aggregation in cone photoreceptors but without proteostatic impairment of two novel Ranbp2 cyclophilin partners, the cytokine-responsive effectors, STAT3/STAT5. Stress-induced STAT3 activation is also unaffected in Tg-Ranbp22944A-HA::Ranbp2−/−/−. Conversely, proteomic analyses found that the multisystem proteinopathy/amyotrophic lateral sclerosis proteins, heterogeneous nuclear ribonucleoproteins A2/B1, are down-regulated post-transcriptionally only in Tg-Ranbp22944A-HA::Ranbp2−/−/−. This is accompanied by the age- and tissue-dependent reductions of diubiquitin and ubiquitylated proteins, increased deubiquitylation activity, and accumulation of the 26 S proteasome subunits S1 and S5b. These manifestations are absent in another line, Tg-Ranbp2CLDm-HA::Ranbp2−/−/−, harboring SUMO-1 and S1-binding mutations in the Ranbp2 cyclophilin-like domain. These results unveil distinct mechanistic and biological links between PPIase and chaperone activities of Ranbp2 cyclophilin toward proteostasis of selective substrates and with novel therapeutic potential.

Peptidyl cis-trans-prolyl isomerization is a rate-limiting step in protein folding (1–3). The catalysis of the cis-trans interconversion of the peptidyl-prolyl isomers is catalyzed by peptidyl-prolyl cis-trans isomerases (PPIase)5 (4–6). PPIases compose three families of structurally unrelated proteins, the cyclophilins (CyP), FK506-binding proteins (FKBP), and parvulins (7).

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1 Both authors contributed equally to this work.

2 Supported by Deans’ Summer Undergraduate Research Fellowships at Duke University.

3 Present address: Dept. of Pediatrics, Duke University Medical Center, Durham, NC 27710.

4 Jules and Doris Stein Research to Prevent Blindness Professor. To whom correspondence should be addressed: Duke University Medical Center, DUEC 3802, 2351 Erwin Rd., Durham, NC 27710. Tel.: 919-684-8457; Fax: 919-684-3826; E-mail: paulo.ferreira@duke.edu.

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The abbreviations used are: PPIase, peptidylprolyl cis-trans isomerase; Ranbp2, Ran-binding protein 2; RBD4, Ran-binding domain 4; BAC, bacterial artificial chromosome; STAT, signal transducer and activator of transcription; Cy, cyclophilin domain of Ranbp2; CsA, cyclosporin A; FKBP, FK506-binding protein; CyP, cyclophilin protein; CLD, cyclophilin-like domain; HDAC4, histone deacetylase-4; RanGAP, Ran GTPase-activating protein; UCH-L, ubiquitin C-terminal hydrolase; DUB, deubiquitylase; mRFP, monomeric red fluorescent protein; 2D-DIGE, two-dimensional difference in-gel electrophoresis; ALS, amyotrophic lateral sclerosis; ERG, electoretinogram; VEP, visual evoked potential; idv, integrated density values; qRT, quantitative RT; IF, immunofluorescence; IB, immunoblot; TRITC, tetramethylrhodamine isothiocyanate; pNA, p-nitroanilide; Suc, succinyl; cd, candela; Tg, transgenic; hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1.
CyPs and FKBP s are designated also as immunophilins, because they mediate immunosuppression (8, 9). This effect is achieved by a gain-of-function mechanism upon binding of the immunosuppressive metabolites, cyclosporin A (CsA) or FK506, to the PPIase active site and formation of a ternary complex with the serine/threonine phosphatase, calcineurin, whose sequestration and inhibition prevents the dephosphorylation and activation of the nuclear factor for activation of T-cells (9–12). However, subsequent work showed that the PPIase activity of the immunophilin and major cellular CsA target, cyclophilin A (CyPA/PPIA), contributes also to the immunomodulatory properties in CD4+ T-cells by negatively regulating Itk kinase via cis-trans isomerization of a proline in its Src homology 2 domain (13, 14). This notion of regulation of protein activity by immunophilin-mediated conformational switches of proline isomers (15, 16) was also found by previous and subsequent studies, in which distinct immunophilins were shown to promote the association of substrates to protein or DNA partners (17–19), formation of oligomeric complexes (20), or regulation of receptor and channel activities (21–23).

Another critical function associated with immunophilins, such as cyclophilins, is that of a chaperone (17, 24–28). Chaperones facilitate protein folding and prevent protein misfolding and aggregation and thus enhance the yield of properly folded proteins without affecting their folding rates (29). Impairment of protein chaperoning is thought to disturb the assembly of protein complexes, protein sorting, or degradation (26–28, 30–35). For example, mutations affecting the cyclophilin, NinaA, of Drosophila impair selectively the biogenesis of two opsin receptor subtypes (28, 30, 31). Recent evaluation of a large number of mutations affecting NinaA (36) found that none of these overlapped with key catalytic residues (37). Instead, the mutations were clustered near the catalytic pocket (S2/S2e) or these overlapped with key catalytic residues (37). The broader cellular expression of NinaA and CyPB/PPiB than those of their physiological substrates strongly support that the catalytic or chaperone activities of cyclophilins act on a more limited pool of physiological substrates than previously predicted from biochemical studies on cyclophilins. This notion is also supported by the apparent and restrictive nephrotoxic effects of CsA (41) and by the nonessential role of all eight CyPs and four FKBP s in yeast (42).

These and other studies raise important questions about the following: (i) the molecular bases of the substrate-selective effects of NinaA and CyPB, and possibly of other cyclophilins; (ii) the functional relationships between chaperone and PPIase activities of immunophilins, and importantly, (iii) the physiological and pathobiological roles of all other single and multidomain cyclophilins (~19) in health and disease. These issues assume even higher significance, because of recent reports that viral agents (e.g. HIV-1 and hepatitis C virus) exploit poorly defined activities of cyclophilins to promote infectivity (43–54), that a number of novel immunophilin-binding drugs present distinct pharmacological and therapeutic properties from CsA and FK506 (55), that CsA promotes prion protein aggresomes (56), and that undefined cyclophilin (CyPA) activities promote neuroprotection against mutations associated with familial amyotrophic lateral sclerosis (ALS) in CuZn superoxide dismutase (57). Hence, CyPA/PPIA has emerged as a major target in these and other pathobiological processes, but its high intracellular concentration and high homology to another member of the cyclophilin family of proteins raise the possibility that a number of roles attributed to CyPA may be carried out completely or in part by other poorly characterized cyclophilin member(s).

Growing evidence supports that the large multimodal and pleiotropic Ran-binding protein-2 (Ranbp2) via its C-terminal cyclophilin domain (CY), which has the highest homology to CyPA (58–60), facilitates viral infectivity alone or in cooperation with other modules of Ranbp2 (46, 48, 54, 61). The localization of Ranbp2 at cytoplasmic fibrils emanating from the nuclear pore complex supports that Ranbp2 also plays a prominent role in the modulation of the nucleocytoplasmic shuttling of substrates, a process thought to require the partial unfolding of shuttling substrates for their passage through the nuclear pore complex (48, 62–66). However, the CY of Ranbp2 was first shown to associate directly and selectively with L/M-opsin and promote the interconversion of L/M-opsin isoforms (17, 18). This interaction involves the CY-dependent association of L/M-opsin to the adjacent RBD4 of Ranbp2 and the enhancement of production of functional L/M-opsin receptor (pigment) in a heterologous expression system (17, 18). Although Ranbp2 is essential to the viability of mouse (67, 68) and cone photoreceptors (69), the physiological and biological roles and substrates of CY of Ranbp2 and the contribution of CY to the pleiotropic and pathobiological roles of Ranbp2 remain elusive. Here, we report the application of combined genetic, cellular, biochemical, and electrophysiological approaches in the mouse models of Ranbp2 with impaired PPIase or chaperone activities to uncover mechanistic and biological links between idiosyncratic activities of CY of Ranbp2, novel physiological substrates and regulation of their proteostasis, and promising venues toward novel therapeutic interventions.

**EXPERIMENTAL PROCEDURES**

**BAC Recombineering—** A bacterial artificial chromosome (BAC) clone, RP24-347K24, from the RPCI mouse BAC library 24 and ~151 kb in size, was identified in silico, by DNA sequencing and restriction mapping, to contain the complete structural Ranbp2 gene (~65 kb) and with its upstream (~45 kb) and downstream (~50 kb) regulatory sequences. The BAC RP24-347K24 clone was obtained from the BACPAC Resource Center at the Children’s Hospital Oakland Research Institute. BAC recombineering was used to generate BAC constructs from the parental BAC clone as described previously with the following modifications (70). Briefly, *Escherichia coli* SW102 cells (gift from Neal G. Copeland) were electroporated with 1 µg of purified BAC DNA (RP24-347K24), grown at 32 °C to an *A*₂₆₀ of 0.6, induced at 42 °C for 15 min, washed, and resuspended in ice-cold 10% glycerol. Competent cells were electroporated with 200 ng of double-stranded DNA oligonucleotide
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containing an HA tag sequence flanked at the 5’ and 3’ end with 50 bp of homologous recombination sequences of the terminal exon and 3’UTR of Ranbp2, respectively. After 1 h of recovery in 1 ml of Super Optimal Broth with glucose at 32 °C, the cells were washed three times with Luria Broth, diluted to ~30–40 cells per ml of Luria Broth (20 μg/ml chloramphenicol) into several tubes, and grown for 18–20 h at 32 °C, and cell pellets were collected from 40-μl aliquots. To identify the targeted clone, PCR was performed with external primers flanking the recombination site. Identified culture clones underwent serial dilutions and PCR to obtain individual colonies of recombinant BAC. Targeting was confirmed by dideoxy sequencing and NotI restriction mapping of recombinant BAC DNAs purified with Nucleobond-BAC 100 kit (Macherey-Nagel, Germany) as per the manufacturer’s instructions. Identical procedures were used to generate recombinant BACs comprising mutations in Ranbp2 (GenBank™ accession number NP_035370.2) with the exception that purified 500-bp amplicons with each mutation were used for electroperoration and recombination. Mutant amplicons were generated by a two-step PCR with one pair of complementary primers comprising the point mutation(s) and another pair flanking the mutated primers upstream and downstream. Freshly purified and recombinant BAC DNA was diluted in microinjection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 30 μM spermine, 70 μM spermidine, 100 mM NaCl) until microinjection.

Generation of Transgenic Mice—Recombinant BACs were injected into pronuclei to generate transgenic mice after implantation into pseudo-pregnant females. Microinjections were performed at the Transgenic Mouse Facility and Duke Neuroscience Laboratory of Duke University Medical Center. Positive F0 founders and expressers were identified by PCR from genomic DNA of tail biopsies and immunoblot analyses of tail homogenates with anti-HA antibodies. Positive founders were then mated with Ranbp2+/pGT0pfs630Wcs/Gt/H9262 mice to generate BAC transgenic mice on a homozygous background. Genomic PCR, RT-PCR, and immunoblot analyses of F1 and F2 progenies confirmed the transmission of the BAC transgenes. Generation of floxed Ranbp2 mice were described previously (67, 69). Mice were raised in a pathogen-free transgenic barrier facility at 37 °C in presence of 5% CO2 and 100% humidity. Exponentially growing cells were transiently transfected with pDest-733 (mRFP) vector fused in-frame to wild-type CY and mutant CYR2944A and CYS3036E of Ranbp2 using Lipofectamine-2000 (Invitrogen). Upon 24 h of incubation, cells were harvested, washed three times with Luria Broth, diluted to 100 mM Tris, 150 mM NaCl, 1% Nonidet P-40, mini-complete protease inhibitor tablet (Roche Applied Science, King of Prussia, PA). Substrates were dissolved to a concentration of 2.6 mM in tetrahydrofuran containing 0.25 mM LiCl (73). Bovine pancreas a-chymotrypsin type 1-S (Sigma) was dissolved in 2 mg/ml Tris buffer (100 mM Tris, pH 7.8, 0.1 mM MgCl2, 1 mM EGTA). All solutions were kept on ice. 50 μl of 2 mM wild-type or mutant CY or RBD4-CY constructs and 920 μl of chymotrypsin were mixed in the cuvette first. The reaction was initiated by adding 30 μl of 2.6 mM solution of the peptide substrate. The hydrolysis of the peptide was monitored by collecting the absorbance at 400 nm for 5 min with a SpectraMax M5 spectrophotometer (Molecular Devices). Readings at time 0 were subtracted as baseline from all subsequent readings. The data were then analyzed, and the first-order rate constants, k, were obtained by nonlinear curve fitting to the equation, A = A0(1 − e−kt), using SigmaPlot 8.0 (Systat Software Inc., San Jose, CA). Five measurements were performed for each substrate. The catalytic efficiency kcat/Km was obtained by the equation, kobs = (k_cat/K_m)[E] + k_sp where [E] is the concentration of the PPIase (CY); kobs is the measured apparent first-order rate constant when a PPIase is present, and k_sp is the measured apparent first-order constant when PPIase is absent (spontaneous isomerization) (74). Under our assay conditions, molar absorption coefficient at 400 nm was determined experimentally as 𝜀 = 0.011 μM⁻¹ cm⁻¹.

Cell Culture and Transfections—HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum at 37 °C in presence of 5% CO2 and 100% humidity. Exponentially growing cells were transiently transfected with pDest-733 (mRFP) vector fused in-frame to wild-type CY and mutant CYR2944A and CYS3036E of Ranbp2 using Lipofectamine-2000 (Invitrogen). Upon 24 h of incubation, cells were harvested, washed with 1× PBS, and lysed in Nonidet P-40 buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, mini-complete protease inhibitor tablet (Roche Applied Science)). After 30 min of incubation at 4 °C, the cell lysates were passed through 21½-gauge syringes and centrifuged for 10 min at 10,000 × g, 4 °C. The supernatant was collected and used for Western blot analysis or immunoprecipitation assays.

Antibodies—To generate rabbit antibodies, Ab-W1W2#9 and #10, against the homologous internal repeat (IR) IR1 + 2 domain of human RANBP2 (59, 60), a Bsu36I/BamHI restriction fragment comprising the W1/W2 domain of bovine Ranbp2 (58) was subcloned into pGEX-KG vector, expressed, purified, and cleaved from the GST moiety with thrombin (72). Two antibodies were independently generated from two rabbits upon four booster shots with recombinant protein (~100 μg) and Hunter’s TiterMax Gold adjuvant (CytRx Corp., Norcross, GA) followed by affinity purification against the same antigen under non-denaturing conditions according to the manufacturer’s instructions (Stereogene, Arcadia, CA). Both antibodies produced identical results in immunohistochemistry, and no signal was detected in tissues of transgenic Ranbp2 mouse lines lacking expression of Ranbp2 in selective tissues.
Ab-W1W2#10 was employed at 8 µg/ml for immunofluorescence. Other antibodies used for immunofluorescence (IF) or immunoblots (IB) were as follows: rabbit anti-DsRed (1:1,500 (IB), Clontech); rat anti-HA (1:500 (IB), Roche Applied Science); mouse anti-HA (1:100 (IF), Abcam, Cambridge, MA); mouse anti-ubiquitin (1:1,000 (IB), Santa Cruz Biotechnology); rabbit anti-hsc70 (1:3,000 (IB), ENZO Life Science, Farmingdale, NY); monoclonal mouse anti-actin (α-tubulin (1:40,000 (IB), Sigma); rabbit anti-L/M opsin no. 21069 (1:500 (IF), 1:1000 (IB)) (75); rabbit anti-S opsin (1:2,500 (IB), 1:100 (IF), JH455, gift from Jeremy Nathans); goat anti-S opsin (1:100 (IF) Millipore, Temecula, CA); peanut agglutinin TRITC conjugate (1:100 (IF), Sigma); rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:500 (IB), Covance, Emeryville, CA); mouse anti-Lamin A/C (1:1,000 (IB), BD Biosciences); rabbit anti-19 S proteasome S1 (1:1000 (IB), Invitrogen); rabbit anti-Nr2e3 (1:1,000 (IB), Proteintech); rabbit anti-RANGAP1 (1:1,000 (IB), Invitrogen); rabbit anti-Nr4r2e3 (1:1,000 (IB), Proteintech); rabbit anti-COUP-TF1 (1:1,000 (IB), Abcam); rabbit anti-STAT3 (1:1,000 (IB), Abcam); rabbit anti-phospho-STAT3 (1:1,000 (IB), Abcam); rabbit anti-phospho-STAT5 (1:1000 (IB), Proteintech); rabbit anti-COUP-TF1 (1:1,000 (IB), Abcam); rabbit anti-γ-crystalline (gift from Vasanth Rao, originated by Dr. Sam Zigler); mouse PSR-45 (anti-phosphoserine antibody, 1:1000 (IB), Abcam ab6639); Alexa Fluor-conjugated secondary antibodies and Hoechst 33,342 were from Invitrogen.

Co-immunoprecipitations and Western Analysis—Nonidet P-40 extracts of transiently transfected HeLa cells were adjusted to 500 µg of protein per reaction and pre-cleared with nonimmune IgG and protein A/G-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. Immunoprecipitations were performed with 2 µg of rabbit-DsRed antibody and 20 µl of 50% protein A/G-agarose beads at 4 °C and overnight. Beads were washed three times with Nonidet P-40 buffer, and proteins were eluted with Laemmli buffer. Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE and blotted on PVDF membrane. Membranes blocked with nonfat dry milk block solution (Bio-Rad) were used to probe all antibodies as described elsewhere (72) with the exception of anti-phosphoserine antibody, which was probed overnight at 4 °C in 0.5% BSA on membranes blocked with 5% BSA (25 °C/1 h). Membranes were probed with anti-phosphoserine, anti-STAT3, anti-phospho-STAT3, anti-phospho-STAT5, or anti-DsRed antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody (25 ng/ml). The immunoreactive proteins were visualized by incubating with enhanced chemiluminescence reagent (Pierce) and were exposed to X-ray Hyperfilm (Amershams Biosciences). For densitometric analysis, integrated density values (idv) for the representative bands were normalized to the background and RFP idv. For graphical representation of amount of phosphorylation in CY constructs, the phosphoserine immunoreactive band was normalized to RFP.

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For co-immunoprecipitation assays with mouse tissues, midbrain and retinal extracts were solubilized in Nonidet P-40 buffer using Bullet Blender BBX24 (Next Advance Inc., Averill Park, NY) at 4 °C in the presence of 0.5-mm zirconium oxide beads (Next Advance Inc.). 3–4 and 1.25 µg of midbrain and retinal extracts, respectively, were used for immunoprecipitation reactions after preclearing with 2 µg of nonimmunized IgG and 50 µl of 50% protein A/G bead slurry (Santa Cruz Biotechnology) at 4 °C. Immunoprecipitations were carried out using mouse anti-HA antibody (Abcam ab18181; 3.5–4 µg per reaction) at 4 °C. Co-immunoprecipitate complexes were resolved by SDS-PAGE and processed for Western blot analysis as described (72).

Subcellular Fractionations—The nuclear and non-nuclear subcellular fractionations of one frozen retina of 5-week-old transgenic and nontransgenic mice were prepared with the Qproteome cell compartment kit as described by the manufacturer’s instructions (Qiagen, Valencia, CA) with the following two exceptions: 1) the cytosolic fraction was collected from the retinal total lysate upon centrifugation at 1,000 rpm, and 2) the volumes for cytosolic, membrane, and cytoskeleton fractions were 50 µl each and they were later combined as one fraction; whereas the total volume of the nuclear fraction was 100 µl per one retina. Protein concentrations of samples were determined by micro-BCA kit (Pierce). All subcellular fractions were solubilized in SDS sampler buffer and resolved by SDS-PAGE, and immunoblots were carried out with antibodies against proteins of interest and markers to each subcellular fraction. The idv of the immunoblot bands was first corrected by subtracting the idv background of the same area in the corresponding lane. Then they were normalized to the idv of Nup62 of the same fraction. Upon normalization, the idv of nuclear and non-nuclear fractions for each of protein in transgenic and nontransgenic mice were transformed into a percentage scale (total protein = 100%). Averages of the values of transformed percentage data were compared using two-sample t test with assumption of unequal variance at the minimum significance level of 0.05 (Origin8.5).

Immunohistochemistry—Procedures for eyecup and retinal tissue collection, fixation, processing, and microscopy imaging, were carried out exactly as described previously (69). For hnRNP A2/B1 immunostaining, eyeballs were fixed instead with 1% paraformaldehyde for 1 h at room temperature. The 10-µm-thick retinal cryosections were incubated in blocking buffer for 1 h at room temperature followed by treatment with proteinase K (20 µg/ml, Promega, Madison, WI) for 9 min and standard immunostaining protocols as described elsewhere. Images were acquired with a Nikon C1+ laser-scanning confocal microscope coupled with an LU4A4 launching base of four solid-state diode lasers (407 nm/100 milliwatts, 488 nm/50 milliwatts, 561 nm/50 milliwatts, 640 nm/40 milliwatts) and controlled by the Nikon EZC1.3.10 software (version 6.4).

Morphometric Analyses—Morphometric analyses of cone photoreceptors immunolabeled for M- and S-opsin and peanut agglutinin were performed from 127 × 127-µm image fields captured with a Nikon C1+ laser-scanning confocal microscope essentially as described previously (69). Briefly, immunostained retinal flat mounts were used to capture optical slices.
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for three-dimensional reconstruction throughout the whole length of outer segments (~25 μm, step size of 0.5 μm). Cone photoreceptors were tallied from three image fields for each region of the retina with the post-acquisition Nikon Elements AR (version 3.2) software. Two-tailed equal or unequal variance t test statistical analysis was performed. p ≤ 0.05 was defined as significant.

Light Treatment of Mice and TUNEL Assays—Ten-12-week-old wild-type and Tg-Ranbp2R2944A-HA::Ranbp2-/- in a heterozygous Rpe65<sup>L450Q/M450Q</sup> background were placed in a white reflective cage with bedding, food, water, and a panel of dimmable light-emitting diode lights mounted at the top of the cage. After 18 h of dark adaptation, the pupils of the mice were dilated by applying 1% atropine sulfate and 10% phenylephrine hydrochloride (HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA) into their eyes. Mice were exposed to 5,000 lux of continuous white light-emitting diode for 24 h followed by 24 h in the dark. Mice were killed, and the eyeballs were immediately collected and processed for immunohistochemistry and morphometric analyses. TUNEL assays were performed with the DeadEnd Fluorometric TUNEL System (Promega, Madison) as described previously.

Modeling of CY of Ranbp2—The CY model of Ranbp2 was built using a stochastic global energy optimization procedure in internal coordinate mechanics (ICM) in the ICM-Pro desktop modeling package version 3.7 (MolSoft LLC, San Diego). A Blast search identified the template cyclophilin structure in the Protein Data Bank with the highest sequence similarity and best coverage compared with CY of Ranbp2 primary sequence. Based on this analysis the CyPA/PPIA structure with Protein Data Bank code 1w8m was chosen as the template for modeling. An alignment was generated between CY and the template sequence using an adaptation of the Needleman and Wunsch algorithm. The model was refined by globally optimizing the side chains and annealing the backbone. The iterative refinement procedure contains three major steps as follows: (i) random sampling of the dihedral angles according to the biased probability Monte Carlo method; (ii) a local minimization step, and (iii) the Metropolis criterion is then used to accept or reject a conformation. The lowest energy structure was selected as the final CY model.

2D-DIGE Protein Expression Profiling—Global protein profiling between nontransgenic and transgenic mice was carried out by 2D-DIGE of retinal homogenates solubilized in RIPA buffer followed by buffer exchange in two-dimensional lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.8). Samples were provided to Applied Bioscics (Hayward, CA) for CyDye labeling and resolving in analytical and Prep gels. Spots with expression variation between genotypes were identified with DeCyder “in-gel” analysis software, and protein spots of interest were picked for protein identification by mass spectrometry (MALDI-TOF/TOF) and database search for protein ID. Data analyses and validation of mass spectrometry data were performed by the Ferreira laboratory.

Quantitative RT-PCR—For total RNA isolation, tissues were collected and stored in RNAlater<sup>®</sup> (Invitrogen) at ~80 °C until use. After recovering from RNAlater<sup>®</sup>, tissues were homogenized with TRIzol reagent (Invitrogen) using Bullet Blender BBX24 (Next Advance Inc., Averill Park, NY) in the presence of 0.5-mm zirconium oxide beads (Next Advance Inc.) for 3 min at 8,000 rpm. mRNA was reverse-transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen), oligo(dT) primer, and the two 5’ Ranbp2 gene-specific primers, Rbp2ex19, CAGACCGCTGCTAATTGTAACCTCCA, and Rbp2ex20, TGGGCCCAGTTTCTGTAAGTATT, as per the manufacturer’s instructions. The quantitative RT-PCR were carried out with 20 ng of cDNA, 800 fm forward and reverse primers, 10 μl of 2× SYBR<sup>®</sup> Green PCR Master Mix (Invitrogen) in a 20-μl final volume in 48-well plates using the ECO<sup>™</sup> real time PCR system (Illumina, San Diego). The amplification was carried out by the following: (i) polymerase activation at 95 °C for 10 min, and (ii) PCR cycle at 95 °C for 15 s and 60 °C for 1 min, 40 cycles. The relative amount of transcripts was calculated by the ΔΔC<sub>T</sub> method using Gapdh as reference (n = 3–4). Primer sequences used for the experiment were as follows: Gapdh-f, GCAGTGGGAAGGTGGAGTT, and Gapdh-r, GAATTTGCCGATGATGGAGT; hnRNPA2/B1-f, TTTGTAGCCATGACCTCCTGT, and hnRNPA2/B1-r, CTCTGAACTTCTCGATTTCTC; S-opsin-f, GCTTGACCTATGGCTTGGAC; and Opsin-r, CAGTGAGTTCTGGTGAC. Amplification was carried out by the following: (i) polymerase activation at 95 °C for 10 min, and (ii) PCR cycle at 95 °C for 15 s and 60 °C for 1 min, 40 cycles. The relative amount of transcripts was calculated by the ΔΔC<sub>T</sub> method using Gapdh as reference (n = 3–4). Primer sequences used for the experiment were as follows: Gapdh-f, GCAGTGGGAAGGTGGAGTT, and Gapdh-r, GAATTTGCCGATGATGGAGT; hnRNPA2/B1-f, TTTGTAGCCATGACCTCCTGT, and hnRNPA2/B1-r, CTCTGAACTTCTCGATTTCTC; S-opsin-f, GCTTGACCTATGGCTTGGAC; and Opsin-r, CAGTGAGTTCTGGTGAC.

Deubiquitylation and Proteasome Assays—Retinas were flash-frozen on dry ice, and each retina was resuspended in 60 μl of homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA and 0.0025% digitonin) and incubated on ice for 5 min. The extracts were then centrifuged at 20,000 × g for 15 min at 4 °C, and supernatants were transferred to new tubes (76). 1 μg of total protein from the supernatant was incubated with or without 20 S proteasome inhibitor (20 μM epoxomicin) or DUB inhibitors (25 μM PR-619 and/or 5 μM 1,10-phenanthroline) for 30 min at 37 °C. Then 100 μM 7-aminomethylcoumarin-conjugated substrates, 100 nM rhodamine-coupled Lys-63- or Lys-64-linked tetra-ubiquitin substrates were added and incubated for 45 min at 37 °C. Free AMC and rhodamine were measured at excitation/emission/cutoff = 380/460/455 and excitation/emission/cutoff = 485/535/530, respectively, with a SpectraMax M5 spectrophotometer (Molecular Devices). Specific proteolytic activities contributed by the 20 S proteasome were determined by subtracting the activities measured in the presence of epoxomicin from those in its absence. Specific DUB activities were determined by subtracting the activities measured in the presence of PR-619, 1,10-phenanthroline, or both, from those in their absence. The fluorescence intensity was normalized against the amount of S1 subunit of the 19 S regulatory particle in the soluble fraction that was detected by immunoblotting of 10 μg of supernatant (PA1-973, Thermo Fisher Scientific, Rockford, IN). Reagents used were the follow-
ing: epoxomicin (Calbiochem); PR-619 (Sigma); 1,10-phenanthroline (Sigma); Suc-LLVY-AMC (Boston Biochem, Cambridge, MA); Ac-RLR-AMC (Boston Biochem); Ac-nLPnLD-AMC (ENZO Life Science, Farmingdale, NY); Lys-63-linked tetraubiquitin-rhodamine 110 and Lys-48-linked tetraubiquitin-rhodamine 110 (LifeSensors, Malvern, PA).

**Visual Electrophysiology**—Mice were run using two recording protocols, designed to evaluate function of the outer retina or visual pathway (77). All studies were conducted following overnight dark adaptation, after which the mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) and placed on a temperature-regulated heating pad. The pupils were dilated with eye drops (2.5% phenylephrine HCl, 1% cyclopentolate, 1% tropicamide); for ERG studies, the corneal surface was anesthetized with 1% proparacaine HCl eye drops.

**Dark- and Light-adapted ERG**—We used a conventional strobe-flash ERG protocol to evaluate responses of the outer retina. The ERG was recorded using a stainless steel wire active electrode that was referenced to a needle electrode placed in the cheek; and a needle electrode placed in the tail served as ground. Responses were differentially amplified (0.3–1,500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). White light strobe flashes were initially presented in darkness within a Ganzefeld bowl. Stimuli were presented in order of increasing luminance and ranged in flash luminance from $-3.6$ to 2.1 log cd s/m². Cone ERGs were isolated by superimposing stimuli upon a steady adapting field (20 cd/m²). Flash luminance ranged from $-0.8$ to 1.9 log cd s/m². The amplitude of the $a$-wave was measured 7 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 prestimulus baseline.

**Visual Evoked Potential**—To measure responses of the visual cortex, VEPs were recorded using an active electrode positioned along the midline of the visual cortex and referenced to a needle electrode placed in the cheek. The ground electrode was placed in the tail, and the amplifier bandpass filter settings were 1–100 Hz. VEPs were initially recorded under dark-adapted conditions to strobe flash stimuli that ranged in luminance from $-2.4$ to 2.1 log cd s/m². A light-adapted stimulus series was then run, using stimuli superimposed upon a steady 20 cd/m² adapting field. The mouse VEP was dominated by a negative component that is referred to as N1. The implicit time of the N1 component was measured at the negative peak. The amplitude of the VEP was measured from the N1 negative peak to the ensuing positive peak (P2).

**Statistical Analysis**—Two-way repeated measure analyses of variance were used to analyze luminance-response functions for measures of dark- and light-adapted ERG or VEP and VEP timing. Two-tailed equal or unequal variance $t$ test statistical analysis was performed for qRT-PCR or immunoblot quantitations. $p \leq 0.05$ was defined as significant. Non-parametric Kruskal–Wallis test for group analysis and the Mann–Whitney $U$ test for two group comparisons were carried out for TUNEL analyses of light-treated mice.

**RESULTS**

**Mutations Causing Loss of PPlase Activity of CY of Ranbp2**—To investigate the physiological and biological role(s) of the PPlase activity of CY of Ranbp2 (Fig. 1A), we generated and examined CY constructs for loss of PPlase activity. Alignment of the primary structures between CY of Ranbp2, CyPA, and CyPB shows that CY has higher homology to CyPA than CyPB (66% versus 44% identity), although the catalytic PPlase residues are highly conserved between these three cyclophilins (data not shown) (37, 58). CyPA was chosen as the model template, and thus the predicted structure of CY of Ranbp2 resembles more closely the known structure of CyPA than CyPB, and the differences with the latter are mainly restricted to their loop regions, such as the loop between the a2 and b8 structures (Fig. 1B, data not shown). The molecular model led us to examine the effects of two changes in highly conserved residues of CY on its PPlase activity (Fig. 1C) (58). The first was an a change in the highly conserved catalytic residue, CY$^{R2944A}$, of Ranbp2, because the counterpart R55A mutation in human CyPA leads to loss of its PPlase activity (78). The second was a post-translational modification by phosphorylation in the consensus Ca$^{2+}$/calmodulin-dependent protein kinase II site of CY$^{S3036}$, because recent analysis of the cellular phosphoproteome supports that CY of Ranbp2 may undergo phosphorylation, and one of these post-translational modifications sites is in close proximity ($\sim 7$ Å) to its active site (Fig. 1C) (79). As shown in Fig. 1D, immunoblots with a Ser(P) antibody of immunoprecipitates of mRFP-fused CY, CY$^{R2944A}$ and CY$^{S3036E}$ expressed in HeLa cells, showed that the levels of phosphorylated CY were not affected by the CY$^{R2944A}$ mutation, whereas the CY$^{S3036E}$ substitution caused an $-60\%$ reduction of the amount of phosphorylated CY (Fig. 1D). Then we examined the effect of the CY$^{R2944A}$ and CY$^{S3036E}$ mutations and the adjacent RBD4 on the PPlase activity of CY against several cis-trans-peptidylprolyl substrates (Fig. 1E and Table 1). No PPlase activity was detected with CY$^{R2944A}$ in the presence of any substrate, whereas the phosphomimetic substitution, CY$^{S3036E}$, led to a generalized and significant $60-100\%$ decrease of the PPlase activity of CY across various prolyl substrates (Fig. 1E and Table 1). Furthermore, the upstream and adjacent RBD4 caused a tenuous decrease of the CY PPlase activity against two of the five substrates tested (Fig. 1E and Table 1). Hence, these data support that the CY$^{R2944A}$ does not affect the phosphorylation of CY, but the CY$^{R2944A}$ and CY$^{S3036E}$ mutations result in complete and partial loss of catalytic function of CY, respectively. Furthermore, consistent with prior studies (58), the RBD4 has a small but significant effect on the PPlase activity of CY toward selective prolyl substrates, suggesting that RBD4 may affect the access/release of selective substrates to/from the adjacent CY domain.

**Generation of Null Ranbp2 Mice with Transgenic Expression of Ranbp2 without and with PPlase and Impaired C-terminal Chaperone Activities**—We employed the CY$^{R2944A}$ mutation, which abolished completely the PPlase activity of CY of Ranbp2, to generate transgenic mice expressing Ranbp2 without PPlase activity in a null Ranbp2 background (Fig. 2, A and B). A bacterial artificial chromosome (BAC) construct encompassing the structural and regulatory sequences of the Ranbp2
gene (80) was used to introduce the CYR2944A mutation by BAC recombineering (Tg-Ranbp2\textsuperscript{R2944A-HA}) (81). This Ranbp2 construct and another without the R2944A mutation in CY were also engineered with a C-terminal HA tag in CY (Tg-Ranbp2\textsuperscript{WT-HA}; Fig. 2B). In addition to its use as a molecular tool, the C-terminal HA tag serves to examine also the chaperone function of the C-terminal region of CY independently of its PPIase activity, because recent analyses of mutagenesis studies of the cyclophilin of Drosophila, NinaA (30, 31, 36), support the existence of a distinct C-terminal surface patch, P\textsubscript{m}, which is physiologically critical to chaperone opsin biogenesis, and it is localized away from the PPIase catalytic domain (37). Hence, we reasoned that the insertion of a tag sequence (e.g. HA tag) in the counterpart C-terminal end sequence of CY of Ranbp2 may sterically hinder the interaction(s) between the putative C-terminal chaperone domain of CY and its substrates, even though the C-terminal chaperone domains of NinaA and CY of Ranbp2 appear to be topologically distinct. Two independent transgenic (Tg) lines were generated for each transgenic construct in a null Ranbp2 background by intercrossing the transgenic lines, Tg-Ranbp2\textsuperscript{WT-HA} and Tg-Ranbp2\textsuperscript{R2944A-HA} with the line Ranbp2\textsuperscript{+/Gt(pGT0pfs)630Wcs} (hereafter denoted as Ranbp2\textsuperscript{+/−})
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(68, 69), which harbors a constitutively disrupted allele of Ranbp2 (Fig. 2, A and B). All Tg lines on a null Ranbp2 −/− background (Tg-Ranbp2 WT-HA::Ranbp2 −/− and Tg-Ranbp2 R2944A-HA::Ranbp2 −/−) rescued the embryonic lethality of the Ranbp2 −/− line and did not present overt anatomical or behavioral phenotypes at least up to 24 weeks of age. The transcriptional and protein expressions of the Tg-Ranbp2 R2944A-HA in Tg-Ranbp2 R2944A-HA::Ranbp2 −/− mice were comparable with wild-type mice, whereas those of Tg-Ranbp2 WT-HA in Tg-Ranbp2 WT-HA::Ranbp2 −/− mice were 4–6-fold higher than wild-type Ranbp2 (Fig. 2, C and D). Comparison of the subcellular localization of the transgenic and native Ranbp2 proteins across the neurons of the retina showed no prominent differences between Tg-Ranbp2 R2944A-HA and native Ranbp2 (Fig. 3). Both proteins localize to nuclear pores of neurons, such as retinal ganglion cells, where such structures and Ranbp2 are known to be extremely abundant (Fig. 3, A and B) (75), and they also localize clearly to the connecting cilia of photoreceptor neurons (Fig. 3A). In contrast to the nontransgenic and Tg-Ranbp2 R2944A-HA::Ranbp2 −/− mice, Tg-Ranbp2 WT-HA::Ranbp2 −/− presented a noticeable accumulation of Tg-Ranbp2 WT-HA in the connecting cilia of photoreceptors even though all other retinal neurons presented no remarkable differences in localization and expression of Ranbp2 (Fig. 3A).

Tg-Ranbp2 R2944A-HA::Ranbp2 −/− and Tg-Ranbp2 WT-HA::Ranbp2 −/− Selectively Promote M-opsin Aggregation and Accumulation in Cone Photoreceptors—We began the examination of the effects of the expression of Tg-Ranbp2 R2944A-HA::Ranbp2 −/− and Tg-Ranbp2 WT-HA::Ranbp2 −/− in M- and S-cone photoreceptors, because of the following: (i) the concerted action of the CY and RBD4 of Ranbp2 were previously found to mediate directly the PPIase-dependent interconversion of M-opsin isoforms and to enhance M-opsin biogenesis in heterologous expression systems (17, 18), and (ii) Ranbp2 expression is critical to the survival of all cone photoreceptor types (69). Furthermore, examination of Ranbp2 flox/flox mice supported that Ranbp2 controls the development of M-cone photoreceptors, because Ranbp2 flox/flox mice lack M-cone photoreceptors in the most ventral (inferior) region of the retina, where M- and S-cones are typically less and more abundant, respectively, in wild-type mice (data not shown). However, morphometric analyses of Tg-Ranbp2 R2944A-HA::Ranbp2 −/−, Tg-Ranbp2 WT-HA::Ranbp2 −/−, and Ranbp2 +/+ mice showed that none of the genotypes presented differences in number and apparent development of M- and S-cone photoreceptors in any regions of the retina (Fig. 4; data not shown). However, we found that compared with Ranbp2 +/+ mice, there was prominent aggregation and mislocalization of M-opsin, but not S-opsin, typically at the base and edges of the outer segments of M- and M/S-cone photoreceptors of Tg-Ranbp2 R2944A-HA::Ranbp2 −/− and Tg-Ranbp2 WT-HA::Ranbp2 −/− mice (Fig. 5, A and B). This aggregation of M-opsin was accompanied by an almost 3-fold increase of the levels of M-opsin, but not S-opsin, in Tg-Ranbp2 R2944A-HA::Ranbp2 −/− and Tg-Ranbp2 WT-HA::Ranbp2 −/− mice compared with those of the nontransgenic wild-type mice (Fig. 5C), despite a compensatory decrease in expression...
FIGURE 3. Immunolocalization of native Ranbp2, Tg-Ranbp2R2944A-HA, and Tg-Ranbp2WT-HA proteins in radial retinal sections (A) and retinal flat mounts of cell bodies of ganglion neurons (B). Endogenous and transgenic Ranbp2 proteins of 4–6-week-old mice were detected with antibodies against the IR domain of Ranbp2 and HA tag, respectively. The native localization of Ranbp2 (A, 1st row panel) was distributed throughout the cell bodies of retinal neurons with prominent expressions in the ciliary region of photoreceptors and cell bodies of ganglion neurons. A similar subcellular distribution of transgenic Ranbp2 was observed with Tg-Ranbp2R2944A-HA and Tg-Ranbp2WT-HA, but there is an accumulation of Tg-Ranbp2WT-HA in the ciliary region of photoreceptors (A, 2nd row panel). Insets are higher magnifications of the boxed ciliary regions. B, high magnifications of ganglion neurons captured from retinal flat mounts and depicting the localization of native and transgenic Ranbp2 at the nuclear pores of the nuclear rim. No discernible differences were observed between nontransgenic and transgenic genotypes. −/−, Ranbp2−/−; Tg-R2944A-HA, Tg-Ranbp2R2944A-HA; Tg-WT-HA, Tg-Ranbp2WT-HA; OS, outer segments of photoreceptors; IS, inner segments of photoreceptors; ONL, outer nuclear layer (cell bodies of photoreceptors); INL, inner nuclear layer (cell bodies of second-order neurons); GC, ganglion cells. Scale bars, 50 μm (A), 20 μm (A, inset), and 10 μm (B).
of the transcriptional levels of M-opsin (Opn1mw) in Tg-Ranbp2R2944A-HA::Ranbp2−/− and S-opsin (Opn1sw) in both transgenic genotypes (Fig. 5D). Taken together, these data support that the insertion of the C-terminal HA tag in CY of Ranbp2 disrupts its chaperone activity, which is indispensable for the biogenesis of M-opsin, whereas the PPIase activity of CY plays a nonessential physiological role in M-opsin biogenesis.

STAT3 and STAT5 Associate with CY of Ranbp2 Independently of Its PPIase and C-terminal Chaperone Activities—In cultured cells, CyPB/PPIB associates with STAT3 and STAT5 (82, 83). The association of CyPB/PPIB with STAT3 is likely transient and is reduced, but not abolished, by CsA (83). CyPB/PPIB interactions with STAT3/STAT5 modulate the nuclear transcriptional activities of STAT3 and STAT5 in cytokine-mediated signaling, a process that appears to require the PPIase activity of CyPB (83). However, the highly related CyPA/PPIA does not appear to associate with STAT3 and STAT5 but instead is necessary to regulate the tyrosine phosphorylation of STAT3, an event that is critical for its nuclear translocation (83). In light of the high homology of CY of Ranbp2 with CyPB/PPIB and CyPA/PPIA (58), we probed the association of CY of Ranbp2 with STAT3/STAT5 and its activated phosphorylated isoforms and the effect of mutant isoforms of CY on these associations or proteostasis of STAT3 under normal and disease conditions.
states. We first examined whether STAT3 and STAT5 were substrates of CY upon expression of wild-type and mutant isoforms of CY alone (CYR2944A and CYS3036E) in cell culture. Qualitative and quantitative co-immunoprecipitation assays showed that CY associates with STAT3 and STAT5. The PPIase-deficient mutant, CYR2944A, significantly reduced such
associations, whereas CY might have had a significant impact (Fig. 6A). Then we examined the physiological effects of the transgenic lines, Tg-Ranbp2::R2944A-HA::Ranbp2–/– and Tg-Ranbp2::WT-HA::Ranbp2–/–, on the proteostasis of STAT3 and STAT5. However, we found no changes in the retinal levels of STAT3 and STAT5 between the transgenic and wild-type lines (Fig. 6B). To determine whether STAT3, STAT5, or their tyrosine-phosphorylated isoforms associate in vivo with the transgenic constructs of Tg-Ranbp2::R2944A-HA::Ranbp2–/– and Tg-Ranbp2::WT-HA::Ranbp2–/–, we carried out qualitative and quantitative co-immunoprecipitation assays with extracts of the midbrain, where the basal expression levels of STAT3 and STAT5 are much higher than in the retina (Fig. 6C). As shown in Fig. 6D, STAT3, STAT5, and their activated tyrosine-phosphorylated isoforms associated with the transgenic proteins, Tg-Ranbp2::R2944A-HA and Tg-Ranbp2::WT-HA of Tg-Ranbp2::R2944A-HA::Ranbp2–/– and Tg-Ranbp2::WT-HA::Ranbp2–/– mice, respectively. Furthermore, we found both STAT3 and STAT5 associate with the Tg-Ranbp2::R2944A-HA and Tg-Ranbp2::WT-HA proteins at comparable levels (Fig. 6D). Finally, we examined the role of PPIase activity of Ranbp2 in Tg-Ranbp2::R2944A-HA::Ranbp2–/– in the homeostasis of activated STAT3 and photoreceptor cell death upon chronic light exposure, a powerful disease stress stimulus known to promote the degeneration of photoreceptors and up-regulation of cytokine signaling (84–86). We found that phototoxicity was a powerful inducer of STAT3 activation in cell bodies of photoreceptors, but there were no changes in the levels of such activation between wild-type and Tg-Ranbp2::R2944A-HA::Ranbp2–/– (Fig. 6E). Even though phototoxicity induced a robust increase in the cell death of photoreceptors, there were also no differences in the amount of death of these neurons between wild-type and Tg-Ranbp2::R2944A-HA::Ranbp2–/– mice (Fig. 6F). Collectively, these data suggest that the CY of Ranbp2 mediates the physiological association of STAT3, STAT5, and their activated isoforms, but the PPIase and C-terminal chaperone activities of CY of Ranbp2 play no roles in STAT3 binding, activation, and protection of photoreceptors from light-mediated injury.

Control of hnRNPA2/B1 Proteostasis Selectively by CY PPlase Activity of Ranbp2—Although M-opsin biogenesis was selectively dependent on the C-terminal chaperone activity of CY, neither M-opsin nor STAT3/STAT5 proteostasis of transgenic mice required physiologically the PPIase activity of CY of Ranbp2. Hence, we took an unbiased proteomic approach to screen and identify proteins with proteostatic changes between wild-type and Tg-Ranbp2::R2944A-HA::Ranbp2–/– mice. This survey also included another transgenic line with a mutation in the cyclophilin-like domain (CLD) of Ranbp2 (58), Tg-Ranbp2::CLDm-HA::Ranbp2–/–, which is described later in this study. The CLD of Ranbp2 is thought to modulate the degradation of properly folded proteins by the 26 S proteasome (87, 88). The Tg-Ranbp2::CLDm-HA::Ranbp2–/– served also as another control line for the screening and identification of substrates uniquely affected by the loss of PPIase activity of CY of Ranbp2. To this end, SDS-solubilized retinal homogenates of wild-type, Tg-Ranbp2::R2944A-HA::Ranbp2–/–, and Tg-Ranbp2::CLDm-HA::Ranbp2–/– mice were prepared, labeled with distinct CyDye fluorescent dyes, mixed, and resolved by 2D-DIGE. Pairwise comparison of changes in protein expression (3–fold) between retinal homogenates of wild-type, Tg-Ranbp2::R2944A-HA::Ranbp2–/–, and Tg-Ranbp2::CLDm-HA::Ranbp2–/– mice identified 23 proteins, whose levels were changed in Tg-Ranbp2::R2944A-HA::Ranbp2–/–; but of these 23 proteins, only two candidate proteins appeared to be uniquely affected in Tg-Ranbp2::CLDm-HA::Ranbp2–/– mice. These were the heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNPA2/B1) and cytosolic malate dehydrogenase. Validation of changes in proteostasis of these proteins selectively in Tg-Ranbp2::R2944A-HA::Ranbp2–/– mice was followed up by immunoblot analyses of retinal homogenates. These analyses showed that only hnRNPA2/B1 was down-regulated by >2-fold in Tg-Ranbp2::R2944A-HA::Ranbp2–/– compared with wild-type and Tg-Ranbp2::WT-HA::Ranbp2–/– mice (Fig. 7A, data not shown). The down-regulation of hnRNPA2/B1 in Tg-Ranbp2::R2944A-HA::Ranbp2–/– occurred at post-transcriptional levels, because wild-type and Tg-Ranbp2::R2944A-HA::Ranbp2–/– mice presented comparable transcriptional levels of hnRNPA2/B1 (Fig. 7A). The post-transcriptional changes of hnRNPA2/B1 in Tg-Ranbp2::R2944A-HA::Ranbp2–/– were highly selective, because no changes were observed in the levels of other partners of Ranbp2, such as SUMO-1-RanGAP, Nr2e3, and COUP-TFI (data not shown) (89).

Previously, we have shown that CY of Ranbp2 enhances the direct association between RBD4 of Ranbp2 and M-opsin, an effect consistent with a chaperone role of CY in opsin biogenesis (17, 18). To probe whether hnRNPA2/B1 stably associates with Ranbp2, we carried out co-immunoprecipitation assays of Ranbp2 with retinal extracts by employing similar assays as those described previously for STAT3/STAT5. However, and in contrast to STAT3/STAT5 (Fig. 6D), we found no evidence of a stable association in vivo between hnRNPA2/B1 and transgenic Ranbp2 constructs of Tg-Ranbp2::R2944A-HA::Ranbp2–/– and Tg-Ranbp2::WT-HA::Ranbp2–/– (data not shown). To determine the tissue and cellular origins of the down-regulation of hnRNPA2/B1, we found that hnRNPA2/B1 was expressed at much higher levels in the retina than in other regions of the brain, such as the midbrain (Fig. 7B). The localization of...
hnRNPA2/B1 was prominent in the cell bodies of the inner retinal and ganglion neurons (Fig. 7C). The RNA-binding proteins, hnRNPA2/B1, are implicated in mRNA processing, and their dysregulation affects cytosolic RNA granule assembly (stress granules) and causes ALS and multisystem proteinopathy upon mutations in a prion-like domain prone to aggregation (90). Hence, we assessed the nuclear-cytosolic partitioning of hnRNPA2/B1 and other molecular partners of Ranbp2 between genotypes. There were no selective differences of hnRNPA2/B1 levels between the nucleus and all other subcellular compartments combined of the retinas of wild-type, Tg-Ranbp22944A::Ranbp2−/−, and Tg-Ranbp22944A::Ranbp2−/− mice (Fig. 7D). This analysis was also extended to histone deacetylase-4 (HDAC4), a protein whose self-aggregation was shown to be a prerequisite for its Ranbp2-dependent sumoylation, degradation, and nuclear import in cell culture (91, 92). Although the physiological role of HDAC4 in retinal degeneration is still unclear (93, 94), dysregulation of its nuclear-cytosplasmic partitioning and levels is associated with ataxia telangiectasia-mutated and brachydactyly mental retardation syndrome, respectively (95, 96). In comparison with the other genotypes, we found that the levels of HDAC4 were sig-
nificantly and equally reduced and increased in the non-nuclear and nuclear fractions, respectively, of Tg-Ranbp2R2944A-HA::Ranbp2R2944A-HA mice (Fig. 7D), and we did not find physiological evidence of HDAC4 sumoylation. Hence, Ranbp2 and its PPIase and/or chaperone activity regulate the nucleocytoplasmic shuttling of HDAC4.

**Tissue- and Age-dependent Dysregulation of Ubiquitin Proteostasis in Tg-Ranbp2R2944A-HA::Ranbp2R2944A-HA**—Protein misfolding because of the loss of biological activities linked to chaperones and folds, such as immunophilins, is thought to cause or increase the susceptibility to various pathologies compromising neuronal survival (31, 37, 57, 97, 98). Protein aggregation caused by protein misfolding is often associated with the formation of ubiquitylated bodies, a hallmark feature of many neurodegenerative diseases (99). Furthermore, prior studies have implicated a role of Ranbp2 in the degradation of properly folded proteins by the 26 S proteasome as reflected by the accumulation of ubiquitylated substrates and reporters of the ubiquitin-proteasome system upon ectopic expression of CLD alone of Ranbp2 in culture cells (88). Hence, we tested the effect of loss of PPIase activity of CY of Ranbp2 in ubiquitin homeostasis across different tissues and at various ages. Changes
in ubiquitin homeostasis were assessed by quantifying the levels of free and substrate-conjugated ubiquitin of various tissue homogenates of 4- and 24-week-old wild-type and Tg-Ranbp2R2944A-HA mice. At 4 weeks of age, we found no differences in the levels of free ubiquitin and ubiquitylated protein species in the retina, retinal pigment epithelium, or liver (Fig. 8). By 24 weeks of age, however, the diubiquitin levels were completely suppressed and there was a strong reduction of the levels of ubiquitylated proteins, but not monoubiquitin, selectively in the retina, while the retinal pigment epithelium had reduced levels of ubiquitylated proteins (Fig. 8A and B). The liver presented no changes in ubiquitin homeostasis (Fig. 8C).

**FIGURE 8. Age- and tissue-dependent deficits of ubiquitin homeostasis in Tg-Ranbp2R2944A-HA::Ranbp2−/−.** Compared with wild-type mice, the levels of diubiquitin (Ub2) or conjugated polyubiquitin (P-Ub) are significantly decreased in the retina (A) and RPE (B), but not liver (C), of Tg-Ranbp2R2944A-HA::Ranbp2−/− at 24 weeks, but not 4 weeks of age. GADPH or hsc70 are used as loading controls. Quantitative analyses are shown next to the immunoblots. Data shown represent the mean ± S.D., n = 4. n.s., nonsignificant; −/+; Tg-R2944A-HA; Tg-Ranbp2R2944A-HA::Ranbp2−/−; Tg-Ranbp2WT-HA; Tg-WT-HA. Scale bar, 25 μm.

**FIGURE 7. Post-transcriptional down-regulation of hnRNPA2/B1 and impairment of the subcellular partitioning of HDAC4 selectively in Tg-Ranbp2R2944A-HA::Ranbp2−/−.** A, 2D-DIGE of retinal homogenates of 4- and 6-week-old wild-type (+/+) and Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Insets are volumetric analyses of spot intensity marked in yellow; there was an ~3-fold difference between wild-type (+/+) and Tg-Ranbp2R2944A-HA::Ranbp2−/−. Spot identification (marked in yellow) by mass spectrometry analysis showed that it was hnRNPA2/B1. Validation and comparison of changes in hnRNPA2/B1 expression between genotypes show selective and about 60% reduction of hnRNPA2/B1 expression between 4- and 6-week-old mice. Localizations of hnRNPA2/B1 are prominent in cell bodies of inner retinal neurons (INL) and ganglion cells (GC). There were no discernible differences in hnRNPA2/B1 localization between wild-type and Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. D, selective shift in subcellular partitioning of HDAC4, but not Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. Immunoblot analysis of nuclear and non-nuclear retinal fractions of Tg-Ranbp2R2944A-HA::Ranbp2−/− mice.
Tg-Ranbp2<sup>CLDm-HA::Ranbp2<sup>−/−</sup></sup> mice to examine the physiological effect(s) of CLD of Ranbp2 on the molecular processes described and extended our analysis of its role in ubiquitin homeostasis. Furthermore, the Tg-Ranbp2<sup>CLDm-HA::Ranbp2<sup>−/−</sup></sup> line serves also as an additional control to discern potential effects between loss of PPIase and chaperone activities of CY
linked to its catalytic and C-terminal chaperone domains, respectively, in ubiquitin homeostasis, because the \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) line contains an intact catalytic PPIase domain.

Like the \( \text{Tg-Ranbp2}^{\text{R2944A-HA}} \cdot \text{Ranbp2}^{-/-} \) mice, the expression of \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \) rescued the embryonic lethality of \( \text{Ranbp2}^{-/-} \) mice, and no overt phenotypes were apparent in \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) up to 24 weeks of age. However, genotyping of 149 offspring of the cross between \( \text{Ranbp2}^{-/-} \) and \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice produced 25 instead of the expected 50 \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice (\( p = 0.00015 \)). This significant deviation from the expected Mendelian ratio supports that about 50% of \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice die embryonically. Measurements of \( \text{Tg-Ranbp2} \) transcript(s) with 3′ end primers of surviving \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice found that \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \) was expressed at ~4-fold higher levels than the wild-type transcript in the retina and liver, but this change was not observed with primers against the 5′ end of \( \text{Ranbp2} \) transcripts from either tissue (Fig. 9B), likely because of transcriptional differences between alternatively spliced transcripts. However, the expression levels of wild-type and \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) proteins were comparable between wild-type and \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice, respectively (Fig. 9C). The subcellular expression and distribution of \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \) in \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice also resembled those observed for \( \text{Tg-Ranbp2}^{\text{WT-HA}} \cdot \text{Ranbp2}^{-/-} \) (Fig. 9D, see also Fig. 3). The \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \) exhibited prominent accumulation in the connecting cilium of photoreceptors, whereas its localization and expression in other neurons, such as retinal ganglion cells, was unremarkable in comparison with nontransgenic wild-type mice (Fig. 9D, see also Fig. 3).

Then we examined the effect of \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \) on the levels of SUMO-1-\text{RanGAP}, HDAC4, S1 subunit (Rpn2 by the yeast nomenclature) of the 19 S cap of the 26 S proteasome, and ubiquitin homeostasis (Fig. 9, E and F). Compared with wild-type, \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice present significantly reduced levels of SUMO-1-RanGAP and HDAC4, but not of S1, and no transcriptional changes in HDAC4, in the retina (Fig. 9E). Conversely to \( \text{Tg-Ranbp2}^{\text{R2944A-HA}} \cdot \text{Ranbp2}^{-/-} \) mice, there were no changes of the levels of free ubiquitin and conjugated polyubiquitin in retinal and liver homogenates of 24-week-old \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice (Fig. 9F, see also Fig. 8, A and C).

Deregulation of the Ubiquitin-Proteasome System between \( \text{Tg-Ranbp2}^{\text{R2944A-HA}} \cdot \text{Ranbp2}^{-/-} \) and \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice—In light of the role of the domains, CY and CLD, of Ranbp2 in proteostasis and ubiquitin homeostasis, we compared distinct 26 S proteasome activities of soluble fractions of digitonin-permeabilized retinas between 24-week-old wild-type and \( \text{Tg-Ranbp2}^{\text{R2944A-HA}} \cdot \text{Ranbp2}^{-/-} \) and \( \text{Tg-Ranbp2}^{\text{CLDm-HA}} \cdot \text{Ranbp2}^{-/-} \) mice. We first compared the deubiquitination activity of the S13 (Rpn11 by the yeast nomenclature), and possibly other loosely associated isopeptidases, of the 19 S cap of the 26 S proteasome. The isopeptidase activity of DUB enzymes is critical to deubiquitylate substrates followed by their unfolding and degradation in the 26 S proteasome (101). The activities of Jab1/Mov34/Mpr1 Pad1 N-terminal (JAMM)-type isopeptidases, such as of the S13 subunit, and cysteine-type isopeptidases, were differentiated by their sensitivity to the inhibitors, 1,10-phenanthroline and PR-619, respectively (102, 103). We also employed two types of polyubiquitin chains, Lys-48- and Lys-63-linked ubiquitin chains, to measure DUB activity. Protein substrates conjugated with the former are targeted for proteasomal degradation (104), whereas the latter is thought to be linked to nonproteolytic functions, such as intracellular signaling (105). Compared with other genotypes, there was an ~2-fold increase of deubiquitylase activity toward Lys-63-linked ubiquitin chains in \( \text{Tg-Ranbp2}^{\text{R2944A-HA}} \cdot \text{Ranbp2}^{-/-} \) mice upon inhibition of cysteine-type isopeptidases, whereas no differences were observed for JAMM-type isopeptidase
activity between any genotype (Fig. 9G). In contrast, there was an ~1.5-fold increase of deubiquitylase activity toward Lys-48-linked ubiquitin chains selectively in Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice upon inhibition of JAMM-type isopeptidase activity, whereas there was no change in cysteine-type isopeptidase activity in any genotype (Fig. 9G). Next, we compared the three proteolytic activities of the 20 S proteasome between the three genotypes. As shown in Fig. 9H, there was a selective reduction of the chymotrypsin-like activity by ~25% in Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice. Finally, we examined shifts in the subcellular partitioning of components of the 19 S regulatory cap, such as the S1 subunit, which interacts with CLD of Ranbp2, and of the ubiquitin receptor, S5b subunit, which interacts with S1 (106), because they may contribute to changes in activities linked to the 26 S proteasome (88). Notably, Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> compared with all other genotypes exhibits a marked accumulation of the S1 and S5b subunits of the 19 S regulatory particle of the proteasome in the pellet of digitonin-solubilized retinal extracts, although no changes in S1 levels were observed in the cytosolic/soluble fraction used for the deubiquitylation and 20 S proteasome assays across all three genotypes (Fig. 9I).

**Disturbances in Visual Evoked Potentials in** Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> — The selective aggregation and down-modulation of M-opsin in cone photoreceptors and reduced levels of hnRNPA2/B1 in inner retinal neurons of Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice prompted us to examine if these and other molecular and subcellular manifestations were accompanied by electrophysiological changes of the retina or visual pathway. Disturbances in transmission through this pathway are manifested in neurodegenerative diseases, such as ALS, multiple sclerosis, optic atrophies, and several retinopathies (107–110). We examined cone and rod photoreceptor functions by light- and dark-adapted ERGs and transmission through the visual pathway, including retinal ganglion cells, in VEPs. Fig. 10, A and B, presents strobe flash ERG results obtained with 8-week-old mice under dark-adapted or light-adapted conditions, respectively. There was no significant difference (all p > 0.05) between the dark-adapted (Fig. 10A) or light-adapted ERG amplitudes (Fig. 10B) of Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice in comparison with Ranbp2<sup>2P944A-HA::Ranbp2<sup>+/+</sup></sup> or Ranbp2<sup>+/+</sup> littermates. Similar results were obtained from 24-week-old animals (data not shown). In comparison with littermates of other genotypes, the VEP amplitudes of 24-week-old Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice were not significantly different under dark-adapted (Fig. 10C) or light-adapted (Fig. 10D) conditions. Under dark-adapted conditions, however, VEP implicit times were significantly shorter in Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice in comparison with Ranbp2<sup>2P944A-HA::Ranbp2<sup>+/+</sup></sup> (p < 0.05) or Ranbp2<sup>+/+</sup> (p < 0.01) genotypes (Fig. 10E). There was no significant difference for the light-adapted VEP implicit times of Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> mice in comparison with mice of the other genotypes (Fig. 10F).

**DISCUSSION**

These studies uncover novel and distinct functional, physiological, and pleiomorphic properties of the cyclophilin domain of Ranbp2 with implications for our understanding of the pleiotropic role of other cyclophilins and Ranbp2. As depicted in Fig. 11, these properties are manifested by the following: (i) the PPlase-independent and selective C-terminal chaperone activity of CY of Ranbp2 in M-opsin, but not S-opsin, biogenesis; (ii) the even association of latent and activated STAT3 and STAT5 to CY of Ranbp2 regardless of the loss of its PPlase and C-terminal chaperone activities; (iii) the selective PPlase-dependent post-transcriptional down-regulation of hnRNPA2/B1 with the concomitant dysregulation of HDAC4 subcellular partitioning between the nuclear and non-nuclear compartments; (iv) the selective PPlase-dependent down-regulation of diubiquitin and ubiquitylated substrates in a tissue- and age-dependent fashion; and (v) the down-regulation of the PPlase activity of CY upon phosphorylation, a modification that is likely promoted by selective extracellular stimuli (79, 111). Furthermore, these studies show that the CLD of Ranbp2 selectively and physiologically modulates the proteostasis of SUMO-1-RanGAP and nonsumoylated HDAC4 and the chymotrypsin-like activity of the 20 S proteasome without impairment of the proteostasis of the S1 subunit of the 26 S proteasome. Instead, loss of PPlase activity of CY of Ranbp2 promotes the accumulation of the S1 and the ubiquitin receptor, S5b, subunits of the 19 S cap of the 26 S proteasome, an effect that may contribute to changes in activities associated with the ubiquitin-proteasome system.

**PPlase-independent Chaperone Activity of CY of Ranbp2 Is Critical to M-opsin Biogenesis**—The first physiological role of the CY domain of Ranbp2 uncovered by this study was its selective effect in M-opsin biogenesis, which was characterized by the post-transcriptional accumulation of M-opsin and its aggregation in M- and M/S-cone photoreceptors. In contrast to mice lacking expression of Ranbp2 in cones (69), these manifestations did not trigger the degeneration of cone photoreceptors across the retina nor cause discernible deficits in the light-adapted ERG, an electrophysiological measure that is dominated by bipolar cell activity and a feature that may underlie its relatively lower sensitivity as compared with the other biological measures employed here. Remarkably, the cellular and molecular manifestations developed by M- and M/S-cone photoreceptors were indistinguishable between Tg-Ranbp2<sup>2P944A-HA::Ranbp2<sup>−/−</sup></sup> and Tg-Ranbp2<sup>WT-HA::Ranbp2<sup>−/−</sup></sup>. This supports that the PPlase activity of CY is not essential for M-opsin biogenesis. Instead, the C-terminal domain of CY plays an important chaperone role in M-opsin proteostasis. These observations are surprising, because they unveil physiological idiosyncratic properties of CY and expose important mechanistic differences between in vivo functional studies and prior studies using heterologous expression systems, where the PPlase activity of CY of Ranbp2 was found to promote the interconversion and association of M-opsin isoforms with the RBD4 of Ranbp2 (17, 18). In this regard, our work supports that the absence of a free C-terminal domain in CY of Ranbp2 promotes the accumulation of M-opsin regardless of the PPlase activity of CY because the addition of the HA tag at the C-terminal end of CY promotes the loss or disturbance of its chaperone activity toward M-opsin. Furthermore, this chaperone activity may be conserved between homologous domains of CY of Ranbp2 and NinaA (37). Hence, the chaperone activity of CY of Ranbp2
represents a determinant activity of CY toward the increase in M-pigment production observed with heterologous expression systems (17).

The notion that the chaperone activity of a cyclophilin is critical for opsin biogenesis is also supported by the analyses of mutations from an exhaustive saturation mutagenesis screen for \textit{ninaA} of \textit{Drosophila} (36, 37). Structural mapping of all mutated residues in NinaA that impair opsin biogenesis demonstrates that many were clustered to a structurally disorganized C-terminal region (Pm) away from the PPIase site (37). Although NinaA and CY of Ranbp2 are topologically distinct (27, 30, 31, 58–60), these studies indicate that they may share similar activities in subdomains at their C-terminal regions for the chaperoning of opsin biogenesis. Although NinaA chaper-

![Electrophysiological responses of rod and cone photoreceptors and inner retinal neurons of Tg-Ranbp2\textsuperscript{R2944A-HA}::Ranbp2\textsuperscript{+/−} mice. Luminance-response functions for the dark-adapted (A) and light-adapted (B) ERGs obtained from 8-week-old mice. Luminance-response functions for VEP amplitudes (C and D) and implicit times (E and F) obtained from 24-week-old mice under dark-adapted (C and E) and light-adapted (D and F) stimulus conditions. No significant differences between genotypes were found in the scotopic (A) and photopic (B) \(a\)- and \(b\)-waves and VEP amplitudes (C and D). The VEP implicit times under dark-adapted (E), but not light-adapted (F) stimulus conditions, were reduced in Tg-Ranbp2\textsuperscript{R2944A-HA}::Ranbp2\textsuperscript{+/−} mice in comparison with Ranbp2\textsuperscript{+/−} (\(p < 0.05\)) or Ranbp2\textsuperscript{−/−} (\(p < 0.01\)) genotypes. Data points indicate the average ± S.E.; \(n = 9–10\). −/−, Ranbp2\textsuperscript{−/−}; +/-, Ranbp2\textsuperscript{+/−}; Tg-R2944A-HA, Tg-Ranbp2\textsuperscript{R2944A-HA}.](https://www.jbc.org/content/289/8/4618)
Our data indicate also that the selective accumulation and aggregation of M-opsin may be caused by an impairment of its polyribosome formation. In this regard, it is noteworthy that the cyclophilin-binding activity of M-opsin triggers the compensatory down-modulation of transcriptional levels of M- and S-opsins co-expressed in M/S-cones, because these mice present reduced transcriptional levels of M-opsin translation, where mRNA stabilization and translational efficiency are known to promote the destabilization of nuclear immunosuppressor, CsA, and proline isomerization of RNA polymerase II are known to promote the destabilization of nucleocytoplasmic export, translation, or trafficking of M-opsin transcripts. To this effect, Ranbp2 is implicated in potentiating the translation of mRNA encoding secretory proteins or eIF4E-mediated nuclear export and translation of selective transcripts (123, 124). Neither of the scenarios described are mutually exclusive, and follow-up studies are needed to examine these mechanisms in greater depth.

**FIGURE 11.** Model depicting idiosyncratic and physiological activities of CY of Ranbp2 toward the proteostasis of distinct substrates. The CY of Ranbp2 presents three distinct biological activities toward physiological substrates. First, the C-terminal domain of CY of Ranbp2 harbors chaperone activity selectively toward M-opsin. Impairment of the C-terminal chaperone activity of CY of Ranbp2 promotes M-opsin aggregation and accumulation in cone photoreceptors. Second, the PPlase activity of CY of Ranbp2 is required for the proteostasis of hnRNPA2/B1. Suppression of the PPlase activity of CY leads to the post-transcriptional down-regulation of hnRNPA2/B1. This is also accompanied by the down-regulation of diubiquitin, an effect that promotes the activation of selective deubiquitylases and a reduction of the levels of ubiquitylated substrates. Third, the CY of Ranbp2 presents another subdomain, which mediates the binding of latent and activated STAT3/STAT5. This STAT3/STAT5-binding domain in CY is distinct from its PPlase and C-terminal chaperone domains and has not yet been defined molecularly. Finally, this work supports that the PPlase activity of CY is modulated by phosphorylation of at least a residue (Ser-3036) near its active PPIase site. The phosphorylation of CY is likely modulated by extracellular stimuli (e.g., cytokines), and the physiological implications of the post-translational modification of CY have yet to be defined. CY of Ranbp2 is depicted with a ribbon representation. Residues Ser-3036 and Arg-2944 are shown as orange sticks and other residues of the catalytic PPlase site of CY are shown as green sticks.

**Proteostasis Regulation by Cyclophilin of Ranbp2**

The ciliary localization of Ranbp2 suggests a role for its CY chaperone activity in the ciliary transport of M-opsin to the outer segments. Interestingly, emerging evidence supports that some features of cargo trafficking through ciliary structures and nuclear pores share similar components and mechanisms, even though Ranbp2 is described typically as a nucleo-cytoplasmic (component of the nuclear pore complex) (114). In this regard, the nuclear pore complex and the cilium constitute diffusion barriers for the selection of cargos for nucleocytoplasmic and ciliary trafficking, respectively. The regulatory mechanisms of these trafficking processes are shared by nuclear-cytoplasmic and ciliary-cytoplasmic gradients driven by the small GTPase, Ran, whose Ran-GTP concentration is high in the nuclear and ciliary compartments and low in the cytosol (115, 116). Two high affinity Ran GTPase-binding partners, Ranbp1 and Ranbp2, are pivotal components that contribute to the establishment of the Ran GTPase gradient and the loading and release of cargoes from receptors or chaperones upon docking to Ranbp2 (117–120). Furthermore, Ranbp2 interacts with Rpgrip1, an essential ciliary component for the elaboration of the modified cilium (outer segment) of photoreceptors (121, 122). Hence, impairment of the chaperone activity of CY of Ranbp2 may deregulate M-opsin trafficking and cause its aggregation in the outer segment of photoreceptors, as observed by this study. A critical role of Ranbp2 in ciliary trafficking is also supported by prior ultrastructural studies of mice lacking Ranbp2 in cone photoreceptors, because these mice present massive accumulation of electrodense material at the connecting cilium prior to cone degeneration (69). Another possibility is that CY of Ranbp2 causes the post-transcriptional down-regulation of M-opsin upon its aggregation because of the down-modulation of nucleocytoplasmic export, translation, or trafficking of M-opsin transcripts. To this effect, Ranbp2 is implicated in potentiating the translation of mRNA encoding secretory proteins or eIF4E-mediated nuclear export and translation of selective transcripts (123, 124). Neither of the scenarios described are mutually exclusive, and follow-up studies are needed to examine these mechanisms in greater depth.

**CY of Ranbp2 Presents Unique Interacting Subdomains toward STAT3/STAT5—**CyPB interacts with STAT3 and STAT5. CyPB, like NinaA, is an endoplasmic reticulum-resident cyclophilin (125), but the interaction of CyPB with STAT3 takes place in the nucleus upon seconion and cellular re-uptake in response to cytokines, such as IL-6 (83). Interestingly, STAT3 trans-activating potential is dependent on the PPlase activity of CyPB in culture cells (83). However, inhibition of CyPA (and CyPB) by CsA suppresses STAT3 activation by IL-3, an effect reflected by the IL-3-induced phosphorylation and nuclear translocation of STAT3 and recapitulated also by CyPA knockdown (83). Hence, STAT3 signaling appears to be dependent on complementary signaling pathways mediated by CyPA and CyPB. However, it is unclear whether the PPlase and chaperone activities of CyPA and CyPB play complementary roles in IL-3 signaling. Further, physiological validation of their roles in IL-3 signaling is lacking. This is of high relevance, because of the high homology of CyPA and CyPB to CY of Ranbp2 and its role in nucleocytoplasmic trafficking of substrates via the direct interaction of Ranbp2 with nuclear import proteins.
Proteostasis Regulation by Cyclophilin of Ranbp2

and export receptors (62, 63). Hence, it is possible that CY of Ranbp2 harbors functional properties similar to those attributed to CyPB and/or CyPA in STAT3/STAT5 signaling. Indeed, our studies show that STAT3/STAT5 associates with CY alone of Ranbp2 in cultured cells and physiologically with the transgenic Ranbp2 regardless of the loss of its PPIase and C-terminal chaperone activities. Furthermore, impairment of these activities did not affect the robust stress-dependent activation of STAT3 and development of stress-induced apoptotic photoreceptor cell bodies between wild-type and Tg-Ranbp2R2944A-HA::Ranbp2−/− mice. These data reveal another idiosyncratic feature of the CY of Ranbp2, whereby STAT3/STAT5 association to CY is independent of its PPIase and C-terminal chaperone activities. Hence, these results strongly support the existence of another subdomain in CY, which is critical for STAT3/STAT5 interactions and distinct from the CY C-terminal subdomain, which is central for M-opsin biogenesis. Additional studies will address the identity of the STAT3/STAT5-binding subdomain in CY and to what extent its loss-of-function is physiologically compensated by CyPA and/or CyPB.

PPIase Activity of CY of Ranbp2 Is Critical to hnRNPA2/B1 Proteostasis—In this study, we searched for substrates whose proteostasis depended on the PPIase activity of CY of Ranbp2, because there were no differences in M-opsin biogenesis and STAT3/STAT5 proteostasis between the Tg-Ranbp2R2944A-HA::Ranbp2−/− and Tg-Ranbp2WT::Ranbp2−/− lines. We found that hnRNPA2/B1 is highly expressed in inner retinal neurons and their post-transcriptional levels, but not transcriptional levels, are significantly and specifically reduced in Tg-Ranbp2R2944A-HA::Ranbp2−/− compared with the Tg-Ranbp2WT::Ranbp2−/− and nontransgenic lines. In contrast to M-opsin and STAT3/STAT5, we found that hnRNPA2/B1 does not form a stable complex in vivo with Ranbp2, supporting that the interaction between hnRNPA2/B1 and Ranbp2 is likely transient, an effect consistent with the catalytic activity of CY on a substrate. This result is apparently at odds with a study where CyPA was found to associate with hnRNPA2 in HeLa cells, an effect that is strongly enhanced by the stimulation with the chemokine, CXCL12 (111). However, our studies cannot exclude an indirect effect of CY of Ranbp2 on hnRNPA2/B1 proteostasis or that the association of hnRNPA2/B1 with CY of Ranbp2 is dependent on physiological stimuli controlling the PPIase activity of CY as supported by the decrease of PPIase activity of the phosphomimetic mutant, CY53036E. Notably, the electrophysiological responses of the outer retina (photoreceptors) between any of the lines was unremarkable, although the dark- but not light-adapted VEPs elicited by inner retinal neurons had a significantly shorter latency in Tg-Ranbp2R2944A-HA::Ranbp2−/−. This indicates that the rod pathway is selectively affected, but the exact underlying mechanisms impairing this neural pathway will require further study. However, the shorter dark-adapted VEP latency is surprising, because it indicates that a PPIase-dependent deficit in hnRNPA2/B1 promotes a reduction of neural transmission through the retina and/or increase of conduction velocity along ganglion cell axons, perhaps due to increased myelination. Because we did not discern any ERG and hnRNPA2/B1 changes in the outer retina, the most likely scenario is that a decrease of hnRNPA2/B1 levels in the inner retina upon loss of Ranbp2 PPIase activity selectively potentiates the activity of hnRNPA2/B1 leading to a shorter dark-adapted VEP latency. Furthermore, the following observations also support the notion that changes in hnRNPA2/B1 proteostasis are a major contributor to the reduced latency of dark-adapted VEPs: (i) Ranbp2 is a docking site for the nuclear export receptor, CRM1, exportin-1 (63); (ii) Ranbp2 affects selectively the levels of the RNA-binding proteins, hnRNPA2/B1, as shown here; (iii) Ranbp2 binds single-stranded RNA (126); and (iv) Ranbp2 stimulates the export of mRNA and translation of endoplasmic reticulum-targeted and likely mitochondrially targeted proteins in cell culture (123, 124). Finally, although no PPIase activity was detected consistently in vitro with CYR2944A, it is arguably possible that Tg-Ranbp2R2944A-HA::Ranbp2−/− mice may still harbor vanishingly low levels of CY PPIase activity. For example, a mutant CyPA with the counterpart R55A substitution presents in vivo 0.1% of PPIase activity of the wild-type protein, which has higher catalytic efficiency than CY of Ranbp2 toward peptidyl-prolyl substrates (78). In addition, vanishingly low levels of PPIase activity of the essential yeast parvulin, Ess1, are still thought to be required for the biological function of Ess1 (127), even though loss-of-expression of its mammalian ortholog, Pin1, does not affect mouse viability (97, 128, 129). Future studies on mice lacking expression of CY of Ranbp2 will help to discern potential hypomorphic manifestations linked to Ranbp2R2944A-HA expression and CY substrates identified by this work.

As described earlier, mutations affecting hnRNPA2/B1 in a prion-like domain prone to aggregation cause multisystem proteinopathy and ALS and promote changes in the nucleocytoplasmic partitioning of hnRNPA2/B1 and homeostasis of stress granules (90). Notably, CyPA associates also with hnRNPA2 and promotes its nuclear export (111), whereas CsA induces the formation of prion protein-aggresomes in cell culture (56). In this study, we found that the relative subcellular partitioning of hnRNPA2/B1 did not change physiologically across all genotypes. Hence, even though the PPIase activity of CY of Ranbp2 regulates the proteostasis of hnRNPA2/B1 without impairing their nucleocytoplasmic shuttling, it is likely that other domains and partners of Ranbp2 modulate this shuttling process (63). Finally, ALS patients also present profound changes in VEPs before the clinical onset of motor symptoms ensue (107). Thus, it will be important to find whether similar or analogous Ranbp2-dependent pathomechanisms are shared between ganglionic retinal and motor neurons and the contribution of other factors modulated by Ranbp2, such as HDAC4, to disease expression.

PPIase Activity of Ranbp2 Down-modulates Ubiquitin Homeostasis—Loss of catalytic activity by immunophilins is thought to reduce the folding rates of proteins and increase their propensity to misfolding, misassembly, and aggregation. The formation of these misfolded by-products is thought to impair proteasome activity and lead to the formation of ubiquitylated bodies, a cardinal feature of many neurodegenerative diseases (99). However, monoubiquitin and diubiquitin appear to control selectively the deubiquitinating activities of ubiquitin C-terminal hydrolases-L1 (UCH-L1) and -L3 (UCH-L3),
respectively (130). UCH-L1 and UCH-L3 are implicated in familial Parkinson disease (131), gracile axonal dystrophy (132), and retinal degeneration (133), respectively. Collectively, these underscore the importance of ubiquitin homeostasis in neuronal function. This study found that loss of PPIase activity causes the age- and tissue-dependent deregulation of ubiquitin homeostasis as reflected by the reductions of free diubiquitin and ubiquitylated protein species observed in Tg-Ranbp2^{2944A-HA::Ranbp2^{-/-}}, but not Tg-Ranbp2^{CLDm-HA::Ranbp2^{-/-}} and wild-type mice. This selective and prominent PPIase-dependent change in ubiquitin homeostasis was unexpected, because in cell culture only CLD of Ranbp2 was thought to promote accumulation of ubiquitylated substrates, an effect that was suppressed by the same mutations harbored by Tg-Ranbp2^{CLDm-HA::Ranbp2^{-/-}} mice (88). Again, our data support differences in biological behavior between domains of Ranbp2 when expressed alone in cell culture and when expressed as part of the whole protein in an intact physiological system, where cross-talk between domains and partners of Ranbp2 take place in their native physiological environment. This and other studies support that inter-domain cross-talk does occur in Ranbp2 (17, 18, 134). For example, this study shows that the CY and CLD modules of Ranbp2 play complementary roles in the subcellular partitioning and proteostasis of HDAC4, respectively. In addition, compensatory mechanisms are also triggered upon loss of selective activities of domains of Ranbp2, such as CLD, as shown by the incomplete penetrance of the lethality of Tg-Ranbp2^{CLDm-HA::Ranbp2^{-/-}} during development but not postnatally.

Prior findings in vitro and with cell cultures support that diubiquitin suppresses the deubiquinating activity of the cysteine protease, UCH-L3 (130). In agreement with these data, our results show that retinas of Tg-Ranbp2^{2944A-HA::Ranbp2^{-/-}} with low levels of diubiquitin exhibit physiologically an increase of DUB activity toward Lys-63-linked ubiquitin chains by DUBs of the cysteine protease family. Our findings also support that loss of PPIase function of Ranbp2 promotes the selective and differential deubiquitylation of Lys-63- and Lys-48-linked ubiquitin chains by DUBs of the cysteine protease and metalloprotease (MPN-Lys-48-linked ubiquitin chains by DUBs of the cysteine protease family. Our findings also support that loss of PPIase activity of CY of Ranbp2 reflect novel cell context-dependent roles of different subdomains of CY of Ranbp2 on physiological substrates, whose functions are dependent on the chaperone and PPIase activities of CY of Ranbp2. It is also possible that distinct conformational sub-states of CY of Ranbp2 contribute to its discernible idiosyncratic activities (135, 136). Regardless, the implication of these activities on substrates underpinning multiple diseases and pathogenic processes will pave the way to development of novel pharmacological strategies to harness and unveil novel therapeutic potentials of cyclophilins.

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