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RESEARCH ARTICLE

Neuroprotection resulting from insufficiency of RANBP2 is associated with the modulation of protein and lipid homeostasis of functionally diverse but linked pathways in response to oxidative stress

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SUMMARY

Oxidative stress is a deleterious stressor associated with a plethora of disease and aging manifestations, including neurodegenerative disorders, yet very few factors and mechanisms promoting the neuroprotection of photoreceptor and other neurons against oxidative stress are known. Insufficiency of RAN-binding protein-2 (RANBP2), a large, mosaic protein with pleiotropic functions, suppresses apoptosis of photoreceptor neurons upon aging and light-elicited oxidative stress, and promotes age-dependent tumorigenesis by mechanisms that are not well understood. Here we show that, by downregulating selective partners of RANBP2, such as RAN GTPase, UBC9 and ErbB-2 (HER2; Neu), and blunting the upregulation of a set of orphan nuclear receptors and the light-dependent accumulation of ubiquitylated substrates, light-elicited oxidative stress and *Ranbp2* haploinsufficiency have a selective effect on protein homeostasis in the retina. Among the nuclear orphan receptors affected by insufficiency of RANBP2, we identified an isoform of COUP-TFI (Nr2f1) as the only receptor stably co-associating in vivo with RANBP2 and distinct isoforms of UBC9. Strikingly, most changes in proteostasis caused by insufficiency of RANBP2 in the retina are not observed in the supporting tissue, the retinal pigment epithelium (RPE). Instead, insufficiency of RANBP2 in the RPE prominently suppresses the light-dependent accumulation of lipophilic deposits, and it has divergent effects on the accumulation of free cholesterol and free fatty acids despite the genotype-independent increase of light-elicited oxidative stress in this tissue. Thus, the data indicate that insufficiency of RANBP2 results in the cell-type-dependent downregulation of protein and lipid homeostasis, acting on functionally interconnected pathways in response to oxidative stress. These results provide a rationale for the neuroprotection from light damage of photosensory neurons by RANBP2 insufficiency and for the identification of novel therapeutic targets and approaches promoting neuroprotection.

INTRODUCTION

Cell proliferation and death often reflect antagonistic biological outcomes produced by the stimulation or inhibition of signaling pathways in the presence or absence of a wide variety of biological and stress factors (Campisi, 2005; Johnstone et al., 2002). The RAN-binding protein-2 (RANBP2) is a large, mosaic protein (Ferreira et al., 1995; Wu et al., 1995; Yokoyama et al., 1995), whose pleiotropic functions are reflected by its interaction with a set of well-defined partners implicated in a wide variety of biological processes, such as nucleocytoplasmic (Bernad et al., 2004; Chi et al., 1996; Delphin et al., 1997; Engelsma et al., 2004; Forler et al., 2004; Singh et al., 1999; Vetter et al., 1999) and cytoplasmic (Cai et al., 2001; Cho et al., 2007; Cho et al., 2009a) trafficking, protein modification through sumoylation (Lee et al., 1998; Mahajan et al., 1997; Mahajan et al., 1998; Matunis et al., 1996), protein turnover and biogenesis (Ferreira et al., 1995; Ferreira et al., 1996; Ferreira et al., 1997; Ferreira et al., 1998; Yi et al., 2007), and energy homeostasis (Aslanukov et al., 2006). A growing body of evidence supports the view that RANBP2 has crucial physiological roles in the control of cell proliferation and death and that various stressors play a determinant role in modulating such RANBP2-dependent physiological activities (Cho et al., 2009b; Dawlaty et al., 2008; Neilson et al., 2009). For example, infectious diseases of various etiologies and febrile states together with otherwise asymptomatic heterozygous mutations in the leucine-rich domain of RANBP2 promote rampant necrosis of neurons of the basal ganglia and other regions of the brain, which is clinically manifested as acute necrotizing encephalopathy 1 (ANE1) (Gika et al., 2009; Neilson et al., 2009; Suri, 2009). By contrast, insufficiency of RANBP2 promotes age-dependent missegregation of chromosomes (aneuploidy) and an increase in spontaneous oncogenesis and susceptibility to carcinogen-elicited tumorigenesis (Dawlaty et al., 2008). Haploinsufficiency of *Ranbp2* also confers age-dependent neuroprotection to photosensory neurons upon light-elicited oxidative stress (Cho et al., 2009b), a deleterious stressor known to promote the death of these neurons and to be a key risk factor in the pathogenesis of neurodegenerative disorders of the retina (Imamura et al., 2006; Noell et al., 1966; Reme, 2005; Yamashita et al., 1992). Finally, insufficiency of RANBP2 also promotes physiological deficits in glucose and lipid metabolism (Aslanukov et al., 2006; Cho et al., 2009b). However, what biological activities linked to RANBP2 contribute to its physiological roles in the regulation of cell survival and proliferation and allied pathophysiologies remains largely unexplored.

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A variety of stressors, including age-induced oxidative stress, are known to induce alterations in the nucleocytoplasmic gradient of the GTPase Ras-related nuclear protein (RAN) and to impair nucleocytoplasmic transport, a process thought to constitute an intrinsic signal for apoptosis, and to contribute to aging manifestations and pathogenesis of human diseases (Casanova et al., 2008; Crampton et al., 2009; D'Angelo et al., 2009; Hirano et al., 2006; Kodiha et al., 2004; Wong et al., 2009; Yasuda et al., 2006). At least two key partners of RANBP2 – RAN GTPase and the ubiquitin-conjugating enzyme UBC9 – have been found to mediate novel oxidative stress and apoptotic signaling events (Bossis and Melchior, 2006; Heo, 2008; Kodiha et al., 2004; Yasuda et al., 2006). Recent evidence indicates that RAN GTPase acts as a sensor of oxidative stress via modulation of its conformation and nucleotide-bound state (Heo, 2008). In addition, UBC9, the sole E2-conjugating enzyme in the sumoylation of proteins, which is thought to be important for subcellular localization and nucleocytoplasmic shuttling of sumoylated substrates (Ayaydin and Dasso, 2004; Melchior, 2000; Seeler and Dejean, 2003), was also shown to be modulated by oxidative stress in macrophages (Bossis and Melchior, 2006). However, the biological role(s) of the sumoylation-dependent effects of UBC9 are unclear for several reasons. First, UBC9 acts as a co-transcriptional regulator with other nuclear factors, such as COUP transcription factor I (COUP-TFI; also known as Nr2f1) (Kobayashi et al., 2004; Kurihara et al., 2005), and sumoylation-deficient mutants of UBC9 do not impair transcriptional activity and the subcellular localization of UBC9 (Chakrabarti et al., 1999; Kobayashi et al., 2004; Kurihara et al., 2005; Kurtzman and Schechter, 2001; Poukka et al., 1999). Second, deficiency of UBC9 causes lethality and it is important for cell cycle progression and growth, probably by modulation of the degradation of mitotic cyclins (Nacerddine et al., 2005; Nowak and Hammerschmidt, 2006; Seufert et al., 1995). Yet, in contrast to the loss of UBC9 (Alkuraya et al., 2006), the loss of the UBC9 substrate SUMO1 has been shown to not cause any overt physiological and morphological phenotypes in the mouse (Evdokimov et al., 2008; Zhang et al., 2008), with the exception of a discordant study (Alkuraya et al., 2006).

In this study, we molecularly define the neuroprotective effects of insufficiency of RANBP2 in response to light-elicited oxidative stress by showing that this is caused by the differential deregulation of the expression levels of a subset of known and new partners of RANBP2 and differential accumulation of lipid metabolites between retinal neurons and the retinal pigment epithelium (RPE).

RESULTS

Selective perturbation of proteostasis in retinal neurons upon gene dosage of *Ranbp2* and photo-oxidative stress

We have shown previously that the cyclophilin-like domain (CLD) of RANBP2 associates specifically with subunits of the 19S cap of the proteasome (Ferreira et al., 1998) and that ectopic expression of the CLD of RANBP2 inhibits the ubiquitin-proteasome system (UPS) by promoting the accumulation of ubiquitylated and properly folded substrates (Yi et al., 2007). Furthermore, haploinsufficiency of *Ranbp2* causes reduced levels of RANBP2 (Aslanukov et al., 2006; Dawlaty et al., 2008) and confers neuroprotection to photosensory neurons upon aging and light-elicited oxidative stress (Cho et al., 2009b), which are known to promote deregulation of the UPS and neurodegeneration of photoreceptors (Andersen, 2004; Breusing and Grune, 2008; Ethen et al., 2007; Hollyfield et al., 2008; Kapphahn et al., 2007; Reinheckel et al., 1998; Vigouroux et al., 2004). Hence, to probe the molecular bases of the neuroprotective effects of insufficiency of RANBP2 upon light-elicited oxidative stress, we examined whether partial deficits in RANBP2 in the presence and absence of light stress promote changes in the homeostasis of partners known to associate directly with selective domains of RANBP2 or of substrates possibly affected by functions linked to RANBP2.

As shown in Fig. 1A,B, among the partners known to associate with RANBP2, we found that the steady-state levels of RAN GTPase, UBC9 and the S1 subunit of the 19S cap of the proteasome were selectively and strongly downregulated upon light-elicited stress in *Ranbp2*$^{+/-}$, but not wild-type, mice. By contrast, increases in the levels of the orphan nuclear receptors COUP-TFI, COUP-TFII (Nr2f2), and NeuroD were found only in wild-type mice upon prolonged light treatment, whereas levels of Nr2E3 were increased in wild-type mice and decreased in *Ranbp2*$^{+/-}$ mice under the same light treatment. Notably, no changes were found in others partners of RANBP2 (e.g. KIF5B, KIF5C, COX11, importin-β; K.C., H.Y. and P.A.F., unpublished) and the pro-apoptotic mitochondrial releasing factor AIF (Klein et al., 2002; Lipton and Bossy-Wetzel, 2002), but there was a genotype-independent and light-dependent increase in the levels of the cytosolic and mitochondrial chaperones Hsc70 (HSPA8) and mHsp70, and of the α-subunit of the mitochondrial ATP synthase (Fig. 1A,B). We examined also whether light stress and insufficiency of RANBP2 affected the levels of the orphan transmembrane tyrosine kinase receptor ErbB-2 (also known as HER2 or Neu). Although ErbB-2 is thought to not be expressed in the mature retina (Bermingham-McDonogh et al., 1996), RANBP2 was found to mediate the nucleocytoplasmic trafficking of ErbB-2 upon endocytosis in cultured cells (Giri et al., 2005). As shown in Fig. 1C, ErbB-2 was upregulated in *Ranbp2*$^{+/-}$ mice reared under cyclic light conditions, whereas chronic light treatment reversed the genotype-dependent increase in ErbB-2 to levels comparable to those in wild-type mice reared under cyclic light conditions.

Next, we ascertained whether the effects of light treatment and insufficiency of RANBP2 observed in the retina extended also to its supporting tissue, the RPE, whose phagocytic activity is crucial to the renewal of damaged outer segments of photosensory neurons (Young and Bok, 1969). As shown in Fig. 2, no significant changes in the expression levels of partners or possible substrates of RANBP2 were observed in RPE cells in the different genotypes and light conditions tested, except for S1, the expression of which rose in *Ranbp2*$^{+/-}$ mice under cyclic light conditions, but such an effect was significantly suppressed in the same genotype when reared under prolonged light exposure.

Hence, the data indicate that the combination of light stress and insufficiency of RANBP2 induces tissue-selective perturbations of proteostasis, and that a partial deficit of RANBP2 by itself is sufficient to upregulate reversibly the levels of ErbB-2 in retinal neurons.

Insufficiency of RANBP2 suppresses a global increase in the accumulation of ubiquitylated substrates in the retina upon photo-oxidative stress

The findings that (1) ectopic expression of the CLD of RANBP2 selectively suppresses the activity of the UPS in vivo (Yi et al., 2007), (2) oxidative stress impairs the UPS (Breusing and Grune, 2008), and (3) the combination of light stress and insufficiency of RANBP2

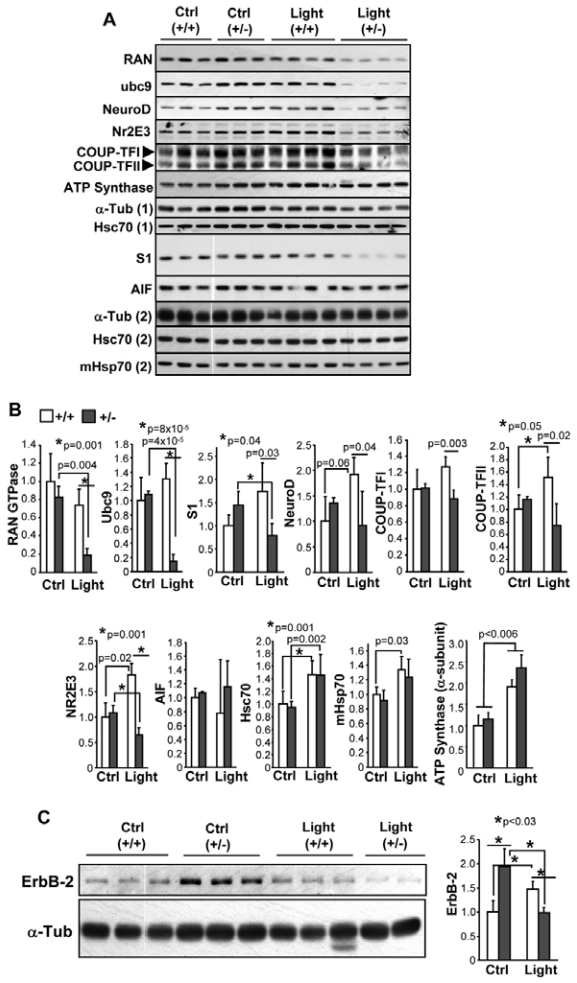

Fig. 1. Prolonged light exposure and insufficiency of RANBP2 differentially perturb protein homeostasis in retinal neurons. (A) Immunoblot analyses of retinal extracts of 24-week-old wild-type (+/+) and *Ranbp2*$^{+/-}$ (+/–) mice reared under cyclic (12:12) light conditions (Ctrl) and prolonged light treatment (Light), with antibodies against RAN GTPase, UBC9, NeuroD, Nr2E3, COUP-TFI, COUP-TFII, ATP synthase α subunit, cytosolic Hsc70 (Hsc70), S1 subunit of the 19S cap of the proteasome (S1), mitochondrial apoptosis inducing factor (AIF) and mitochondrial Hsp70 (mHsp70). Acetylated α-tubulin was used as a loading control. (B) Quantification of immunoblots in A by densitometry and normalized against α-tubulin expression. Relative protein levels in wild-type mice under cyclic light conditions were arbitrarily set as 1. In comparison with mice under cyclic (12:12) light conditions (Ctrl), prolonged light exposure (Light) induces a significant decrease in the levels of RAN GTPase, UBC9 and S1 in *Ranbp2*$^{+/-}$ mice, whereas it causes the upregulation of NeuroD, COUP-TFI and COUP-TFII in wild-type mice. Prolonged light treatment causes an increase and decrease of Nr2E3 levels in wild-type and *Ranbp2*$^{+/-}$ mice, respectively. No expression change was observed for AIF, whereas chronic light exposure induces an increase in Hsc70, mHsp70 and ATP synthase, regardless of the genotype. (C) Immunoblot (left) and densitometry (right) analyses of ErbB-2 expression (molecular mass ~185 kDa) in retinal extracts of 24-week-old wild-type (+/+) and *Ranbp2*$^{+/-}$ (+/–) mice reared under mice under cyclic (12:12) light conditions (Ctrl) and prolonged light treatment (Light). Note that insufficiency of RANBP2 promotes the upregulation of ErbB-2, whereas prolonged light treatment causes a decrease of ErbB-2 levels in both genotypes, but this is more pronounced in *Ranbp2*$^{+/-}$ mice. Densitometry values are normalized against α-tubulin expression and expressed as arbitrary units as described in B. Densitometry results shown represent the mean ± s.d. of retinas of four mice per group (*n=4*). Only changes considered significant ($P \leq 0.05$) are noted.

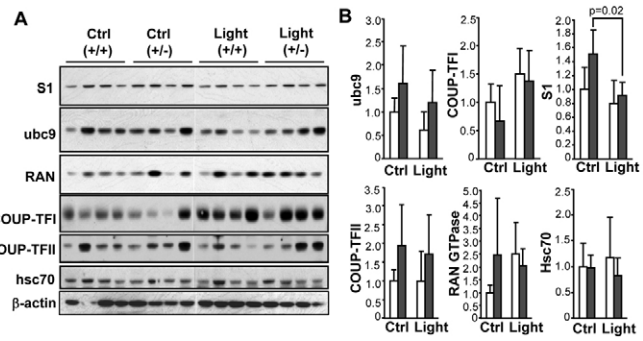

Fig. 2. Cyclic and prolonged exposures to light have no effect on the expression levels of several RANBP2 partners in the RPE. (A) Western analyses of RPE extracts of 24-week-old wild-type (+/+) and *Ranbp2*$^{+/-}$ (+/–) mice reared under cyclic (12:12) light conditions (Ctrl) and prolonged light treatment (Light), with antibodies against proteins employed in Fig. 1. β-Actin was used as loading control. (B) Quantification of immunoblots in A by densitometry and normalized against β-actin expression. Relative protein levels in wild-type (white bars) mice under cyclic light conditions were arbitrarily set as 1. Black bars: *Ranbp2*$^{+/-}$ mice. With the exception of S1, the expression levels of partners of RANBP2 and cytosolic Hsc70 (Hsc70) did not change significantly under any light conditions. Densitometry values are expressed as arbitrary units. Results shown represent the mean ± s.d. of RPE of four mice per group (*n=4*). Only changes considered significant ($P \leq 0.05$) are noted.

downregulates the homeostasis of selective partners of RANBP2 (Fig. 1) led us to examine whether insufficiency of RANBP2 in the presence of photo-oxidative stress promotes an overall reduction in ubiquitylated substrates as a result of the upregulation of UPS activity (which is normally suppressed by RANBP2). Indeed, immunblotting using an anti-ubiquitin antibody of retinal extracts of wild-type and *Ranbp2*$^{+/-}$ mice reared under cyclic and chronic light revealed that, in comparison with cyclic light conditions, chronic light induced an increase of a wide spectrum of ubiquitylated substrates in wild-type mice, including a prominent ubiquitylated substrate, p97 (Fig. 3A, arrowhead), whereas insufficiency of RANBP2 suppressed such an effect (Fig. 3A,C).

An isoform of COUP-TFI co-associates in vivo with RANBP2 and distinct UBC9 isoforms

Despite the findings that UBC9 acts as a co-transcriptional regulator with orphan nuclear receptors, such as COUP-TFI (Kobayashi et al., 2004; Kurihara et al., 2005), and insufficiency of RANBP2 modulates the proteostasis of a set of orphan nuclear receptors upon oxidative stress, very few transcriptional-regulator and nucleocytoplasmic-shuttling substrates of RANBP2 have been identified in cell lines (Forler et al., 2004; Giri et al., 2005), and, to our knowledge, none has been validated in a physiological setting. Hence, we examined whether any of the orphan nuclear receptors modulated by RANBP2 and light stress (Fig. 1) associates in vivo with RANBP2 in retinal extracts. As shown in Fig. 4, we found that among all orphan nuclear receptors examined only COUP-TFI co-immunoprecipitated with RANBP2 in retinal extracts. The 64 kDa COUP-TFI isoform complexed with RANBP2 represents an isoform of larger mass and lower abundance than the major 50 kDa COUP-TFI isoform present in retinal extracts. Hence, the 64 kDa COUP-TFI isoform bound to RANBP2 probably represents a post-translationally modified form of COUP-TFI. We examined whether

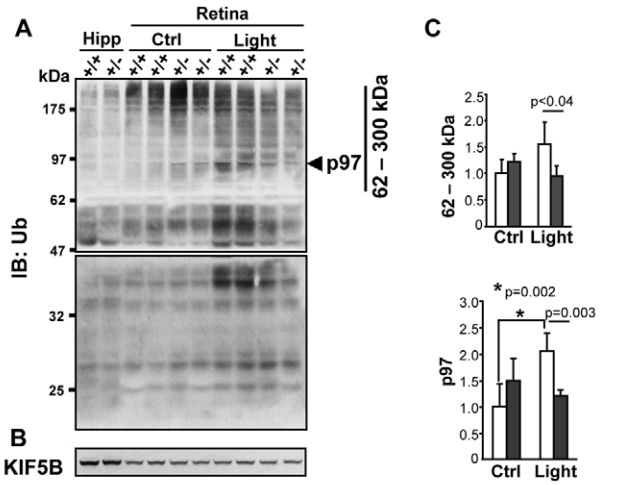

Fig. 3. Insufficiency of RANBP2 suppresses the accumulation of ubiquitylated substrates in response to prolonged light exposure. Immunoblot analyses of retinal and hippocampal (Hipp) extracts of 24-week-old wild-type (+/+) and *Ranbp2*$^{+/-}$ (+/–) mice reared under cyclic (12:12) light conditions (Ctrl) and prolonged light treatment (Light), with antibodies against ubiquitin (A) and KIF5B (B). In comparison with wild-type mice, a decrease of the levels of ubiquitylated substrates was detected in *Ranbp2*$^{+/-}$ mice reared under prolonged exposure to light (A). The levels of the RANBP2 partner KIF5B also remained unchanged (B). (C) Quantification by densitometry of ubiquitylated substrates ranging between the apparent molecular masses of 62 and ~300 kDa [excluding p97, arrowhead (A)], and of a prominent ubiquitylated substrate, p97 [arrowhead, (A)], that are normalized against KIF5B expression. Relative ubiquitin levels in wild-type mice under cyclic light conditions were arbitrarily set as 1. Densitometry values are expressed as arbitrary units. Results shown represent the mean ± s.d. obtained from retinas of four mice per group (n=4) under prolonged light treatment, except for wild-type and *Ranbp2*$^{+/-}$ mice control groups, where n=7 and n=5, respectively. Only changes considered significant (P≤0.05) are noted. White bars: wild-type mice; black bars: *Ranbp2*$^{+/-}$ mice.

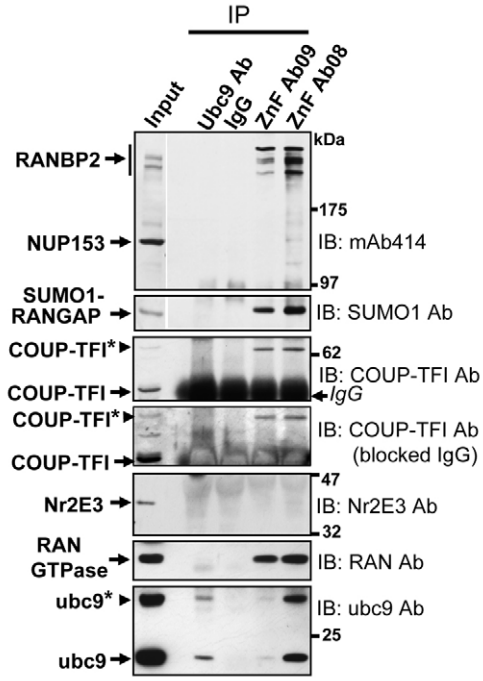

Fig. 4. RANBP2 selectively associates in vivo with COUP-TFI. Immunoprecipitation of RANBP2 with antibodies against its zinc-finger-rich domain (ZnF Ab09, ZnF Ab08) coprecipitates selectively with a modified isoform of COUP-TFI, but not the homologous nuclear receptor Nr2E3 or an unmodified isoform of COUP-TFI (blocked IgG panel). The RANBP2 partners RAN GTPase, SUMO1-RANGAP and UBC9 also coprecipitate with RANBP2 together with a modified isoform of UBC9 (*). Note the antibody employed against UBC9 is not suitable for immunoprecipitation assays as reflected by the detection of very low levels of self-precipitated UBC9 isoforms. The ZnF antibodies selectively immunoprecipitate RANBP2 (NUP358), but not NUP153 and NUP62. mAb414 is a monoclonal antibody that detects RANBP2, NUP153 and NUP62.

the 64 kDa COUP-TFI isoform bound to RANBP2 represented a sumoylated COUP-TFI isoform. The 64 kDa COUP-TFI isoform was not immunoreactive to either SUMO1 or its paralogs SUMO2 and SUMO3 (data not shown). By contrast, only sumoylated RANGAP associated with RANBP2, an observation consistent with other reports (Fig. 4) (Mahajan et al., 1997; Matunis et al., 1996). Immunoblot analysis of RANBP2 coprecipitates also found that the COUP-TFI modification did not reflect ubiquitylation (data not shown). Hence, the 64 kDa COUP-TFI isoform associated with RANBP2 undergoes a yet to be determined modification that is probably crucial to COUP-TFI binding to RANBP2. Interestingly, two isoforms of UBC9, of ~20 and 28 kDa, also coprecipitated with RANBP2 (Fig. 4). The lower isoform is consistent with the apparent mass of UBC9, whereas the latter probably represents an isoform of UBC9 that has a modification other than sumoylation, because it is not immunoreactive to SUMO1, SUMO2 and SUMO3 (data not shown).

Deficits in RANBP2 suppress the accumulation of neutral lipophilic deposits in the RPE upon light damage

In contrast to observations obtained from retinal neurons (Fig. 1), insufficiency of RANBP2 did not affect the steady-state levels of selective partners of RANBP2 in the RPE (Fig. 2). However, we extended our analyses of the RPE to examine the role of RANBP2 in the breakdown of phagocytized membrane-rich outer segments shed daily by photoreceptors damaged by photo-oxidation (Young and Bok, 1969). We employed the lipophilic dye Bodipy 493/503 to probe the accumulation of lipid deposits in the RPE. As shown in Fig. 5, no significant differences are observed between wild-type and *Ranbp2*$^{+/-}$ mice reared under cyclic light conditions. By contrast, upon light-elicited stress, the RPE cells of wild-type mice present strong accumulation of lipid deposits, whereas no accumulation of such deposits is observed in *Ranbp2*$^{+/-}$ mice. Hence, these data further support the view that insufficiency of RANBP2 prevents the accumulation or promotes the clearance of lipid deposits in the RPE. Interestingly, we also found that the accumulation of lipid deposits correlates with the delocalization of COUP-TFII away from the basal end of the RPE cells, whereas COUP-TFII localization is prominently polarized to the basal end of such cells in *Ranbp2*$^{+/-}$ mice upon light-elicited stress (Fig. 5).

Light-elicited oxidative stress and insufficiency of RANBP2 induce perturbations in lipid metabolites in the RPE

We have reported previously that haploinsufficiency of *Ranbp2* promotes an age-dependent decrease of free fatty acids in the

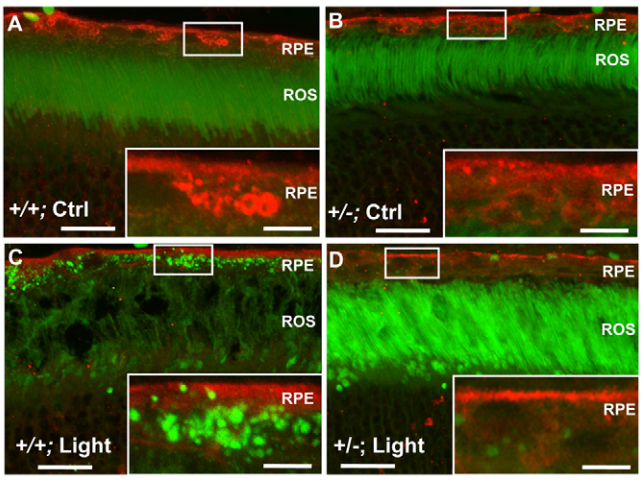

Fig. 5. Prolonged light exposure promotes the RANBP2-dependent accumulation of lipophilic deposits and basal polarization of COUP-TFII in RPE cells. 24-week-old wild-type (+/+) (A,C) and *Ranbp2*$^{+/-}$ (+/–) (B,D) mice reared under cyclic (12:12) light conditions (Ctrl) (A,B) and prolonged light treatment (Light) (C,D) were stained with the lipophilic dye Bodipy 493/503 for neutral lipids (green) and immunostained with anti-COUP-TFII antibody (red). Note the accumulation of lipophilic deposits in the RPE and swollen RPE cells of wild-type mice (C), whereas COUP-TFII is delocalized towards the basal end of the RPE cells in *Ranbp2*$^{+/-}$ mice (D). Inset figures are magnified images of RPE areas in squares. RPE, retinal pigment epithelium; ROS, outer segments of rod photoreceptor neurons (rod outer segments). Scale bars, 20 μm; scale bars in inset figures, 5 μm.

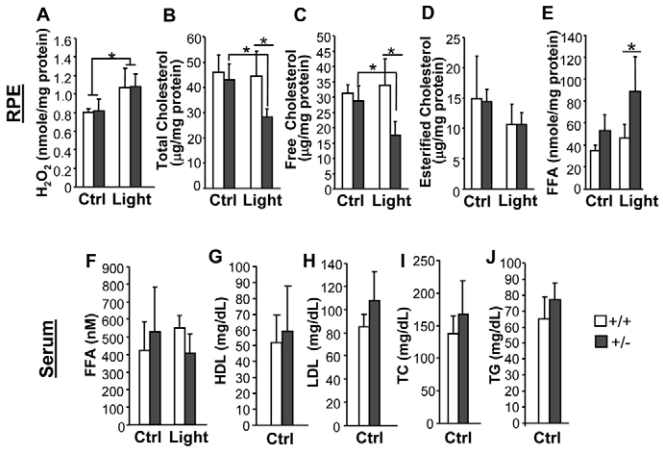

Fig. 6. Perturbation of levels of cholesterol and free fatty acids in the RPE in response to gene dosage of *Ranbp2* and light-elicited oxidative stress. Prolonged light exposure (Light) induces an increase in H_2O_2 in the RPE regardless of the genotype (A). In *Ranbp2*$^{+/-}$ mice, total and free cholesterol levels in the RPE were decreased upon prolonged light exposure (B,C), whereas there was an increase in levels of free fatty acids under the same light conditions (E). The levels of esterified cholesterol in the RPE remain unaltered regardless of the genotype and light conditions (D). Mice of either genotype present no changes in free fatty acids in the serum regardless of the light conditions (F). Wild-type and *Ranbp2*$^{+/-}$ mice also present no innate changes in high-density lipoprotein cholesterol (HDL) (G), low-density lipoprotein cholesterol (LDL) (H), total cholesterol (TC) (I) and triglycerides (TG) (J) in the serum. Results shown represent the mean ± s.d. of RPE or serum of four mice per group (*, $P \leq 0.05$; $n=4$), except for Ctrl in B, C and D ($n=3$). Only changes considered significant ($P \leq 0.05$) are noted. Ctrl, cyclic (12:12) light treatment; Light, prolonged light treatment. +/+ and +/– are wild-type and *Ranbp2*$^{+/-}$ mice, respectively.

retina regardless of the light exposure, whereas polarization of cholesterol lipid droplets toward the basal end of the RPE cells is observed upon light stress (Cho et al., 2009b). These observations, together with the findings reported here of a light-elicited accumulation of lipophilic deposits in the RPE of wild-type but not *Ranbp2*$^{+/-}$ mice, support distinct roles of RANBP2, some of its partners and light in lipid homeostasis of the RPE, which is crucial also to the nourishment of photoreceptor neurons (Young and Bok, 1969). Hence, we examined further the effects of chronic light and deficits in RANBP2 in the accumulation of reactive oxygen species, cholesterol and free fatty acids in the RPE tissue. Like in the retina (Cho et al., 2009b), chronic light induced equally a significant increase of hydrogen peroxide (H_2O_2) in wild-type and *Ranbp2*$^{+/-}$ mice (Fig. 6A). However, chronic light exposure promoted a decrease in total cholesterol in *Ranbp2*$^{+/-}$ but not wild-type mice, whereas there was no difference in total cholesterol levels between these mice under cyclic light conditions (Fig. 6B). This decrease in total cholesterol is accounted for by a decrease in the levels of nonesterified cholesterol, because the levels of esterified cholesterol remained unchanged regardless of the genotype and light treatment (Fig. 6C,D). In addition, these effects were accompanied by a strong trend for a genotype-dependent increase of free fatty acids in the RPE in the absence of light stress ($P<0.06$), an increase that became significant when mice were subjected to prolonged light exposure (Fig. 6E). The rise in free fatty acids in the RPE of *Ranbp2*$^{+/-}$ mice was intrinsic to the RPE, because the levels of free fatty acids in the serum remained unchanged regardless of the genotype and light treatment (Fig. 6F) and there were no innate changes of the levels of triglycerides, total cholesterol and major lipoproteins in the serum of mice of either genotype (Fig. 6G-J).

DISCUSSION

The identification of factors capable of blocking or reversing the multifactorial and pathological effects associated with oxidative stress is of high therapeutic significance. The present study builds upon previous work showing that haploinsufficiency of *Ranbp2* suppresses the apoptosis of photoreceptor neurons under photo-oxidative stress (Cho et al., 2009b). Hence, RANBP2 and its partners emerge as multifunctional and therapeutic targets to counteract or ameliorate the toxic effects of oxidative stress. Pathologies related to oxidative stress and aging are implicated in the downregulation of proteasome activity by mechanisms that are not well understood (Andersen, 2004; Breusing and Grune, 2008; Ethen et al., 2007; Kapphahn et al., 2007; Reinheckel et al., 1998; Vigouroux et al., 2004). Our study shows that insufficiency of RANBP2 combined with light-elicited oxidative stress promotes the downregulation of the levels of selective partners of RANBP2, an outcome that is associated with an increase in 26S proteasome activity – as revealed by the decrease, or suppression of an increase, of the levels of selective partners of RANBP2 and reduction of ubiquitylated substrates in the retina. Strikingly, these effects are selective to the retina, because the levels of the light-stress-

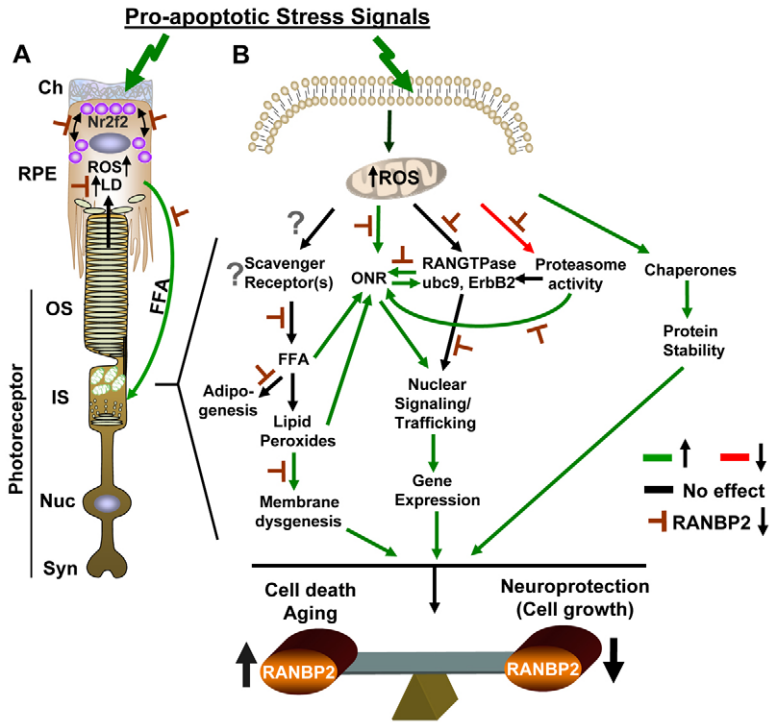

Fig. 7. Overview of a model depicting the effects of oxidative stress and deficits in RANBP2 in the RPE and photoreceptor neurons. (A) Photoreceptor neurons take up nutrients from the choroidal (Ch) circulation via the RPE cells, which phagocytize the shed membrane-rich outer segments (OS) of photoreceptors and also provide recycled lipid metabolites, such as free fatty acids (FFAs), to photoreceptors by an unknown uptake mechanism. Photo-oxidative stress promotes the accumulation of (neutral) lipid deposits (LDs) probably as a result of impairment of phagocytic and/or proteolytic processes. Orphan nuclear receptors, such as COUP-TFII (Nr2f2), might act as signaling sensors of the homeostasis of lipid metabolites (e.g. FFAs). These effects are affected by insufficiency of RANBP2. (B) In photoreceptors, oxidative stress has multiple effects by causing the upregulation of the expression of orphan nuclear receptors (ONRs) and chaperones (e.g. Hsp70, mHsp70), the downregulation of the proteasome activity and lipid-peroxidation-induced dysgenesis of membranes. Toxic metabolites from lipid peroxidation might also serve as pathophysiological ligands to ONRs. These processes promote a cascade of events culminating in cell death. Insufficiency of RANBP2 counteracts several pathophysiological processes elicited by oxidative stress, it promotes neuroprotection of terminal differentiated photoreceptor neurons to light-elicited oxidative stress and it increases the susceptibility of mitotic cells to uncontrolled cell growth. IS, inner segment compartment of photoreceptor neuron; Nuc, nucleus; OS, outer segment compartment of photoreceptor neuron; ROS, reactive oxygen species; Syn, synapse; green line, stimulatory effect; red line, inhibitory effect; upward arrow, increased level; downward arrow, decreased level. Suppressing effects of RANBP2 are marked by a T-bar.

modulated RANBP2 partners in the retina, with the exception of S1, remain unchanged in the RPE. Hence, the data support the down-modulation of cell-type-dependent crosstalk between stress pathways, RANBP2 and some of its partners, which together suppress the deleterious effects associated with photo-oxidative stress in photoreceptors.

Although we have shown that ectopic expression of the CLD of RANBP2 selectively modulates the proteostasis of properly folded proteins in cultured cells (Yi et al., 2007), the physiological effects of deficits of RANBP2 on proteostasis were unknown until this study. Here we identified ErbB-2 as a physiological substrate whose homeostasis depends differentially on RANBP2 or photo-oxidative stress. This outcome suggests that two interconnected mechanisms modulated by RANBP2, such as importin-β-dependent nuclear translocation and ubiquitin-proteasome degradation of ErbB-2 (Giri et al., 2005; Mimnaugh et al., 1996), operate distinctively in the regulation of ErbB-2 levels in the presence and absence of light stress. The upregulation of ErbB-2 by insufficiency of RANBP2 also raises the possibility that ErbB-2 plays a role in the rise of susceptibility of certain tissues to tumorigenesis in mice with insufficiency of RANBP2 (Dawlaty et al., 2008).

The RAN-GTP-dependent association of RANBP2 with the nuclear import receptor importin-β and the nuclear export receptor exportin-1 (CRM1) lends strong support to the RANBP2-mediated regulation of trafficking between the nuclear and cytosolic compartments (Chi et al., 1996; Delphin et al., 1997; Singh et al., 1999; Vetter et al., 1999), even though insufficiency of RANBP2 impairs neither the overall nucleocytoplasmic trafficking (Dawlaty et al., 2008) nor the number of nuclear pores (Aslanukov et al., 2006; Dawlaty et al., 2008). These observations hint that RANBP2 modulates the nucleocytoplasmic shuttling of a restricted set of nuclear factors. In support of this idea, we found that RANBP2 modulates the proteostasis of several orphan nuclear receptors and, among these, a modified isoform of COUP-TFI stably co-associates with RANBP2 and UBC9. These observations raise the possibility that the nuclear shuttling of COUP-TFI depends not just on UBC9 (Kobayashi et al., 2004; Kurihara et al., 2005), but also on RANBP2 and post-translational modification of COUP-TFI by a mechanism independent of sumoylation. Identification of the nature of the modification of COUP-TFI will provide important insights into the mechanisms regulating its homeostasis, nuclear shuttling or interaction with RANBP2. Our data also indicate that a decrease of the levels of RAN GTPase through insufficiency of RANBP2 under photo-oxidative stress does not affect just nucleocytoplasmic trafficking processes dependent on the levels of RAN GTPase, because such effects are also accompanied by the upregulation of UPS activity, as reflected by the reduction of the level of UBC9 and suppression of the rise of the levels of orphan receptors and ubiquitylated substrates. These outcomes are concordant with studies in cultured cells where oxidative stress was found to dissipate the nucleocytoplasmic RAN GTPase gradient by promoting the proteasome-dependent proteolysis of partners of RANBP2, such as RAN GTPase and importin-β (Kodiha et al., 2004). Finally, this and prior work support the view that the down-modulation of the levels of RAN GTPase and nuclear receptors upon oxidative stress combined with the decrease of free fatty acids in the retinas of haploinsufficient-*Ranbp2* mice (Cho et al., 2009b) are determinant factors that collectively contribute to the neuroprotection of photoreceptor neurons against photo-oxidative stress. Such reduced levels of free fatty acids might also contribute to a decrease of the levels of pathophysiological ligands of nuclear

receptors that are known to arise from the radical oxidation of free fatty acids or cholesterol (Gao et al., 2007; Jansen et al., 2004; Szatmari and Nagy, 2008; Zelcer and Tontonoz, 2006).

Notably, our findings show that insufficiency of RANBP2 in the RPE has distinct effects from those observed in the retina in response to light-elicited oxidative stress (Cho et al., 2009b). First, and with the exception of S1, the changes in protein homeostasis observed in the retina did not take place in the RPE. Second, we found a light- and genotype-dependent increase of free fatty acids in the RPE that was accompanied by a decrease in nonesterified cholesterol (Fig. 6C,E). There was also a strong trend for an increase of free fatty acids in the RPE of *Ranbp2*$^{+/-}$ mice compared with wt mice in the absence of light stress (P<0.06) (Fig. 6E). These effects are in contrast to a RANBP2-dependent decrease of free fatty acids without significant changes in the levels of cholesterol metabolites observed in the retina (Cho et al., 2009b). Third, the magnitude of the increase in H_2O_2 production upon light-elicited oxidative stress in the RPE was lower than that observed in the retina (~1.4-fold versus 2-fold, Fig. 6A) (Cho et al., 2009b). These observations together with our prior data (Cho et al., 2009b) suggest that a deficit of RANBP2 impairs the recycling of free fatty acids from the RPE to photoreceptors, an effect that is exacerbated by oxidative stress. A possible explanation is that a deficit in RANBP2 affects the expression of scavenger receptor(s) of free fatty acids in photoreceptors, thus impairing a short-range metabolic and signaling loop between the RPE and photoreceptors (Bazan, 2006). Impairment of such a metabolic loop does not promote the formation of lipid deposits in the RPE of *Ranbp2*-haploinsufficient mice upon oxidative stress probably because of the upregulation of UPS activity, which our data suggest contributes to the clearance of phagocytized outer segment membranes of photoreceptors in the RPE.

Hence, our studies support a model where gene dosage of *Ranbp2,* combined with light-elicited oxidative stress, selectively modulates the homeostasis of a set of partners of RANBP2 and lipid metabolites, affecting multiple but functionally interconnected pathways implicated in various biological processes (Fig. 7). Although the multifactorial impact of RANBP2 is distinct in different tissues, its intrinsic tissue-specific functions might depend partially on paracrine signaling pathways by regulating the production or transport of paracrine signals, such as free fatty acids, and receptors for these. In light of the role of insufficiency of RANBP2 also in oncogenesis (Dawlaty et al., 2008), it is opportune to note that a hallmark feature of cancer cells is their ability to synthesize de novo mono-unsaturated and saturated fatty acids rather than depending on the nutritional supply like normal cells (Greenstein, 1954; Medes et al., 1953; Swinnen et al., 2006). Hence, it is tempting to explore whether the RANBP2-dependent pathways determining neuroprotection of terminally differentiated photoreceptors to oxidative stress also contribute to the increased susceptibility of mitotic cells to oncogenesis (Dawlaty et al., 2008). The heterogeneous nature of stress stimuli converging to the RANBP2 assembly complex is likely to trigger distinct RANBP2-mediated responses across tissues as result of the modulation of stress- and receptor-specific pathways crosstalking to RANBP2 and its partners. Moreover, the likelihood that not all the pathways triggered by a variety of stressors or deficits in RANBP2 operate in any one cell type further contributes to the heterogeneity of RANBP2-mediated phenotypic manifestations. Hence, it will be important in future studies to differentiate and elucidate the exact contribution of each of the RANBP2-regulated pathways and components thereof in the neuroprotection of photosensory neurons to light damage and development of disease manifestations linked to other stressors, such as carcinogens and infectious agents (Cho et al., 2009b; Dawlaty et al., 2008; Neilson et al., 2009). Such studies will also help to identify novel therapeutic targets and approaches to a variety of disorders affecting cell survival and proliferation.

METHODS

Mice and light treatment

Wild-type and *Ranbp2*$^{+/-}$ mice of 24 weeks of age in an inbred 129P2/OlaHsd background were employed in this study as described elsewhere (Cho et al., 2009b). Mice were raised in a pathogen-free transgenic barrier facility at <70 lux and given ad libitum access to water and the chow diet 5LJ5 (Purina). For the light experiments, mice were kept in a standard 12:12 light-dark cycle at <70 lux unless otherwise noted, and prolonged light treatment was performed by exposing mice to 1200 lux of continuous white light for 48 hours as described elsewhere (Cho et al., 2009b). During the light experiments of 48 hours, mice were given ad libitum access to water and the chow diet 5LJ5 (Purina) except for the last 12 hours, when mice were fasted prior to the collection blood and being sacrificed for the collection of tissues. Animal protocols were approved by the Institutional Animal Care and Use Committee at Duke University and the procedures adhered to the ARVO guidelines for the Use of Animals in Vision Research.

Immunochemistry reagents

The antibodies used for immunoblot analyses in this study were mouse monoclonal antibody mAb414 against nuclear-pore-complex proteins RANBP2 (NUP358), NUP153 and NUP62 (400 ng/ml; Abcam), mouse anti-RANGTPase (1:4000; Affinity Bioreagents), mouse anti-UBC9 (1:1000; BD Transduction), goat anti-NeuroD (1:500; Santa Cruz Biotechnology, N-19), rabbit anti-Nr2E3 (1:1000; gift from Anand Swaroop), rabbit anti-COUP-TFI (1:500; gift from Michele Studer), rabbit anti-COUP-TFII (1:1000; Aviva System Biology, ARP39466_T100, San Diego), mouse anti-ATPase syntase subunit α (1:1000; Mitoscience), mouse anti-acetylated α-tubulin (1:40,000; Sigma Aldrich), rabbit anti-Hsc70 (1:3000; Stressgene), mouse anti-mHsp70 (1:3000; Affinity Bioreagent), rabbit anti-S1 (1:2500; Affinity Bioreagent), goat anti-AIF (1:1000; Santa Cruz Biotechnology, D-20), mouse anti-ErbB-2 (2.5 μg/ml; Calbiochem), rabbit anti-HDAC4 (400 ng/ml; Santa Cruz Biotechnology, H-92), mouse anti-KIF5B/KIF5C (1:1500; H2, gift from George Bloom, University of Virginia, Charlottesville, VA), mouse anti-importin-β monoclonal antibody 3E9 (1:2000; gift from Steven Adam), mouse anti-RANGAP1 (1:2000; Zymed), mouse anti-GMP1/SUMO1 (1:1000; Zymed), mouse anti-ubiquitin (1:1000; Santa Cruz Biotechnology, P4D1), rabbit anti-SUMO2/3 C-term (1:1000; Abgent). The rabbit anti-RANBP2 ZnF#20909 (ZnF Ab09) and 20908 (ZnF Ab08) were described elsewhere (Mavlyutov et al., 2002; Singh et al., 1999). For immunohistochemistry, the rabbit anti-COUP TFII (1:200; Aviva System Biology, P100816, San Diego) was employed.

Tissue extracts and immunoblot analyses

Tissue samples were homogenized on ice with a Kontes Microtube Pellet Pestle Rods with Motor in NP-40 buffer with complete protein inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Supernatants of extracts were collected after centrifugation at 10,000 ***g*** for 15 minutes. Protein concentration was measured by the Bradford method using BSA as standard. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and western blotting was carried out as described elsewhere (Ferreira, 2000). Whenever applicable, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific), blocked, and reprobed with different antibodies as described elsewhere (Ferreira, 2000). Densitometry analyses of immunoblots were performed with Metamorph v6.2 (Molecular Devices). Where applicable, two-tailed *t*-test statistical analysis was performed; $P \leq 0.05$ was defined as significant.

Immunoprecipitation assays

Immunoprecipitation of RANBP2 or UBC9 was performed using fresh bovine retinal extracts solubilized in NP-40. 3 µg of rabbit antibodies against the zinc-finger-rich domain of RANBP2 (ZnF Ab09, ZnF Ab08) (Singh et al., 1999) or mouse anti-UBC9 (BD Transduction Laboratories) was incubated with 3 mg of extract for 1 hour at 4°C, and 50 µl of 50% protein A sepharose beads (Amersham Pharmacia) was added and incubated for another hour. Beads were washed three times with NP-40 buffer and then proteins were eluted with SDS-sample buffer, resolved by SDS-PAGE, and blotted onto PVDF membrane as described elsewhere (Cai et al., 2001). To block IgG in immunoprecipitates, membranes were blocked with ReliaBLOT (Bethyl Laboratories, Montgomery, TX) where indicated and as per manufacturer's instructions.

Bodipy 493/503 staining

Radial cryosections of eyecups fixed with 4% paraformaldehyde in PBS solution (pH 7.4) were incubated with Bodipy 493/503 (1 µg/ml; Invitrogen) for 1 hour at room temperature, and then washed several times with PBS solution. Sections were mounted in ProLong Gold antifade reagent (Invitrogen).

Hydrogen-peroxide measurement

Oxidative stress in retina and RPE specimens upon cyclic and prolonged light exposure was always verified by measuring the production of hydrogen peroxide in the respective tissues. The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was employed to measure the hydrogen peroxide levels in NP40-solubilized tissue extracts (10 µl) according to the manufacturer's instructions. Hydrogen peroxide levels were normalized against protein contents in the extract. Two-tailed equal variance *t*-test statistical analysis was performed; $P \leq 0.05$ was defined as significant.

Quantification of free cholesterol, total cholesterol and free fatty acids in the RPE

Regardless of the light conditions, mice were fasted for 12 hours and then sacrificed, and tissues were immediately collected and snap frozen on dry ice. The Cholesterol and Cholesterol Ester Quantitation Kit (Biovision) was employed to measure free cholesterol and total cholesterol in NP40-solubilized tissue extracts (3 µl) diluted with assay buffer as per the manufacturer's instructions. Measurements of fluorescence were performed at excitation/emission/cutoff=560/590/590 nm (SpectraMax M5,

TRANSLATIONAL IMPACT

Clinical issue

Oxidative stress is associated with a plethora of human diseases, aging manifestations and metabolic deficits. Regardless of the etiology, the biological and pathological effects of oxidative stress are linked to dysregulation of various signaling pathways and subcellular processes, such as mitochondrial dysfunction, toxicity caused by the accumulation of metabolic byproducts and impaired functioning of detoxifying subcellular machineries such as the ubiquitin-proteasome system. Accumulating evidence indicates that different populations of neurons show differential vulnerabilities and responses to oxidative stress. In addition, mutations in a widely expressed gene can alter the vulnerability of select neuronal cell types to oxidative stress and can promote neurodegeneration. The identification and characterization of factors and processes that suppress the deleterious effects of oxidative stress can provide insight into mechanisms of neuroprotection and uncover novel therapeutic targets by which the clinical manifestations of various diseases and aging can be modulated.

Results

Light-elicited oxidative stress is a deleterious risk factor linked to retinal dystrophies, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP), which occur owing to the selective degeneration of abundant photoreceptor neurons of the retina. In this work, the authors explore the molecular effects of insufficiency of RAN-binding protein-2 (RANBP2) in protecting photoreceptors and the supporting tissue, the retinal pigment epithelium (RPE), from degeneration induced by light-elicited oxidative stress. By contrasting molecular and subcellular phenotypes of retina and RPE in inbred wild-type and *Ranbp2*-haploinsufficient mice in the absence or presence of light-elicited oxidative stress, the authors show that the expression levels of a subset of functionally diverse partners of RANBP2 – such as RAN GTPase, UBC9, subunits of the 26S proteasome and a set of orphan nuclear receptors – are differentially modulated by *Ranbp2* haploinsufficiency and oxidative stress in the retina. The effects of oxidative stress are also accompanied in *Ranbp2*-haploinsufficient mice by a decrease in the levels of ubiquitylated proteins, thereby supporting the notion that insufficiency of RANBP2 relieves the suppressive effect of this protein on the activity of the 26S proteasome in the presence of oxidative stress. Strikingly, the authors also find that the formation of lipid deposits induced by oxidative stress in the RPE – a hallmark of many neurodegenerative diseases and aging – is strongly suppressed by *Ranbp2* haploinsufficiency. However, in contrast to effects in the retina, the expression levels of most RANBP2 partners are not affected in the RPE.

Implications and future directions

This study establishes a cell-context dependent and direct link between RANBP2, a subset of its binding partners, light-elicited oxidative stress and associated pathological manifestations. These data have multiple implications. First, they establish the *Ranbp2*-haploinsufficient mouse model as a useful tool to study the roles of oxidative signaling pathways and various stressors in the context of aging, neurodegeneration and other diseases that affect photoreceptors. Second, these data might help to unravel the roles of various stressors in other neuronal cell types with differential susceptibility to oxidative stress. Finally, determining the cell-context-dependent effects of the subunits of the pleiotropic complex that is regulated and assembled by RANBP2 will help to unravel further the complexity of responses underpinning stress-signaling pathways, and contribute to developing novel therapeutic approaches to targets with neuroprotective properties.

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Molecular Devices). Total cholesterol was measured with cholesterol esterase added; free cholesterol was measured without cholesterol esterase. The amount of esterified cholesterol was calculated by the subtraction of free cholesterol from total cholesterol. The Free Fatty Acid Quantification Kit (Biovision) was employed to measure C_8 (octanoate) and longer fatty acids in NP40-solubilized tissue extracts (2 μl) as per the manufacturer's instructions. The free fatty acid palmitic acid was used as a standard to measure the free fatty acid content with a colorimetric assay (Ab570). Results were normalized against protein contents in the tissue extracts. Two-tailed equal and unequal variance *t*-test statistical analyses were performed; $P \leq 0.05$ was defined as significant.

Quantification of lipids in the serum

Mice were fasted for 12 hours, then pre-prandial blood was collected from the facial vein into EDTA-containing tubes (final concentration 1 mM) and spun for 2000 ***g*** for 20 minutes; serum was then collected. The Free Fatty Acid Quantification Kit (Biovision) was employed to measure C_8 and longer fatty acids in sera (2 μl) as per the manufacturer's instructions and described before. Colorimetric measurements of high-density lipoprotein (HDL), total cholesterol and triglycerides were performed in a SpectraMax-M5 plate reader (Molecular Device, Sunnyvale, CA). HDL was measured with 2 μl of serum using the HDL Ultra Cholesterol Reagent Kit (Equal Diagnostics/Genzyme Diagnostics, Cambridge, MA). Total cholesterol was measured with 10 μl of serum using the Cholesterol SL-Assay (Diagnostic Chemicals, Oxford, Connecticut). Low-density lipoprotein (LDL) was calculated as the difference between total cholesterol and HDL. Triglycerides were measured with 10 μl of serum using the Triglyceride-SL Assay (Diagnostic Chemicals, Oxford, Connecticut). Two-tailed equal and unequal variance *t*-test statistical analyses were performed; $P \leq 0.05$ was defined as significant.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

K.-i.C., H.Y. and P.A.F. conceived and designed experiments, and edited the manuscript. K.-i.C., H.Y., N.T. and K.S. performed experiments. K.-i.C., H.Y. and P.A.F. analyzed the data. P.A.F. wrote the paper.

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