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Haploinsufficiency of *RanBP2* is neuroprotective against light-elicited and age-dependent degeneration of photoreceptor neurons

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Abstract

Prolonged light exposure is a determinant factor in inducing neurodegeneration of photoreceptors by apoptosis. Yet, the molecular bases of the pathways and components triggering this cell death event are elusive. Here, we reveal a prominent age-dependent increase in the susceptibility of photoreceptor neurons to undergo apoptosis under light in a mouse model. This is accompanied by light-induced subcellular changes of photoreceptors, such as dilation of the disks at the tip of the outer segments, prominent vesiculation of nascent disks, and autophagy of mitochondria into large multilamellar bodies. Notably, haploinsufficiency of *Ran-binding protein-2* (*RanBP2*) suppresses apoptosis and most facets of membrane dysgenesis observed with age upon light-elicited stress. *RanBP2* haploinsufficiency promotes decreased levels of free fatty acids in the retina independent of light exposure and turns the mice refractory to weight gain on a high-fat diet, whereas light promotes an increase in hydrogen peroxide regardless of the genotype. These studies demonstrate the presence of age-dependent and RanBP2-mediated pathways modulating membrane biogenesis of the outer segments and light-elicited neurodegeneration of photoreceptors. Furthermore, the findings support a mechanism whereby the RanBP2-dependent production of free fatty acids, metabolites thereof or the modulation of a cofactor dependent on any of these, promote apoptosis of photoreceptors in concert with the light-stimulated production of reactive oxygen species.

Keywords

Ran-binding protein 2 (RanBP2); neuroprotection; light; free fatty acids; photoreceptor neurons; apoptosis

Introduction

The retina is comprised of a well-defined neurocircuitry mediating the capture, processing and transmission of photon stimuli to high-order processing centers in the brain. The primary neurons of the retina, rod and cone photoreceptors, mediate the physicochemical transduction of light. While several components of the light-transduction cascade promote the degeneration of photoreceptors upon inherited mutations in the cognate genes [1], light acts also as a powerful

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inducer of degeneration of these neurons in wild-type mouse strains.[2] Neurodegeneration elicited by light and age appears to vary in multiple genetic backgrounds, thus supporting the presence of various genetic modifiers of cell death upon selective stressors.[3–5]

To this date, few loci conferring resistance to light damage have been identified in genetically altered mice. These include mice lacking the expression of *Rpe65*, *Rho*, and mice harboring the *Rpe65* Leu450Met mutation.[5–9] Although some of these loci appear to have no impact on age-related retinal degeneration, quantitative trait loci have been implicated in age-related retinal degeneration, but the identities of the genes implicated in this process remain elusive.[3, 4] Regardless, cumulative damage from oxidative stress appears to play a determinant role in the development of age-related phenotypes of photoreceptors in part as the result of marked and uneven oxygen tension, and metabolic demands, across the retina, that make photoreceptors particularly vulnerable to oxidative damage.[10–13] To this effect, overexpression of erythropoietin in transgenic mice is neuroprotective against light-induced but not inherited retinal degeneration.[14] Hence, the data hint of a link between light- and age-dependent death of photoreceptor neurons. On the other hand, the phenotypic analyses of genetically engineered mouse models support that light-induced degeneration is independent of the activation of phototransduction, but dependent on the light-receptor, rhodopsin, and that independent and poorly defined mechanisms triggering apoptosis may operate for light-induced, age-dependent, and inherited forms of retinal degeneration.[5, 8] In addition, light-elicited degeneration of photoreceptors may act synergistically with certain forms of inherited degeneration selectively affecting these neurons, because neurodegeneration is exacerbated by light in certain mouse models with inherited degeneration of photoreceptors.[15–19] Hence, the identification of novel components modulating the death of photoreceptors upon light and aging are likely to provide critical insights to novel pathways underlying the molecular bases of neurodegeneration upon various stress stimuli.

The Ran-binding protein-2 (RanBP2) is at the nexus of multiple subcellular and molecular processes underlying nuclear-cytoplasmic trafficking [20–22], transport and function of mitochondria [23], modulation of proteasome function and protein homeostasis [23–26], and modulation of protein-protein interaction by sumoylation in culture cells.[27–30] Notably, haploinsufficiency of *RanBP2* in combination with diet and genetic background triggers defined age-related phenotypes manifested by perturbation of growth and glucose catabolism.[31] In addition, further decrease of the levels of RanBP2 in a mouse model harboring a hypomorphic allele of *RanBP2* promotes missegregation of chromosomes (aneuploidy) in mitotic cells and carcinogen-elicited and age-dependent tumorigenesis without overall impairment of nuclear-cytoplasmic trafficking, mitotic spindle formation, and protein SUMO-modification.[32] Here, we reveal that light-induced susceptibility to damage and apoptosis of photoreceptor neurons increases prominently between 12- and 24-week old inbred 129P2/OlaHsd mice, and that haploinsufficiency of *RanBP2* in these mice suppresses strongly the age- and light-dependent increase of damage and cell-death of photoreceptors. Moreover, the neuroprotective effects caused by a deficit in RanBP2 is reflected by a significant decrease of free fatty acids, which upon light-induced oxidative stress, may suppress apoptosis and preceding phenotypes such as membrane dysgenesis.

Results

Light-induced morphological changes of photoreceptor neurons by *RanBP2* haploinsufficiency and age

We previously identified phenotypes in *RanBP2* haploinsufficient mice that are manifested in an age-dependent fashion.[31] In light of the role of RanBP2 in retinal function [31] and multiple processes that may contribute to the development of manifestations linked to cell death events, we assessed the role of RanBP2 in the development of light- and age-dependent phenotypes

linked to the damage and death of photoreceptor neurons. Because age-dependent manifestations were observed between 12-and 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice on an inbred 129P2/OlaHsd background [31], we examined the effect of constant white illumination for a period of 48 hours on inducing gross morphological abnormalities of retinal neurons, in particular photoreceptors, between 12- and 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice on the same genetic background. We found that 12-week old *RanBP2*$^{+/-}$ mice presented vacuolization of the outer segment compartment of photoreceptors that was limited to the distal (upper) portion of this compartment. This damage was less apparent in *RanBP2*$^{+/+}$ mice (Figures 1a and b). In addition, few scattered and pyknotic nuclei were visible in both *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice, but no significant changes were observed in the organization of the outer nuclear layer and inner segment compartment of photoreceptors. In contrast, the central regions of the retina of 24-week old *RanBP2*$^{+/+}$ mice presented drastic morphological changes of the outer and inner segment compartments of photoreceptors, including condensed inner segments, overall disorganization of outer segments and outer nuclear layer, the presence of cystic spaces and cellular-like debris in the inner segments, and a strong increase of widespread condensed nuclei of photoreceptors that led to a decrease of the thickness of the outer nuclear layer (rows of nuclei) (Figure 1c, supplementary Figure 1). These phenotypic abnormalities in the central regions of the retina were largely alleviated in *RanBP2*$^{+/-}$ mice (Figure 1d) and peripheral regions of the retina of both genotypes (Figs. 1e and f).

Haploinsufficiency of *RanBP2* alleviates membrane dysgenesis in photoreceptors triggered by prolonged light exposure

We assessed further the abnormalities of photoreceptors upon light-induced degeneration at ultrastructural level. There was a strong increase in the overall disorganization of the rod and inner segment subcellular compartments of photoreceptors in 24-week old *RanBP2*$^{+/+}$ mice compared with 12-week *RanBP2*$^{+/+}$ mice (Figures 2a, b). This increase in disorganization was largely suppressed in *RanBP2*$^{+/-}$ mice (Figures 2c, d). The subcellular changes in photoreceptors induced by light comprised a substantial increase of dilated disks at the tip of the outer segments, the presence of condensed (electrodense) inner segments (star, Figure 2b), large cystic spaces between outer segments and between nuclei (arrowheads, Figure 2b), vacuoles in the inner segments, and migration of mitochondria to the outer nuclear layer. These age-dependent pathological phenotypes were lessened in *RanBP2*$^{+/-}$ mice (Figures 2c, d). In addition, there are at least two unique features at ultrastructural level that were observed in photoreceptors of 24-week old *RanBP2*$^{+/+}$, but not *RanBP2*$^{+/-}$ mice. First, *RanBP2*$^{+/+}$ mice presented a prominent accumulation of multivesicular bodies at the base of the outer segments (Figures 2b, e) that disrupted the stacking of the nascent disks of the outer segments. This was accompanied often by fragmentation of the nascent disks, formation of large lateral disk lamellas and numerous vesicular bodies of various sizes (Figure 2e, inset picture). Conversely, *RanBP2*$^{+/-}$ mice appear largely to present normal formation of nascent disks, but two large vesicular bodies were often visible between nascent disks causing the focal disruption of their stacking (Figure 2f, arrows), and small crescent vesicular bodies appear to bulge also from the rims of some nascent disks (Figure 2f, arrowheads). In both genotypes such vesicular bodies seem comprised of a single membrane leaflet or no membrane could be discerned. Second, the inner segment compartment often presented large multilamellar bodies (MLBs) with loose lamellae of variable interlamellar space surrounding multiple mitochondria, indicating possibly the degeneration of mitochondria into MLBs (Figures 2e, g). This phenotype was not observed in *RanBP2*$^{+/-}$ mice. Mitochondria presented also dilation and fragmentation of the cristae. Thus, the data support the existence of a RanBP2-dependent switch rendering photoreceptors susceptible to damage by light with age and of a neuroprotective mechanism by *RanBP2* that halts the age-dependent effect of light degeneration. Interestingly, all mice exhibited the dilation and disruption of the disks at the tip of the outer segments of photoreceptors

independently of their age and genotype (Figures 2a-d). This phenotype was not observed in *RanBP2*$^{+/+}$ mice reared under low (<70 lux) illumination (supplementary Figure 2). Hence, there is a non-selective RanBP2-independent effect of light in the development of dilated and disorganized disks at the tip of photoreceptors that is common at early stages of light-induced damage of photoreceptors.

Differences in light-induced apoptosis of photoreceptors across the retina caused by aging and *RanBP2* haploinsufficiency

We performed morphometric analyses on the impact of light on eliciting apoptosis of photoreceptor neurons in 12- and 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice. We surveyed retina sections for the presence of DNA fragmentation in nuclei by TdT-mediated dUTP nick end-labeling (TUNEL) assay. Among retinal neurons, light-elicited apoptosis was restricted to nuclei of photoreceptor neurons regardless of the age and genotype of the mice (Figure 3). There was no apparent difference in cell death between 12-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$mice (Figures 3a and b). Conversely, 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$mice presented a substantial difference in the number of apoptotic nuclei in photoreceptors (Figures 3c - f). To this effect, wild-type mice exhibited a strong increase in apoptosis of photoreceptors (Figures 3c, e) in comparison to age-matched *RanBP2*$^{+/-}$ mice (Figures 3d, f). The same was also observed between 12- and 24-week old wild-type mice (Figures 3a, c and e), but not between 12-and 24-week old *RanBP2*$^{+/-}$ mice (Figures 3b, d and f). These observations were confirmed by quantitative analyses of TUNEL-positive nuclei of photoreceptors derived from several sections of the eyecup. A 5-fold significant increase of the total number of apoptotic photoreceptors was observed in 24-week old *RanBP2*$^{+/+}$ mice compared with 12-week *RanBP2*$^{+/+}$ mice (Figure 4a), while there was not a significant difference between 12- and 24-week old *RanBP2*$^{+/-}$ mice (Figure 4b). At the age of 12 weeks, both wild-type and *RanBP2*$^{+/-}$ mice had similar total number of apoptotic cells (Figure 4c). However, *RanBP2*$^{+/-}$ mice exhibited significantly less apoptotic photoreceptors compared with *RanBP2*$^{+/+}$ mice at the age of 24 weeks (Figure 4d). Hence, there is an age-dependent increase in the susceptibility of photoreceptors to undergo cell death upon light-elicited stress that is suppressed by haploinsufficiency of *RanBP2*.

In addition, quantitative morphometric analysis was done on multiple central and peripheral sections of retinas to probe for potential regional differences of susceptibility to apoptosis of photoreceptors. The amount of cell death was normalized against the area of outer nuclear region analyzed. A ~5-fold increase of the total number of apoptotic photoreceptors was observed in 24-week old *RanBP2*$^{+/+}$ mice compared with 12-week *RanBP2*$^{+/+}$ mice (Figure 4e). In contrast, comparison of 12- and 24-week old *RanBP2*$^{+/-}$ mice showed no significant difference in cell death (Figure 4f). Both wild-type and *RanBP2*$^{+/-}$ mice had similar total number of apoptotic cells at the age of 12 weeks (Figure 4g), while there was a significant increase of apoptosis in wild-type, but not *RanBP2*$^{+/-}$mice, at the age of 24 weeks (Figure 4h). On the other hand, similar analyses of the peripheral regions of the retinas showed that there was not a significant age-dependent increase to light-elicited degeneration in these regions of the retinas, although such trend was observed (Figures 4k-n). Finally, analysis of cell death in central and peripheral regions of age- and genotype-matched retinas shows that apoptosis is prominent in the central region of the retina (supplementary Figures 3a-d). Apoptosis increases with age in *RanBP2*$^{+/+}$, but not *RanBP2*$^{+/-}$mice (supplementary Figures 3a-d). Collectively, the data show that the central retina presents the highest susceptibility to cell death upon light-elicited stress and haploinsufficiency of *RanBP2* blocks this effect.

The expression and SUMOylation of Topo IIα and levels of opsin apoprotein are not affected by RanBP2 in retinal neurons

RanBP2 was shown physiologically to modulate the SUMOylation of topoisomerase-IIα (Topo IIα) and its localization [32], whereas reduced levels of opsin apoprotein of rod photoreceptors protects these neurons from light-elicited degeneration.[9] Hence, to gain further insight into the molecular basis of the neuroprotective effects of a deficit of RanBP2 in photoreceptor neurons, we examined whether haploinsufficiency of *RanBP2* affected the levels of SUMOylation of Topo IIα and opsin apoprotein in the absence and presence of light stress. Conversely to other tissues and HeLa cells with high mitotic activity, we found that Topo IIα or its sumoylated isoform is not detectable in retinal neurons (Figure 5a) and haploinsufficiency of *RanBP2* did not cause a change in the levels of opsin apoprotein under either light condition (Figure 5b). Hence, these data do not support Topo IIα- or opsin-dependent effects promoted by RanBP2 in neuroprotection.

RanBP2$^{+/-}$ mice present a deficit in fat mass

Several lines of evidence from this and previous work suggest that lipid metabolism may be deregulated in *RanBP2*$^{+/-}$ mice. First, several facets of membrane dysgenesis are significantly alleviated in *RanBP2*$^{+/-}$ compared to *RanBP2*$^{+/+}$ mice. In particular, the formation large multilamellar bodies and vesicular deposits with single or no discernable membrane leaflet reminiscent of lipid droplets structures are observed often in disorders affecting various facets of lipid metabolism or trafficking.[33–38] Second, *RanBP2* haploinsufficient mice present age-dependent decreased weight gain on a high-energy diet.[31] Finally, gene expression profiling between *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice suggest that lipid metabolism may be deregulated systemically in *RanBP2*$^{+/-}$ mice (unpublished observations). To further probe whether haploinsufficiency of *RanBP2* modulated lipid metabolism, *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice were placed on a high-fat diet (40%) for 12 weeks. In contrast to *RanBP2*$^{+/+}$ mice, *RanBP2*$^{+/-}$ are remarkably refractory to body weight gain (Figure 6a) and present significantly less accumulation of epididymal fat mass (Figure 6b). These results support that lipogenesis is down-regulated in *RanBP2*$^{+/-}$ mice.

Haploinsufficiency of *RanBP2*, age and light promote differential changes in lipid metabolites

Cholesterol and free fatty acids, such as docosahexaenoic acid (DHA), are abundant and critical components of membranes of the outer segments of photoreceptors and other neurons [39–43] and abnormalities in the level of these have been linked to several syndromic and non-syndromic retinal dystrophies.[34, 44–55] Moreover, light-elicited lipid peroxidation and deregulation of the production of free fatty acids (or metabolites thereof) are thought to promote the degeneration of photoreceptors, endothelial cells and pancreatic β-cells, by mechanisms that remain largely elusive.[10, 13, 56–60] Hence, we examined whether the levels and distribution of cholesterol and free fatty acids are deregulated in the retina of haploinsufficient *RanBP2* mice. As shown in Figure 7, we did not discern changes in cholesterol lipid droplets (LD) in photoreceptors between 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice in the absence or presence of chronic light-stress. However, we noted that the distribution of LD was disturbed differently in retina pigment epithelium (RPE) cells located adjacent to the tip of the outer segments of *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice, but only when these were exposed to light-elicited stress (Figure 7a-d). To this effect, *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice on a 12 hour light-dark cyclic illumination presented LD finely distributed throughout the RPE cells (Figures 7a-b). In contrast, upon light-elicited stress the LD deposits (aggregates) were seen uniformly distributed in the RPE of *RanBP2*$^{+/+}$ mice (Figure 7c), whereas such deposits were localized prominently to the basal end of RPE cells of *RanBP2*$^{+/-}$mice (Figure 7d). Examination of cholesterol and free fatty acids (FFA) content of retinas shows that neither the level of total nor free cholesterol were changed between 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice

regardless of the light exposure, although there was a trend for *RanBP2*$^{+/-}$ mice to present lower levels of total and free (non-esterified) cholesterol on a normal 12 hour light-dark cyclic illumination (Figures 7e, f). In contrast, we found that the content of FFA in the retina varied significantly with age and genotype of *RanBP2* mice in a light-independent fashion. There was no difference in FFA content between 12-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice (Figure 7g), whereas 24-week old *RanBP2*$^{+/-}$mice presented reduced levels (~35–40%) of FFA compared to *RanBP2*$^{+/+}$ (Figure 7h). Finally, we probed further whether prolonged exposure to light promotes oxidative stress in the retina in a light-and genotype-dependent manner, since such stress insult is thought to play a role in degeneration of photoreceptors.[10, 13] As shown in Figure 7i, there was a significant increase of hydrogen peroxide (H_2O_2) production in retinal neurons upon light-elicited stress and the levels of H_2O_2 production did not vary with the genotype of *RanBP2* mice.

Discussion

Our studies reveal an age-dependent switch between 12- and 24-week old mice on the 129P2/OlaHsd background that significantly increases the susceptibility of photoreceptors to light-induced degeneration and membrane dysgenesis of photoreceptor neurons. *RanBP2* haploinsufficiency significantly blocks this switch thus rendering older photoreceptors much less susceptible to light-elicited degeneration and suppressing several facets of membrane dysgenesis, such as the formation of multilamellar bodies and disruption of nascent disk formation. Strikingly, a genotype-dependent, but light-independent, decrease in FFA of the retina in 24-week old but not 12-week old *RanBP2*$^{+/-}$ mice correlates well with the morphological phenotypes observed upon light-elicited stress. Hence, the data support a model whereby a RanBP2-dependent reduction of FFA content renders neuroprotection to photoreceptor neurons to light-induced damage (Figure 8). The decrease of free fatty acids in the retina of *RanBP2*$^{+/-}$ mice, and the resistance to weight-gain and formation of adipose tissue of *RanBP2*$^{+/-}$ mice when place on a high fat diet, suggest that FFA uptake is compromised in *RanBP2*$^{+/-}$ mice. The deregulation of FFA uptake may also affect the transport (and distribution) of cholesterol in lipid droplets[61] as observed in retina pigment epithelial cells of *RanBP2*$^{+/-}$ mice, a phenotype likely secondary to deficits in FFA. Lower levels of FFA in FFA-enriched photoreceptor neurons likely decreases lipid peroxidation, such as formation of fatty acid hydroperoxides, which are implicated in the propagation of lipid peroxidation.[62] The major outcomes of lipid peroxidation in photoreceptors are twofold. First, it promotes the generation of toxic FFA metabolites, which by themselves or in concert with other ligands, promote apoptosis.[60, 63–65] Second, changes in the orientation of the acyl chain of peroxidized fatty acids toward the hydrophilic exterior of the lipid bilayer (e.g. lipid whisker model [66]) may disrupt critical protein-protein and protein-lipid contacts required for the membrane biogenesis of nascent disks of photoreceptors or even generate novel pathological ligand(s) contributing to the death of photoreceptors.

The light-elicited pathways and mechanisms leading to the apoptosis of photoreceptors remain largely elusive. In particular, the identity of the FFA or metabolites thereof underlying neuroprotection and exacerbating apoptosis in the central region of the retina upon light-induced damage are unknown. In light of the large content of DHA in the membrane of the outer segment of photoreceptors [43], DHA is a strong candidate to mediate neuroprotection when in deficit or to stimulate apoptosis when at high levels. Indeed, a diet deficit in the linolenic acid precursor of DHA of albino rats confers neuroprotection to photoreceptor neurons upon light-induced damage.[67] Collectively, these observations support that photoreceptors contain an excessive pool of DHA with deleterious implications upon light-elicited damage and that the bioavailability of FFA to photoreceptors differ between the central and peripheral regions of the retina. On the other hand, light-elicited degeneration of photoreceptors of *Drosophila* due to deficits in phosphatidylinositol-phospholipase Cβ4 (PI-

PLCβ4)-mediated production of diacylglycerol (DAG) [68] and generation of FFA and metabolites thereof from DAG by a DAG lipase encoded by *inaE* [59], suggest another vital role of FFA signaling in the survival of photoreceptor neurons (and *Drosophila*). Although it remains unclear whether such PI-PLC-dependent signaling pathway is conserved in vertebrate photoreceptor neurons, the mammalian homolog of the PI-PLCβ4 of *Drosophila* is present in photoreceptor neurons.[69, 70] Hence, this PI-PLC signaling pathway may be another source for the generation of FFA from phosphatidylinositol 4,5-biphosphate (PIP_2).[71–79]

Finally, analysis of haploinsufficiency of *RanBP2* greatly facilitates the identification and dissection of primary phenotypes from confounding secondary phenotypes, which are often difficult to parse in severe phenotypes.[31, 32] However, genetic tools to manipulate and analyze a diverse but limited set of partners, each associating specifically to selective domains of RanBP2, will provide novel insights into what specific biological activities directly linked to each of the functions of RanBP2 [20–23, 31] confers light-elicited neuroprotection and modulates lipid metabolism. The data here presented do not suggest a direct role of sumoylation of Topo IIα[32], and opsin apoprotein[9], in such processes, because of the absence of the former from retinal neurons and the lack of change in the levels of the latter in *RanBP2*$^{+/-}$ mice, respectively. It is of interest to note that an autosomal dominant acute necrotizing encephalopathy triggered by the onset of a febrile illness in the human maps to 2q12.1-2q13, where *RanBP2* is localized [80–82]. Identification of such potential genetic lesion(s) in *RanBP2* may provide new clues of the role of RanBP2 and its partners in neurological function and cell death upon various stressors. Regardless, the *RanBP2* mouse model defines a new genetic tool and framework to partition, isolate, and define, molecularly and genetically, activities and subcellular processes linked to RanBP2 and aging that are important in modulating the survival and death of photoreceptors and other neurons, and manifestations of aging-related diseases affecting the retina (e.g. macular dystrophies) and elsewhere, upon exposure to deleterious stressors.

Material and methods

Mice

RanBP2$^{+/-}$ mice were described elsewhere.[31] *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$mice were in an inbred 129P2/OlaHsd background and reared in 70 lux of diffused white fluorescent light and 12:12 light-dark (LD) cycle. Mice were fed with a standard chow diet (~10% fat; Test Diet 5LJ5, Purina) or placed whenever applicable on a high fat (~40%) diet (Test Diet DIO 58Y1, Purina) for 12 weeks. 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.

Light exposure

Mice were placed in a white reflective cage with food and water and fluorescent light bulbs mounted at the top of the cage. Illuminance was measured at the bottom of the cage with a traceable dual-range light meter (Fisher Scientific). Mice were exposed to 1,200 lux of continuous, cooled, and diffused fluorescent white light for 48 hrs. Mice were sacrificed and the eyeballs immediately collected and processed for histology and morphometric analyses.

Histology

Eyes were prepared for light and electron microscopy by fixing eyecups overnight in 2% glutaraldehyde and 2% paraformaldehyde in 0.1% cacodylate buffer, pH 7.2 at 4°C. Semi-thin sections (0.5 μm) along the vertical meridian were mounted on slides and stained with 1% methylene blue. Light images of the retina were acquired with a Nikon C1-Plus light

microscope equipped with Nomarski optics and coupled to a SPOT RT-SE digital camera (Diagnostic Instruments).

TUNEL Staining and quantitative morphometric analysis of apoptosis

Apoptosis was detected *in situ* with the DeadEnd Fluorometric TUNEL System (Promega) on retinas fixed with 4% paraformaldehyde. Images of TUNEL-positive nuclei of sections of eyecups were captured with a laser confocal Nikon C1-Plus light microscope equipped with epifluorescence and coupled to a Cascade 1K digital camera (Roper Scientific). Images were acquired with 4x and 10x objectives. Digital stitching of composite images acquired with the 4x objective was performed with Photoshop CS (Adobe). Quantitative morphometric analyses of images analyzed with Metamorph v6.2 (Molecular Devices) were performed on eight sections per eye along the vertical meridian with a 10x-objective. Typically, images of the whole section of the retina comprised 7–8 image fields. Peripheral images of the retina containing the marginal areas of the retina comprised the first and last image fields. Central regions of the retina comprised typically the third and fourth image fields of the retina. Statistical analysis of apoptosis was performed with two-tailed equal variance *t*-test.

Transmission electron microscopy

Posterior eyes were processed as described in the Histology section followed by post-fixation in 2% osmium tetraoxide in 0.1% cacodylate buffer and embedded in Spurr resin. Sixty-five nanometer-thick sections were cut with a ultramicrotome and stained with uranyl acetate and lead citrate. Specimens were visualized with JEOL 1200 EX and Philips CM 12 transmission electron microscopes. Low magnification images were captured with the Philips TEM coupled with an AMT camera and processed with the Image Capturing Engine software (version 5.42355). High magnification images were captured with the JEOL TEM and negatives were scanned with Photoshop CS.

Immunoblot analyses

Retina samples were homogenized with a Kontes Microtube Pellet Pestle Rods with Motor in NP-40 buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 (NP-40), with Complete protein inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Samples were centrifuged at 10,000*g* for 15 min and supernatants collected. Protein concentration was measured by Bradford method using BSA as standard. Protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was carried out as described elsewhere [83]. Primary antibodies used: Mouse anti-acetylated tubulin (Sigma, 1:40,000), rabbit anti-rhodopsin (Affinity Bioreagents, 1:20,000), rabbit anti-Hsc70 (Stressgen, 1:3,000), rabbit anti-Topo IIα (Topogen, 1:3,000).

Filipin staining

Radial retinal cryosections (~10 μm thick) mounted on slides were rinsed 3 times with phosphate-saline pH 7.4 (PBS) and incubated with 0.05 mg/ml of filipin (Sigma) for 2hr at room temperature in the dark. Specimens were then rinsed 3 times with PBS and mounted with ProLong Gold antifade (Invitrogen).

Hydrogen Peroxide Measurement

The hydrogen peroxide levels were assayed using the Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (Invitrogen) as per manufacturer's instructions. Ten μl of NP40 extract was used for each measurement. Results were normalized against soluble protein contents in the extract. Two-tailed equal variance *t*-test statistical analysis was performed.

Free cholesterol and total cholesterol quantitation

Free cholesterol and total cholesterol were determined with the Cholesterol and Cholesterol Ester Quantitation kit (Biovision, Mountain View, CA) as per manufacture's instructions. Briefly, 3 μl retinal NP40 extracts was diluted with assay buffer for measurement by fluorescence (Excitation/emission/cutoff=560/590/590 nm; SpectraMax M5, Molecular Devices). Total cholesterol was measured with cholesterol esterase added, free cholesterol was measured without cholesterol esterase. Results were normalized to soluble protein content in the extracts. Two-tailed equal variance *t*-test statistical analysis was performed.

Free Fatty Acid Quantification

Free fatty acids (FFA) were determined with the Free Fatty Acid Quantification kit (Biovision, Mountain View, CA) to detect C-8 (octanoate) and longer fatty acids as per manufacture's instructions. Briefly, retinal NP40 extracts were used for each reaction. FFA content was measured by a colorimetric assay (Ab570) with palmitic acid employed as a standard. FFA results were normalized against soluble protein contents in the extract. Two-tailed equal and unequal variance *t*-test statistical analysis was performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations list

RanBP2
: Ran-binding protein 2

TUNEL
: TdT-mediated dUTP nick end-labeling

MLBs
: multilamellar bodies

SUMO-1
: Small Ubiquitin-like Modifier-1

RPE65
: retinal pigment epithelium-specific protein 65kDa

Topo IIα
: topoisomerase-IIα

LD
: lipid droplets

FFA
: free fatty acids

DHA
: docosahexaenoic acid

DAG
diacylglycerol

H_2O_2
hydrogen peroxide

PIP_2
phosphatidylinositol 4,5-biphosphate

TEM
transmission electron microscope

PI-PLCβ4
phosphatidylinositol-phospholipase Cβ4

DAG
diacylglycerol

ROS
rod outer segment (compartment) of photoreceptors

RIS
rod inner segment (compartment) of photoreceptors

ONL
outer nuclear layer (nuclei of photoreceptors)

RPE
retinal pigment epithelium

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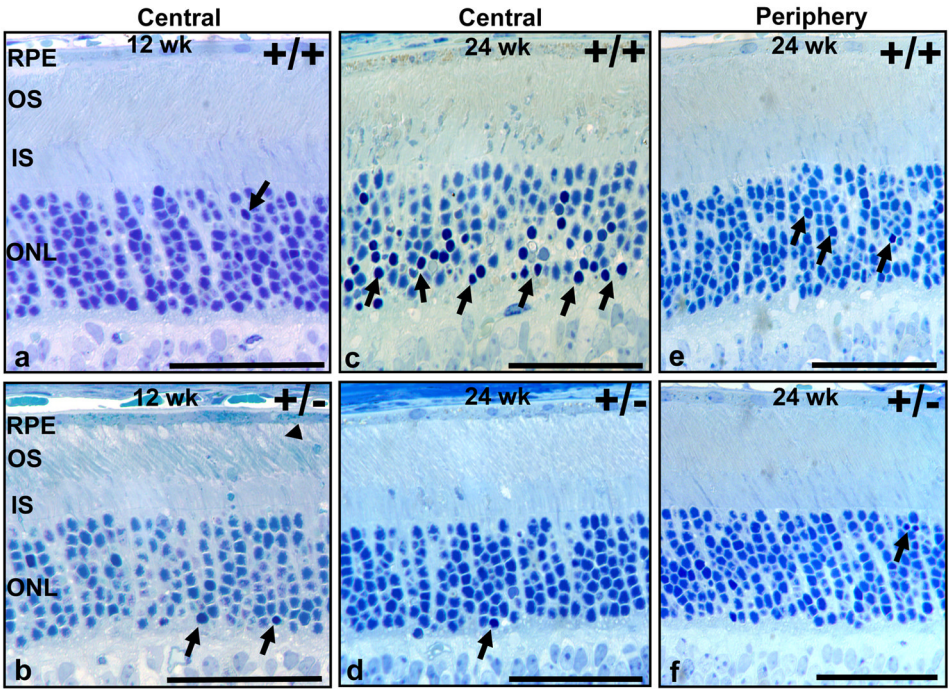

Figure 1.

Age-dependent light damage of photoreceptors is strongly reduced in *RanBP2* haploinsufficient mice. Light photomicrographs of methylene blue-stained sections of central (**a-d**) and peripheral (**e, f**) regions of the retina of 12-(**a, b**) and 24-week old (c-f) *RanBP2*$^{+/+}$ (**a, c, e**) and *RanBP2*$^{+/-}$ mice (**b, d, f**). There is a strong increase in pyknotic nuclei in 24-week old wild-type mice compared with 12-week old wild-type mice (arrows pointing to intense nuclei staining) in the central retina (**a, c**) that is accompanied by the disorganization of the outer nuclear layer. The periphery of the retina is spared largely from pyknosis (**e**). In contrast, no apparent differences in pyknosis are observed between 12- and 24-week old *RanBP2*$^{+/-}$ mice (**b, d, f**), but some vacuolization of the tip of the outer segments can be noted (arrowhead). Legend: RPE, retina pigment epithelium; OS, outer segment of rod photoreceptors; IS, inner segment of rod photoreceptors; ONL, outer nuclear layer (nuclei of photoreceptors). Scale bar, 40 μm.

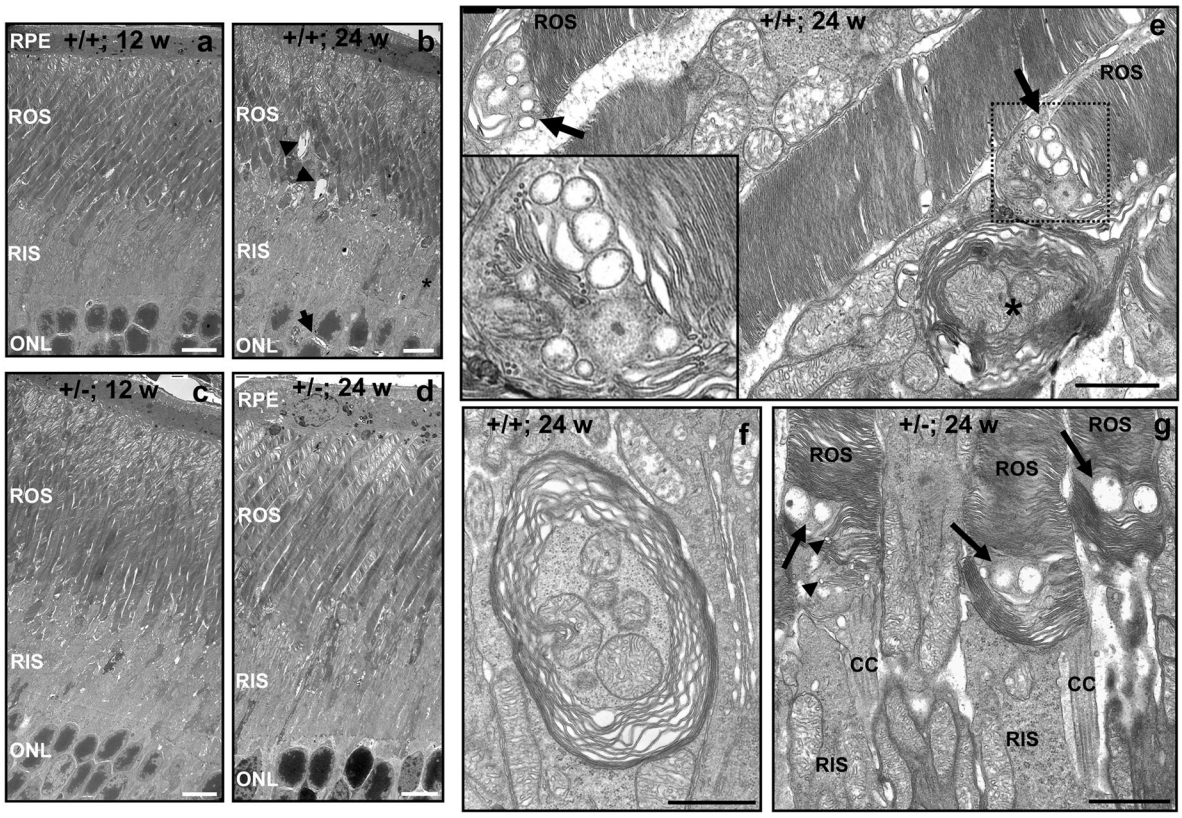

Figure 2.

Electron micrographs depicting the age-dependent ultrastructural changes of inner and outer segments of photoreceptors of 12- and 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice upon prolonged light exposure. There is an overall increase in the subcellular disorganization of morphological features of the inner and outer segments of photoreceptors in 24-week old *RanBP2*$^{+/+}$ mice (**b**) compared to 12-week old *RanBP2*$^{+/-}$ mice (**a**). This is reflected by an increase of cystoid spaces between nuclei (arrow) and outer segments (arrowheads), shrinking and condensed inner segments (star), formation of vacuoles in the inner segment, and dilation of the disks at the tip of the outer segments. The development of these subcellular pathologies, except for the dilation of the disks, was strongly decreased in 24-week old *RanBP2*$^{+/-}$ mice (**d**) compared to 12-week old *RanBP2*$^{+/-}$ mice (**c**). **e**, **f**, and **g**, depict high magnification images of single membrane multivesicular bodies at the base of the outer segments (**e**), large vesicular body duets (arrows) and crescent vesicles in nascent disk rims (arrowheads) at the base of the outer segments of *RanBP2*$^{+/-}$ mice (**f**), and multilamellar bodies engulfing mitochondria in *RanBP2*$^{+/+}$ mice (**e**, star; **g**). Legend: RPE, retina pigment epithelium; ROS, rod outer segment of photoreceptors; RIS, rod inner segment of photoreceptors; ONL, outer nuclear layer (nuclei of photoreceptors); CC, connecting cilium. Scale bar in **a-d**, 6 μm; scale bar in **e-g**, 1 μm.

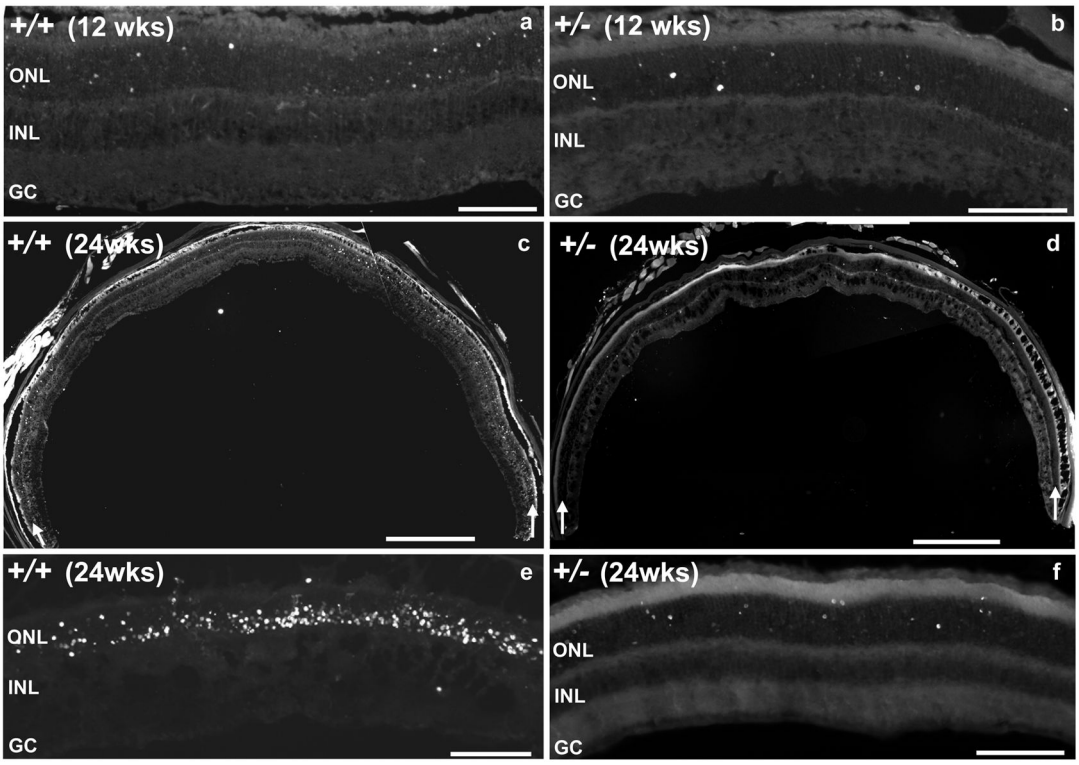

Figure 3.

Light-elicited and age-dependent accumulation of apoptotic nuclei selectively in photoreceptors is substantially reduced in *RanBP2* haploinsufficient mice. TUNEL staining reflecting the nucleosomal DNA fragmentation of nuclei of photoreceptors show comparable cell-death between 12-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice (**a, b**), while there is a substantial increase in apoptosis in 24-week old *RanBP2*$^{+/+}$, but not *RanBP2*$^{+/-}$ mice (**c-f**). **a, b, e** and **f** are high power micrographs of the central region of the retina. **c** and **d** are low power micrographs of the whole retina attached to the eyecup (white arrows point to the outer nuclear layer of photoreceptors). Legend: ONL, outer nuclear layer (nuclei of photoreceptors); INL, inner outer nuclear layer (nuclei of second-order neurons); GC, ganglion cell layer. Scale bar in **a, b, e** and **f**, 100 μm; scale bar in **c** and **d**, 500 μm.

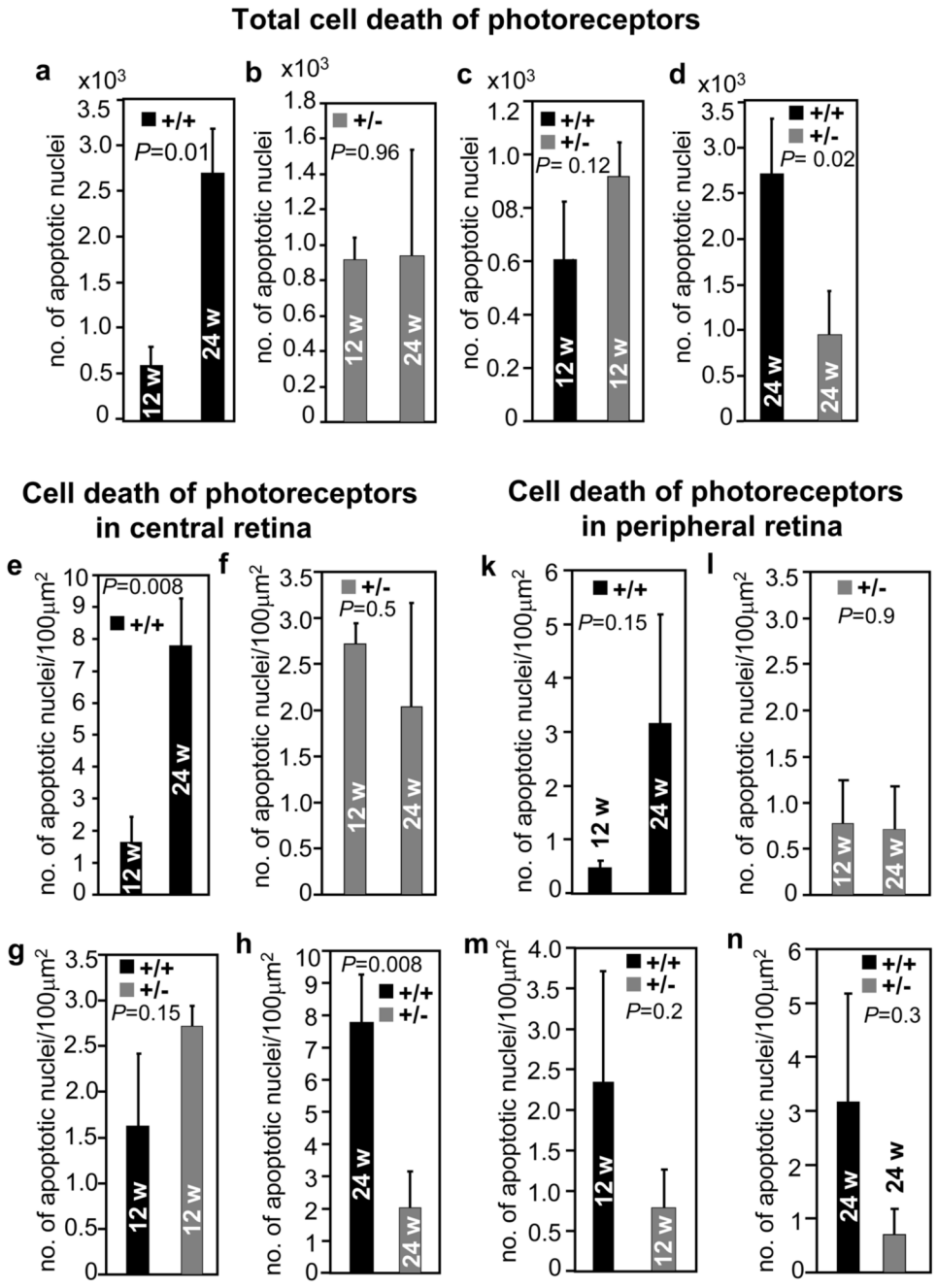

Figure 4.

Quantitative morphometric analyses of apoptosis of photoreceptor neurons in 12- and 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice upon prolonged light exposure. There is a ~5-fold significant increase in the absolute number of apoptotic nuclei in 24-week old *RanBP2*$^{+/+}$ mice compared with 12-week old ones (**a**) that is not observed in *RanBP2*$^{+/-}$ mice (**b**). At the age of 12 weeks, there was no difference in apoptosis between *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice (**c**), but *RanBP2*$^{+/-}$ mice had significantly less apoptotic cells than *RanBP2*$^{+/+}$ mice at the age of 24 weeks (**d**). There were also significant regional differences in apoptosis between the central (**e-h**) and peripheral (**k-n**) sections of retinas. There is a ~5-fold significant increase in the number of apoptotic nuclei from 12- to 24-week old *RanBP2*$^{+/+}$ mice (**e**), but not

RanBP2$^{+/-}$ mice (**f**). No difference in cell death is noticeable between 12-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice (**g**). At the age of 24 weeks, *RanBP2*$^{+/+}$ mice present a significant increase in cell death, which is suppressed in *RanBP2*$^{+/-}$ mice (**h**). Although not significant, similar trends were observed in the peripheral regions of the retina (**k-n**). No difference in apoptosis was observed between 12- and 24-week old *RanBP2*$^{+/-}$ mice in either the central (**f**) or the peripheral (**l**) regions of the retina. Results shown represent the mean ± S.D. (n=4).

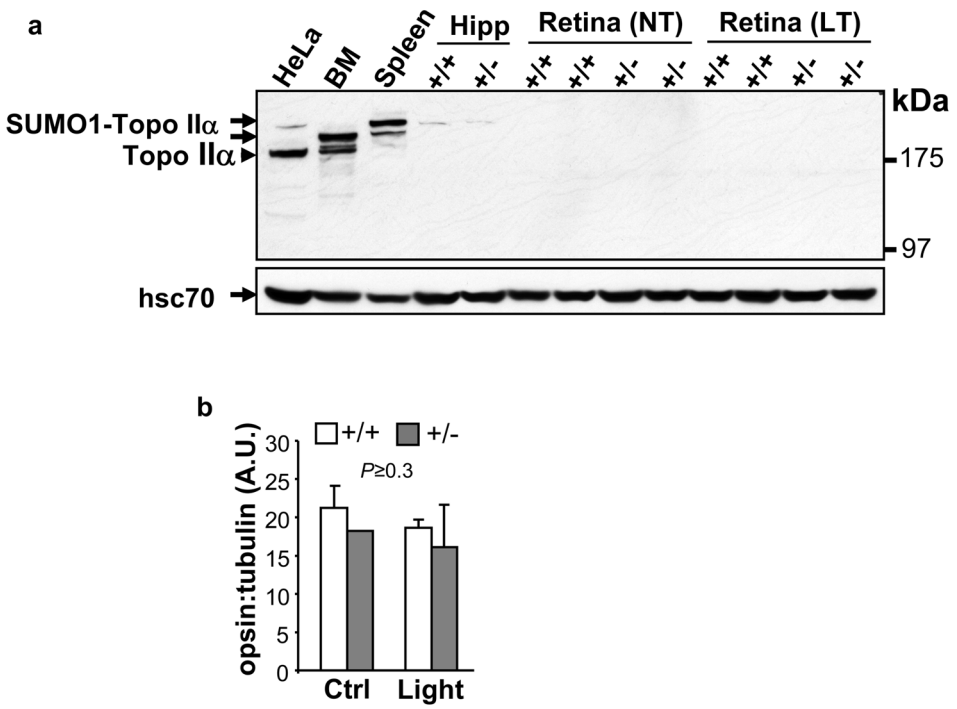

Figure 5.

Haploinsufficiency of *RanBP2* has no effect on the levels of topoisomerase-IIα (Topo IIα), SUMOylated Topo IIα α (SUMO1-Topo IIα), and apoprotein opsin, in the retina. (**a**) The expression of Topo IIα and sumoylated Topo IIα in retinas of non (NT)- and light-treated (LT) 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice was assessed by immunoblot analyses of retinal extracts (100 μg) and compared to extracts (100 μg) of HeLa, bone marrow (BM) from the femoral bone, spleen, and hippocampus (Hipp). Regardless of the light-treatment and genotype, Topo IIα and sumoylated Topo IIα were not detected in the retinas, but they were expressed in HeLa, bone marrow and spleen, whereas traces of sumoylated Topo IIα were detected in the hippocampus. Blot was reprobed for the cytosolic heat shock 70 protein (hsc70) as loading control. (**b**) The expression of the apoprotein opsin in retinas of 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice under normal cyclic (Ctrl) and prolonged light exposure (light) was quantified from immunoblot analyses of retinal extracts (2 μg) by densitometry and normalized for tubulin expression. No changes in apoprotein opsin expression were observed regardless the exposure to light and genotype. Results shown represent the mean ± S.D. (n=4). A.U. arbitrary units.

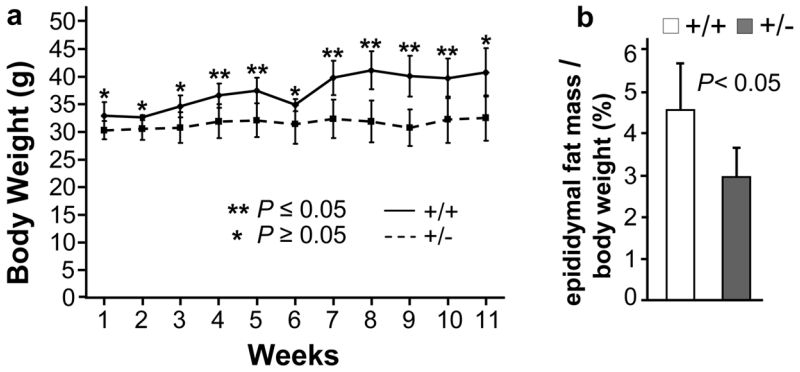

Figure 6.

Haploinsufficiency of *RanBP2* renders 24-week old mice refractory to gain of body weight and epididymal fat mass when placed on a high-fat (40%) diet. *RanBP2*$^{+/+}$ but not *RanBP2*$^{+/-}$ mice gain significant body weight (**a**) and epididymal fat mass (**b**) when placed on a high-fat diet for 12 weeks. In (**a**) double asterisks represent significant differences between the groups (Student's *t* test, *P*=0.05). Statistical significance was also found across the time-course of the experiment (repeated measures 2-way ANOVA, *P*=0.03). Results shown represent the mean ± S.D. (*n*=4).

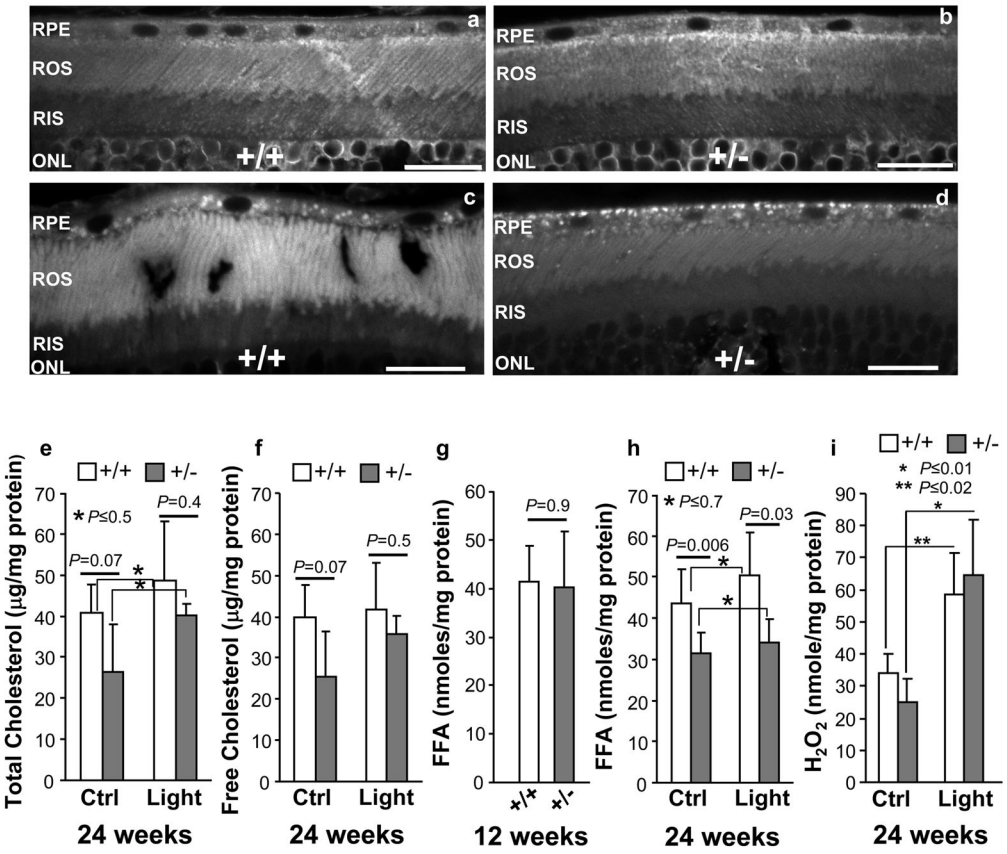

Figure 7.

Lipid metabolic deficits and reactive oxygen species production in 24-week old *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice upon light-elicited stress. Filipin staining of cholesterol/lipid droplets (LD) shows that *RanBP2*$^{+/+}$ (**a**) and *RanBP2*$^{+/-}$ (**b**) under normal cyclic light present no visible difference in LD, whereas under light-elicited stress there is an apparent coalescence of LD throughout the perikarya of the retinal pigment epithelium (RPE) in *RanBP2*$^{+/+}$ mice (**c**) and coalescence and polarized localization of LD to the basal end of the RPE of *RanBP2*$^{+/-}$ mice (**d**). No significant and discernable changes in LD distribution were observed in the outer (ROS) and inner segments (RIS) of rod photoreceptor neurons (**a-d**). Total (**e**) and free (**f**) cholesterol were not significantly altered in retinas of *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice regardless of the light treatment, although *RanBP2*$^{+/-}$ mice exhibited a trend to present lower levels of total (**e**) and free (**f**) cholesterol than *RanBP2*$^{+/+}$ mice when reared under cyclic light, but not prolonged light exposure (n=4). The content of free fatty acids (FFA) in the retina remained unaltered between *RanBP2*$^{+/+}$ and *RanBP2*$^{+/-}$ mice at the age of 12-weeks (n=4) (**g**), whereas 24-week old *RanBP2*$^{+/-}$ mice presented ~ 35% decreased levels of FFA than *RanBP2*$^{+/+}$ mice and this remained unchanged under prolonged light exposure (n=7 for *RanBP2*$^{+/+}$ mice under normal cyclic light, n=6 for *RanBP2*$^{+/-}$ mice under normal cyclic light, n=4 for mice under prolonged light exposure) (**h**). (**i**) The level of hydrogen peroxide (H_2O_2) increases in retinas upon light-elicited stress regardless of the genotype of *RanBP2* mice (n=4). Results shown represent the mean ± S.D.. Legend: ROS, rod outer segment (compartment) of photoreceptors; RIS, rod inner segment (compartment) of photoreceptors; ONL, outer nuclear layer (nuclei of photoreceptors); RPE, retinal pigment epithelium.

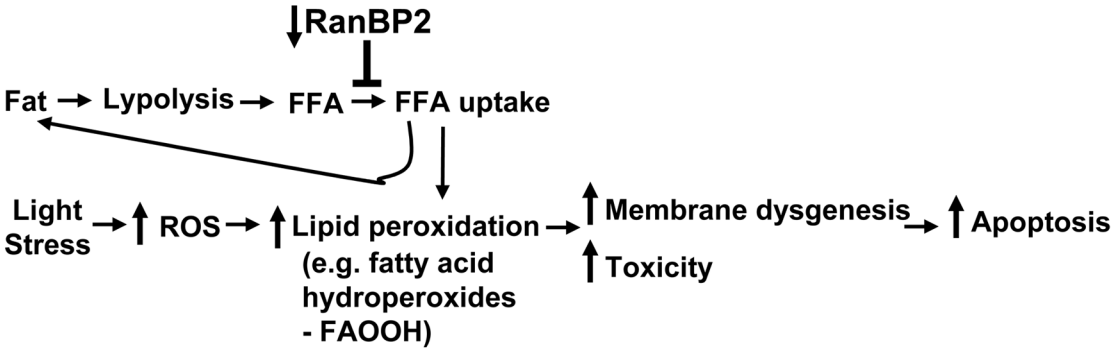

Figure 8.

Model of the effect of haploinsufficiency of *RanBP2* and light-elicited stress, in the production of free fatty acids (FFA), lipid peroxidation by reactive oxygen species (ROS), and downstream effect of these, in membrane dysgenesis, toxicity, and stimulation of apoptosis (see text for details). **Legend**. Upward arrows indicate an increased level/effect; downward arrow indicates a decreased level/effect.