Protection of *Rpe65*-deficient mice identifies rhodopsin as a mediator of light-induced retinal degeneration

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Light-induced apoptosis of photoreceptors represents an animal model for retinal degeneration[1]. Major human diseases that affect vision, such as age-related macular degeneration (AMD) and some forms of retinitis pigmentosa (RP), may be promoted by light[2–7]. The receptor mediating light damage, however, has not yet been conclusively identified; candidate molecules include prostaglandin synthase[8], cytochrome oxidase[9], rhodopsin[10], and opsins of the cones and the retinal pigment epithelium[11] (PE). We exposed to bright light two groups of genetically altered mice that lack the visual pigment rhodopsin (*Rpe65*$^{-/-}$ and *Rho*$^{-/-}$). The gene *Rpe65* is specifically expressed in the PE and essential for the re-isomerization of all-*trans* retinol in the visual cycle and thus for the regeneration of rhodopsin after bleaching[12]. *Rho*$^{-/-}$ mice do not express the apoprotein opsin in photoreceptors, which, consequently, do not contain rhodopsin[13]. We show that photoreceptors lacking rhodopsin in these mice are completely protected against light-induced apoptosis. The transcription factor AP-1, a central element in the apoptotic response to light[14,15], is not activated in the absence of rhodopsin, indicating that rhodopsin is essential for the generation or transduction of the intracellular death signal induced by light.

We exposed wild-type mice to intense white light, resulting in disruption of rod outer segments (ROS) and rod inner segments (RIS), condensation of nuclear chromatin and cell loss in the outer nuclear layer (ONL; Fig. 1*a*,*b*). TUNEL staining and internucleosomal DNA fragmentation demonstrated cell death by apoptosis[14] (data not shown). By seven days after light exposure, most photoreceptor cells had died and degenerated (Fig. 1*c*). In contrast, light exposure did not affect photoreceptors of *Rpe65*$^{-/-}$ mice (Fig. 1*e*,*f*), leaving retinal morphology comparable to that of controls (Fig. 1*d*).

The *Rho*$^{-/-}$ mice lack opsin (and therefore rhodopsin) and develop photoreceptors without ROS (Fig. 2*a*; ref. 13). Light exposure did not affect the retinal morphology of *Rho*$^{-/-}$ mice (Fig. 2). Thus, photoreceptors lacking rhodopsin, regardless of the underlying cause, are equally protected against light damage. Therefore, it is unlikely that secondary alterations caused by the respective gene mutations are responsible for this protection.

The *Rpe65*$^{-/-}$ photoreceptor cells were capable of executing the apoptotic program (Fig. 3) when induced by the DNA alkylating agent N-methyl-N-nitrosourea[15] (MNU). Positive TUNEL staining of photoreceptor nuclei (Fig. 3*c*,*f*) and internucleosomal DNA fragmentation (Fig. 3*b*,*e*) verified the apoptotic nature of MNU-induced cell death. Furthermore, mRNA levels of several pro-apoptotic (*Bad*, *Bax*, *Casp3*) and antiapoptotic (*Bcl2*, *Bcl2l*) genes were similar in retinal cells of wild-type and *Rpe65*$^{-/-}$ mice (Fig. 4). These results suggest that the protection of *Rpe65*$^{-/-}$ mice was not caused by an impaired apoptotic capacity of their photoreceptors.

Exposure of mice to damaging light increases the DNA-binding activity of the transcription factor AP-1 (ref. 16), a prerequisite for light-induced apoptosis: mice lacking the AP-1 component c-Fos are protected[14] and their retinal cells do not show a light-mediated increase in AP-1 activity[15]. Upon light

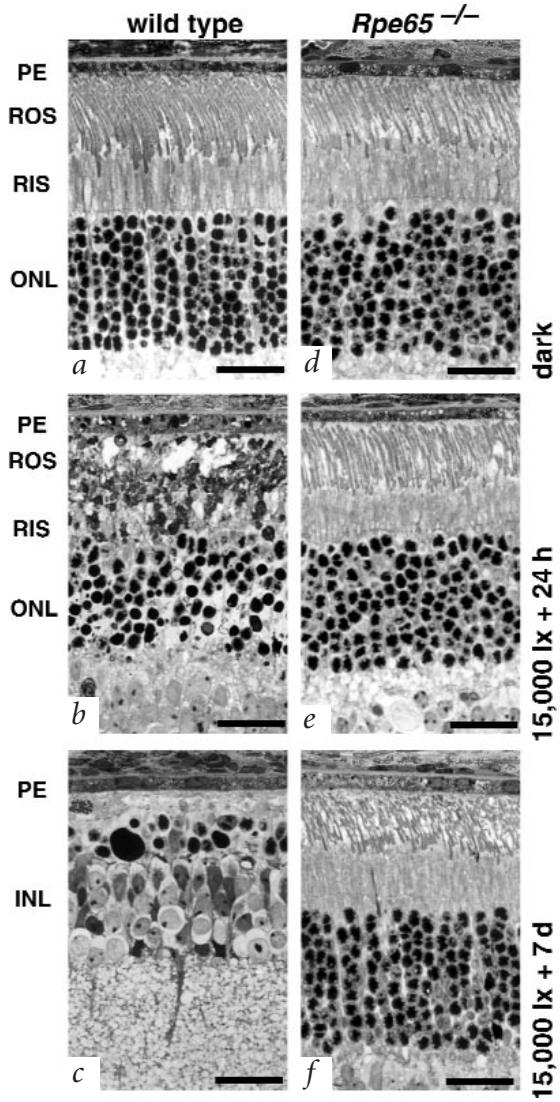

Fig. 1 *Rpe65*$^{-/-}$ mice are protected against light-induced degeneration of photoreceptors. Light microscopic analysis of sections of retinal tissues of wild-type mice (*a*–*c*) and *Rpe65*$^{-/-}$ mice (*d*–*f*) before (***a***,***d***) and after (***b***,***c***,***e***,***f***) light exposure is shown. Dark-adapted animals were exposed for 2 h to white fluorescent light of 15,000 lux and analysed after 24 h (*b*,*e*) or 7 d (*c*,*f*) in darkness. Representative sections of four (recovery period of 24 h) or two (recovery period of 7 d) independent experiments are shown. Scale bars, 25 µm. PE, pigment epithelium; ROS, rod outer segment; RIS, rod inner segment; ONL, outer nuclear layer; INL, inner nuclear layer.

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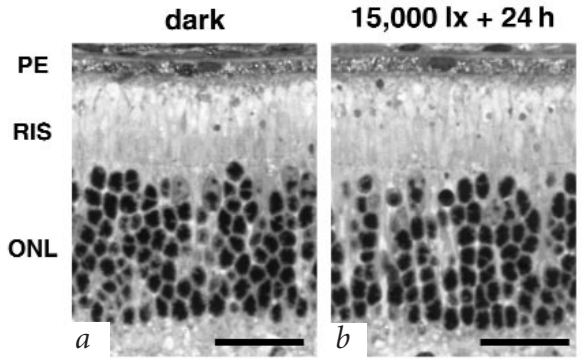

Fig. 2 Light does not induce apoptosis in photoreceptors of *Rho*$^{-/-}$ mice. ***a***, Light microscopic analysis of retinal tissue of *Rho*$^{-/-}$ mice not exposed to light. ***b***, Light microscopic analysis of retinal tissue of *Rho*$^{-/-}$ mice exposed for 2 h to 15,000 lux of white light. Analysis was after a 24-h recovery period in darkness. Scale bars, 25 μm. Abbreviations as in Fig. 1. Representative sections of four independent experiments are shown.

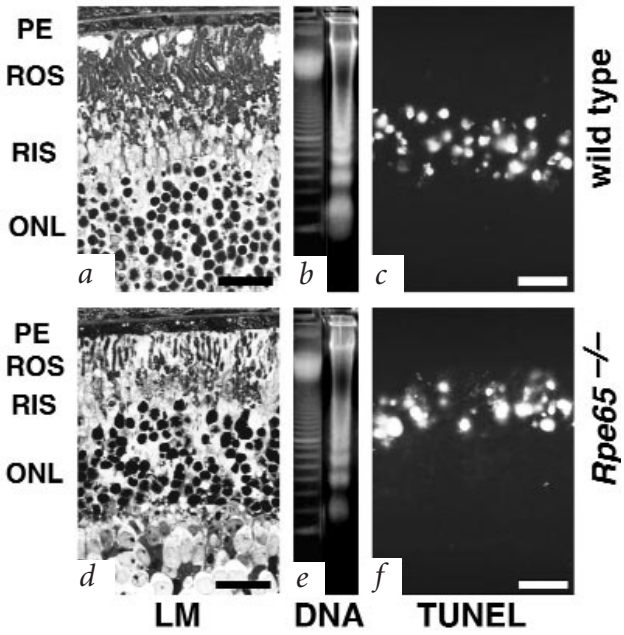

Fig. 3 MNU induces apoptosis of photoreceptors in both wild-type and *Rpe65*$^{-/-}$ mice. *a–c*, Wild-type mice. *d–f*, *Rpe65*$^{-/-}$ mice. MNU (45 mg/kg) was injected intraperitoneally and retinas analysed after 24 h by light microscopy (LM; ***a***,***d***). DNA ladder formation in agarose gel electrophoresis of genomic DNA (DNA; ***b***,***e***) and TUNEL staining (TUNEL; ***c***,***f***) are also shown. Scale bars: LM, 25 μm; TUNEL, 100 μm. Abbreviations as in Fig. 1. Representative sections of three independent experiments are shown.

exposure, AP-1 DNA-binding activity increased in retinal cells of wild-type mice, but not in those of *Rpe65*$^{-/-}$ mice (Fig. 5*a*). Competition experiments with specific and non-specific oligonucleotides demonstrated specificity of the AP-1/DNA complex (Fig. 5*b*) and DNA-binding activity of the transcription factor SP-1 showed the nuclear extracts to be of equal quality (Fig. 5*c*).

AP-1 complexes in the retina mainly consist of c-Fos and Jun heterodimers[16]. Both wild-type and *Rpe65*$^{-/-}$ mice expressed *Fos* mRNA (Fig. 5*d*), and AP-1 complexes in *Rpe65*$^{-/-}$ mice contained c-Fos protein (data not shown). This indicates that the protection of *Rpe65*$^{-/-}$ mice was not due to a lack of c-Fos, as is the case in *Fos*$^{-/-}$ mice. The level of *Fos* mRNA expressed in retinas of *Rpe65*$^{-/-}$ mice was 24% that of wild-type controls (Fig. 5*d*). This might explain the lower basal levels of AP-1 DNA-binding activity in *Rpe65*$^{-/-}$ mice (Fig. 5*a*). Because expression of *Fos* in the retina depends on a light-dark cycle[17], it is conceivable that the lack of rhodopsin prevents formation of a regulatory signal, thus causing a downregulation of *Fos* expression in retinas of *Rpe65*$^{-/-}$ mice. In contrast, both wild-type and *Rpe65*$^{-/-}$ mice expressed *Jun* mRNA at comparable levels (Fig. 5*e*).

We have shown that rhodopsin is essential for light-induced retinal degeneration, indicating that it is the primary receptor mediating the damaging effect of light. Our findings question the importance of other light-absorbing molecules in the induction of retinal damage by exposure to short-term white light.

Diffusible factors can influence physiology and survival of retinal cells[18–21]. Survival of cones, for example, seems to depend on factors originating from rods[18], whereas rods may receive survival factors from the PE (ref. 21). Loss of the ability to recognize such factors may induce retinal degeneration[22]. It is therefore likely that damage to PE cells, which are sensitive to blue light *in vitro*[23], would make them incapable of supporting photoreceptor survival. Furthermore, intercell signalling in the retina may also involve factors that could signal cell death. Our results suggest, however, that light-induced photoreceptor degeneration is mediated neither by death signals originating from other cell types nor by a suppression of survival factors. Rather, the excessive absorption of photons by rhodopsin induces a death cascade in individual rods. Lack of rhodopsin blocks this cascade, leading to a protection against light-induced apoptosis. This block may occur at the level of photon uptake, as Rpe65 deficiency prevented the light-mediated vesiculation of ROS (Fig. 1) and the activation of AP-1 (Fig. 5*a*), a central downstream effector for light-induced apoptosis[14,15]. This implies that photoreceptors lacking rhodopsin cannot absorb sufficient light to cause morphological alterations or to generate a death signal that can be transduced to downstream effector molecules.

The finding that the different susceptibilities to light damage of different mouse strains[24] directly correlate with the levels of Rpe65 protein and with the physiological regeneration rates of rhodopsin (A.W., C.E.R., T.P. Williams, F.H. and C.G., manuscript submitted) substantiates the importance of the availability of rhodopsin for light damage.

An increasing number of animal models supports a role for light in human retinal degenerations[2–5]. These models include RCS rats[25]; mice homo- or heterozygous for mutation of *Prph2* (ref. 26); mice homozygous for *nr* or *pcd* mutations[7]; mice without functional rhodopsin kinase or arrestin as models for Oguchi disease[27,28]; mice lacking Rim protein[29]; and mice carrying *Rho* mutations found also in human RP (ref. 6). Abnormal photoproducts or constant signalling through constitutively active molecules might contribute to the increased light sensitivity of some of these animal models. On the basis of our findings, one might expect that altered rhodopsin physiology would influence prevalence or progression of certain human retinal diseases. Indeed, preliminary data from a clinical study (F.H. *et al.*, manu-

Fig. 4 Pro- and anti-apoptotic genes are similarly expressed in wild-type and *Rpe65*$^{-/-}$ mice. Relative expression of the genes indicated was determined by exponential RT–PCR. Total RNA was prepared from whole retinas of dark-adapted wild-type (wt) and *Rpe65*$^{-/-}$ (m) mice.

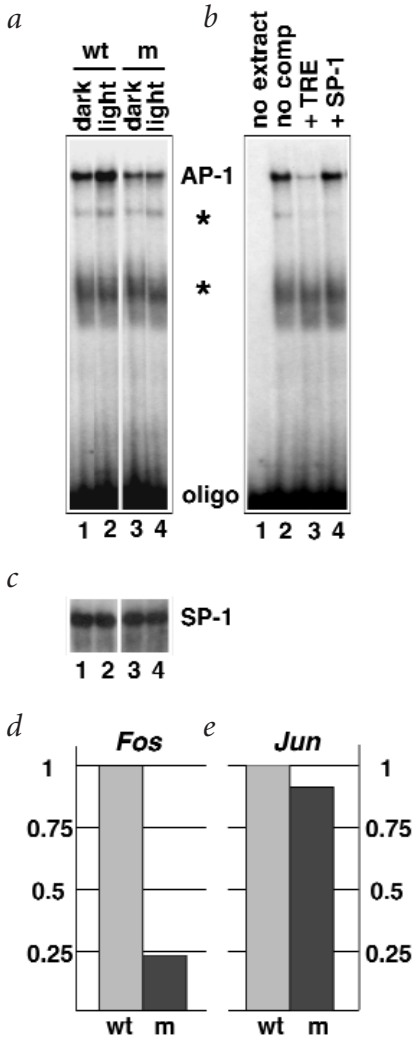

Fig. 5 Light does not induce AP-1 DNA-binding activity in retinas of *Rpe65*$^{-/-}$ mice. ***a***, Retinal nuclear extracts of wild-type (wt, lanes 1,2) or *Rpe65*$^{-/-}$ (m, lanes 3,4) mice were incubated with ^{32}P end-labelled oligonucleotides presenting a binding site (TRE) for AP-1 complexes. Extracts were from mice not exposed to light (lanes 1,3) or from mice exposed to 15,000 lux of white fluorescent light for 2 h followed by a 2-h recovery period in darkness (lanes 2,4). ***b***, Specificity of DNA/protein complexes. Incubation of extracts was with (100-fold excess) or without unlabelled competitor oligonucleotides. Lane 1, oligo incubated with buffer. Lanes 2–4, oligo incubated with nuclear extract without (lane 2) or with (lane 3) specific competitor or non-specific (lane 4) competitor. Asterisks (*) mark non-specific DNA/protein complexes. ***c***, Electrophoretic mobility shift assay for transcription factor SP-1 demonstrating quality of nuclear extracts used in (*a*). ***d***, Semiquantitative determination of *Fos* mRNA levels in retinal cells of dark-adapted wild-type (wt) and *Rpe65*$^{-/-}$ (m) mice by competitive RT–PCR. Levels in wild-type mice were set as 1 (arbitrary units). ***e***, *Jun* mRNA was determined by exponential RT–PCR and levels in wild-type mice were set as 1 (arbitrary units).

script in preparation) support this hypothesis by indicating that a group of patients with slowed rhodopsin regeneration has a distinctly lower prevalence for early and late forms of AMD.

Methods

Animals. All procedures concerning animals adhered to the ARVO statement for the use of animals in ophthalmic and vision research. All mice were bred on a 129sv/C57Bl/6 background, were 4–8 weeks of age and were kept under a 12 h:12 h light:dark cycle (60 lux).

Illumination and analysis of light damage. We dilated pupils of dark-adapted animals in dim red light with Cyclogyl (1%, Alcon) and Phenylephrine (5%, Ciba Vision) before 2 h of exposure to 15,000 lux of diffuse white fluorescent light (TLD-36 W/965 tubes, Philips; ultraviolet-impermeable diffuser). After light exposure, animals remained in darkness until analysed. For light microscopy, we fixed enucleated eyes in 2.5% glutaraldehyde and embedded central regions of eyecups in Epon 812. For TdT-mediated dUTP nick-end labelling (TUNEL), we embedded fixed eyes (2% paraformaldehyde) in paraffin and used the '*in situ* cell death detection kit' (Boehringer) to perform staining on 5-µm sections. For internucleosomal DNA fragmentation analysis, we prepared genomic DNA from isolated retinas and separated the fragments by agarose gel electrophoresis (1.4%).

Photoreceptor apoptosis induction by MNU. We injected a single dose of MNU (45 mg/kg body weight in 0.9% NaCl) intraperitoneally into dark-adapted mice, prepared retinal tissues after 24 h in darkness and analysed retinal morphology by light microscopy as described above.

RT–PCR. We performed reverse transcription on total retinal RNA (400 ng) using oligo(dT) and M-MLV reverse transcriptase (Promega). cDNAs corresponding to total RNA (10 ng) were amplified by PCR using the following primer pairs and cycle numbers (linear range of amplification was determined for each amplified fragment in pre-experiments; data not shown): β-actin (24 cycles), up, 5′–CAACGGCTCCGGCATGTGC–3′, down, 5′–CTCTTGCTCTGGGCCTCG–3′; *Casp3* (30 cycles), up, 5′–AGTCAGTGGACTCTGGGATC–3′, down, 5′–GTACAGTTCTTTCGTGAGCA–3′; *Bad* (32 cycles), up, 5′–AGAGTATGTTCCAGATCCCAG–3′, down, 5′–GTCCTCGAAAAGGGCTAAGC–3′; *Bax* (29 cycles), up, 5′–GCTCTGAACAGATCATGAAG–3′, down, 5′–GATGGTCACTGTCTGCCATG–3′; *Bcl2* (30 cycles), up, 5′–TTGTGGCCTTCTTTGAGTTCG–3′, down, 5′–ATTTCTACTGCTTTAGTGAACC–3′; *Bcl2l* (30 cycles), up, 5′–GACTTTCTCTCCTACAAGC–3′, down, 5′–CGAAAGAGTTCATTCACTAC–3′; *Jun* (28 cycles), up, 5′–GCAATGGGCACATCACCAC–3′, down, 5′–GAAGTTGCTGAGGTTGGCG–3′. We amplified a 189-bp fragment of *Fos* cDNA semiquantitatively in reactions containing increasing amounts of a 219-bp competitor (mimic) DNA. We used 30 cycles and the following primer pair with ^{32}P-end labelled downstream primers: up, 5′–CAACGCCGACTACGAGGCGTCAT–3′; down, 5′–GTGGAGATGGCTGTCACCG–3′. We quantitated amplification products on a PhosphorImager after electrophoresis on a 6% polyacrylamide gel and staining with ethidium bromide.

Electrophoretic mobility shift assay (EMSA). We prepared nuclear extracts from retinal cells as described[16]. We performed EMSAs with nuclear extracts corresponding to 4 µg protein in a reaction containing BSA (24 µg), poly d(IC) (2 µg) and ^{32}P end-labelled, double-stranded oligonucleotide containing the sequence for the AP-1–specific DNA-binding site (TRE; 5′–AAGCATGAGTCAGACAC–3′) or for binding of SP-1 (5′–TCACGGGGCGGGTCAA–3′). Electrophoresis through native 6% polyacrylamide gels (30:0.8) using 0.25×TBE as running buffer resolved protein/DNA complexes. For competition assays, we added a 100-fold excess of unlabelled double-stranded DNA oligonucleotides corresponding to the binding sites for AP-1 or SP-1 to the reactions.

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