



Rod and cone degeneration in the *rd* mouse is p53 independent

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Purpose: To determine whether p53 is required for the death of rod and cone photoreceptors in *rd* mice, a model of human retinitis pigmentosa, and/or for the natural degeneration of inner nuclear layer (INL) cells in the developing retina.

Methods: Rod photoreceptor and INL apoptosis was assessed by TUNEL staining of mouse sagittal sections from post natal day (P) 10, 13, 15, 17, and 20 day *p53*^{+/+} and *p53*^{-/-} *rd* retinas. Cone photoreceptor survival was measured by counting the total number of peanut agglutinin (PNA) positive cells in eighty four 0.25 mm x 0.25 mm bins in each eye, distributed equally across the four quadrants of whole mount retinas from 3 month old *p53*^{+/+} and *p53*^{-/-} *rd* retinas.

Results: Both the kinetics of rod and INL cell death as well as the survival of cones were essentially unaffected by the absence of p53.

Conclusions: Despite established links with retinal apoptosis, p53 is not essential for rod or cone cell degeneration in the *rd* mouse or for the elimination of bipolar and Muller cells during late retinal development.

Retinitis pigmentosa (RP) is a multigenic disorder associated with the loss of photoreceptors [1,2]. The disease usually begins in late adolescence and proceeds slowly over two to three decades causing night blindness and decreased peripheral vision. The most devastating effect comes with subsequent degeneration of cones, which causes loss of color and high acuity vision, ending in total blindness.

Numerous RP genes have been identified, several of which participate in the visual transduction cascade [1,2]. Various animal models have been used to study the role of these genes in RP. The *rd* (retinal dystrophic) mouse, for example, has a recessive null mutation in the β subunit of rod specific cyclic GMP phosphodiesterase (β -PDE) [3,4]. In homozygous animals, rod loss occurs between P10 (post-natal day 10) and P20 when photoreceptors normally develop outer and inner segments. As in humans, rapid rod loss is followed by gradual cone death [5-8].

RP related photoreceptor cell death occurs by apoptosis [9-11]. The molecular pathways involved have not been elucidated. Furthermore, it is not known whether the same or different genes mediate rod and cone degeneration. Genetic experiments are required, therefore, to address this important issue. Knockout and transgenic approaches have been used to examine the role of a few apoptotic regulators in RP related rod cell death [12-18]. Significantly, however, no studies have investigated cone loss.

One gene that could mediate photoreceptor death is the pro-apoptotic transcription factor p53 [19]. p53 can influence

neurogenesis [20,21] and eye development [22,23], and is required for retinal apoptosis induced by either ionizing radiation [24] or viral oncoproteins [25]. Furthermore, in *rds* mice, which develop RP due to a mutation in the *rds*/peripherin gene, rod apoptosis is delayed in the absence of p53 [15]. In a recent study of the *rd* retina, Hopp et al. [26] found that p53 had no effect on rod cell death at post natal day 12 (P12), but noted a small but significant protection in the p53 deficient P14 central and P16 peripheral *rd* retina [26]. In view of the potential clinical importance, we revisited the role of p53 in rod death. In addition, we examined the role of p53 in natural retinal apoptosis. Finally, and most importantly, we provide the first genetic analysis of the role of an apoptotic regulator in cone loss. Our data suggest that rod and cone apoptosis in the *rd* retina, as well as natural cell death in the inner nuclear layer (INL), are all p53 independent.

METHODS

Breeding and genotyping: This study was carried out in accordance with the requirements of the Animals for Research Act, the Guidelines and Policies of the Canadian Council on Animal Care as well as the Institutional (UHN) Animal Care Committee. *rd* mice and *p53*^{-/-} mice, were obtained from Jackson laboratories. The *rd* mutation was on a C57BL/6J background. The *p53*^{-/-} background was predominantly C57BL/6J, although a small percentage (<2%) was 129/SV. These mice were interbred to obtain double heterozygotes, which were further bred to obtain *rd/rd*; *p53*^{+/+}, and *rd/rd*; *p53*^{-/-} mice. Genotyping was performed on tail DNA using the polymerase chain reaction (PCR). The *rd* genotype can be diagnosed either by a DdeI restriction digest that detects a nonsense codon [3], or by amplification of an integrated retrovirus [4]. We

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used the latter approach. Three primers were included in the reaction, Ord3 (AAG CTA GCT GCA GTA ACG CCA T), Ord4 (ATG TCC TAC AGC CCC TCT CCA A), and Ord5 (ACC TGC ATG TGA ACC CAG TAT T). Ord5 and Ord4 hybridize to intron 1 of the β -PDE gene, 5' and 3' of the provirus, respectively. (Ord5 and Ord4 are identical to primers 81 and 82 in [4], except that Ord4 lacks the first four nucleotides of primer 82). Amplification of wild type DNA generates a 247 bp fragment, but the *rd* fragment (8.76 kb) is too large to amplify. Ord3 hybridizes to the viral LTR positions 925-946 in Figure 2B of [4]. Amplification of wild type DNA with Ord3 and Ord4 produces no fragment, while *rd* DNA generates a 570 bp fragment. The *p53* mutation replaces exons 2-6 with a neo cassette, and PCR diagnostics were as described [27].

TUNEL staining and quantification of apoptotic rod and INL cells: *rd/rd; p53+/+* and *rd/rd; p53-/-* mice were cervically dislocated and both eyes removed at postnatal ages 10, 13, 15, 17 and 20 days. The inner edge of the cornea was nicked with a fine knife to allow penetration of the fixative as well as to provide a landmark orientation, and the entire eyes were immersed in 4% paraformaldehyde in PBS for 30 min. The cornea and lens were removed and the eyes fixed for an additional 2 h. Following a PBS rinse, the eyes were placed in a protective 30% sucrose solution in PBS, allowed to sink to the bottom of the container and embedded in OCT. Sagittal cryosections were collected at 10 μ m thickness. All sections analyzed included the optic nerve, located usually 1000-1500 μ m from the edge of the eye.

Following rehydration, frozen retinal sections were treated with 5 mg/ml proteinase K for 2 min at room temperature, rinsed in PBS, and immersed in PBS containing 0.1% Triton-X for 10 min. For additional permeabilization, sections were further incubated in methanol for 15 min. After three 1-min washes in PBS, sections were pre-incubated in TdT reaction buffer (Boehringer Mannheim, Laval, Quebec) containing 5 mM CaCl_2 for 30 min. This was followed by incubation in TdT buffer containing 0.125 units/ml TdT (terminal transferase), (Boehringer Mannheim) and 10 μ M biotin-16-dUTP (Boehringer Mannheim) at 37 $^\circ\text{C}$ for 1.5 h. The reaction was terminated by immersing the slides in 4X SSC that had been prewarmed to 37 $^\circ\text{C}$, for 30 min. To visualize apoptotic nuclei, slides were incubated with FITC-spreptavidin (Jackson ImmunoResearch; distributed by BIO/CAN Scientific, Mississauga, Ontario) for 15 min, rinsed and mounted with mowiol containing an antibleaching agent, DABCO (1,4-Diazabicyclo-[2,2,2]Octane, Sigma).

TUNEL stained sections were analyzed with a Nikon Optiphot microscope with epifluorescence attachment, using a 20x fluor objective. Apoptotic, bright green fluorescent nuclei were easily observed in the outer nuclear layer (ONL) and INL. Slides were coded, and quantified blind by two independent observers. Following an initial period of standardization of morphological criteria, the percent of error among repetitive counts by the observers was consistent at <4%. For each eye, the final number represented the average of two adjacent sections. Cell counts were made along the entire length

of each retinal section, and the number of apoptotic cells in the central and peripheral regions were also determined. The peripheral area included the region from each ora serata to 500 μ m towards the center. The central area included 500 μ m measured in both directions from the optic nerve. Data was analyzed by two way ANOVA and by two tailed unpaired t-test.

PNA staining and quantification of cones in retinal whole mount preparations: Eyes from three month old (P93) cervically dislocated mice were removed and dropped into 4% paraformaldehyde in PBS, pH 7.4, as described above. After 30 min, the cornea and lens were removed and eyes fixed for a total of 3 h. Eyes were transferred to PBS, the retina carefully removed from the eyecup and four radial cuts made, separating the superior, nasal, inferior and temporal quadrants. The retinas were dropped in methanol for 10 min, rinsed, placed in avidin blocking solution for 15 min, followed by biotin blocking solution. They were then incubated in biotinylated PNA (peanut agglutinin; Vector Labs) at a concentration of 1:250 for 30 min at room temperature. The ABC Elite Kit (Vector Labs), with DAB (Sigma) employed as the chromagen, was used to visualize the peroxidase stained cones. PNA binds to glycoconjugates associated with cone cell membranes [28], and has been used by others to study cone survival in the *rd* mouse [6,8].

Cone cells stained with DAB were counted using a square reticule inserted into the microscope eyepiece, using a 10x objective lens. Each quadrant of the eye was further subdivided into 21 squares of dimension 0.25 mm x 0.25 mm (0.0625 mm²), the size of a square of the reticule. A total of nine *p53+/+*, and six *p53-/-* eyes were analyzed. The total counts from all eighty four boxes in one retina were calculated, then a two tail t-test assuming unequal variance was used to compare *p53+/+* and *p53-/-* mice. A one way ANOVA was used to compare the number of cones in superior, temporal, inferior, and nasal quadrants of eyes from both genotypes. Statistical analysis was carried out using the Prism program.

RESULTS

p53 independent rod cell death: Retinal apoptosis was measured at postnatal days P10, P13, P15, P17, and P20 in a total of 38 *rd* eyes, 20 and 18 of which were *p53+/+* or *p53-/-*, respectively (Figure 1). Counts were made across the entire ONL (Figure 1A) and INL (Figure 1B). ONL deaths correspond to rods expressing defective β -PDE, while INL apoptosis represents natural pruning of bipolar and Muller cells [29]. The latter peaks around P8-10. In addition, we also compared the amount of ONL apoptosis in designated central and peripheral regions (Figure 1C,D).

In *p53+/+* *rd* retinas, rod apoptosis peaked around P13-P15, and did so slightly earlier in the central versus peripheral retina (Figure 1A,C,D), as reported previously [9-11]. In the absence of *p53*, the progression of rod cell death was very similar (Figure 1A,C,D). Progression of INL apoptosis also appeared to be unaffected by *p53* (Figure 1B). Interestingly, the mean counts at P13 and P15 in the whole or central retinas, and the P15 peripheral retina (where cell death is delayed)

were slightly higher in $p53^{-/-}$ retina, suggesting that p53 deletion may have a slight protective effect at these time points. However, an analysis of variance found no significant effect of genotype on ONL or INL apoptosis, and by t-test the only data set that showed a significant difference was the P13 central retina counts (Figure 1C; $p=0.0074$). In summary, both abnormal rd -induced photoreceptor death, and naturally occurring INL apoptosis, are p53 independent.

p53 independent cone cell death: While the bulk of rods in the rd mouse die by P20, cone cells take many months to die. Even at 18 months of age, approximately 5% of cones remain in the periphery [5]. A comprehensive temporal assessment of cell death over the complete life span of $p53^{-/-}$ cones is impossible because 50% of p53 deficient mice die from tumors (or must be sacrificed due to ill health) by four months of age. This figure exceeds 90% by 5-6 months [27].

We chose to analyze surviving cone cells in three month old (P93) animals. At this time point, many cones remain, but significant and visually observable losses have occurred [6,8]. Due to the slow disappearance of cones, it is impractical to count dying cells. Instead, we measured the number of surviving cones stained with PNA. Cones represent only 2-3% of all photoreceptors in the normal mouse retina [5]. Thus, rather than counting PNA positive cells in single sections, which could increase error, we determined the number of cones present in eighty four 0.25 mm x 0.25 mm bins distributed throughout the retina of each eye. This approach also allowed us to compare superior, inferior, temporal and nasal quadrants (21 bins per quadrant).

If p53 deletion completely blocked cone cell death, there would be a dramatic difference in cone density between $p53^{-/-}$ and $p53^{+/+}$ retinas. Visual assessment of the nine $p53^{+/+}$

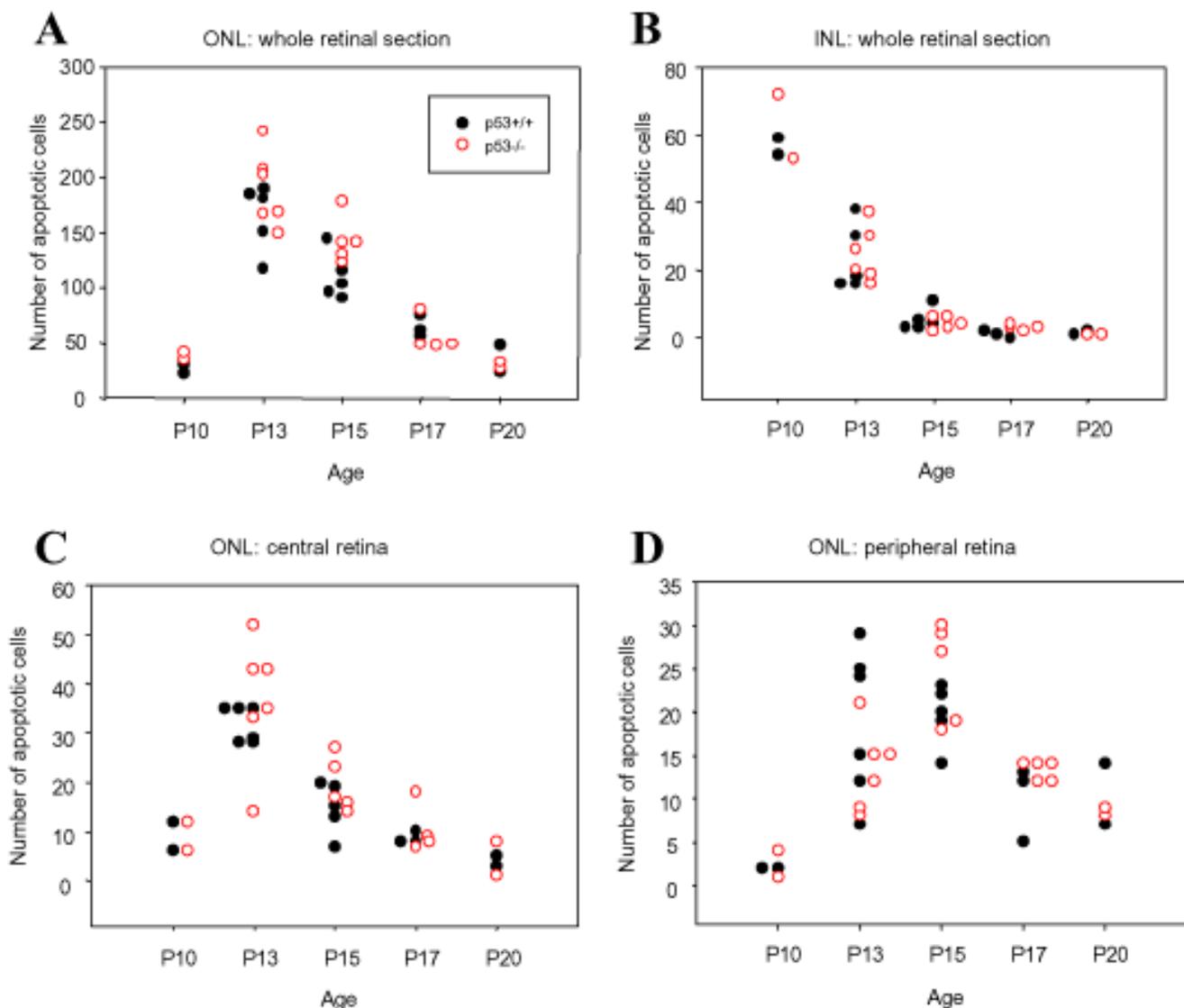


Figure 1. p53 independent rod cell death and INL apoptosis. TUNEL positive cells were counted in retinal sections obtained from rd mice of the indicated age and genotype across the whole ONL (A) and INL (B), or designated central (C) and peripheral (D) regions (see Methods).

and six *p53*^{-/-} PNA stained whole mount *rd* retinas, suggested that this was not the case (Figure 2C). Quantitative analysis confirmed this qualitative assessment (Figure 2A). An unpaired, two tailed t-test found no significant difference between the genotypes ($p=0.71$). In fact, *p53*^{-/-} eyes showed slightly fewer cones (Figure 2A): the average number of cones per mm² in *p53*^{+/+} and *p53*^{-/-} retinas was 705 (SD 144) and 663 (SD 285), respectively. Due to the variability between eyes, much larger numbers would be required to determine

whether p53 has a slight effect on cone death. Our results also do not address the possibility that p53 alters the rate of cone death prior to the three month time point we analyzed. Clearly, however, p53 is not essential for cone degeneration in the *rd* retina.

We also compared the number of cones in different retinal quadrants (Figure 2B). A one way ANOVA showed that there was no significant difference between identical quadrants (e.g., superior versus superior) in *p53*^{+/+} and *p53*^{-/-} eyes.

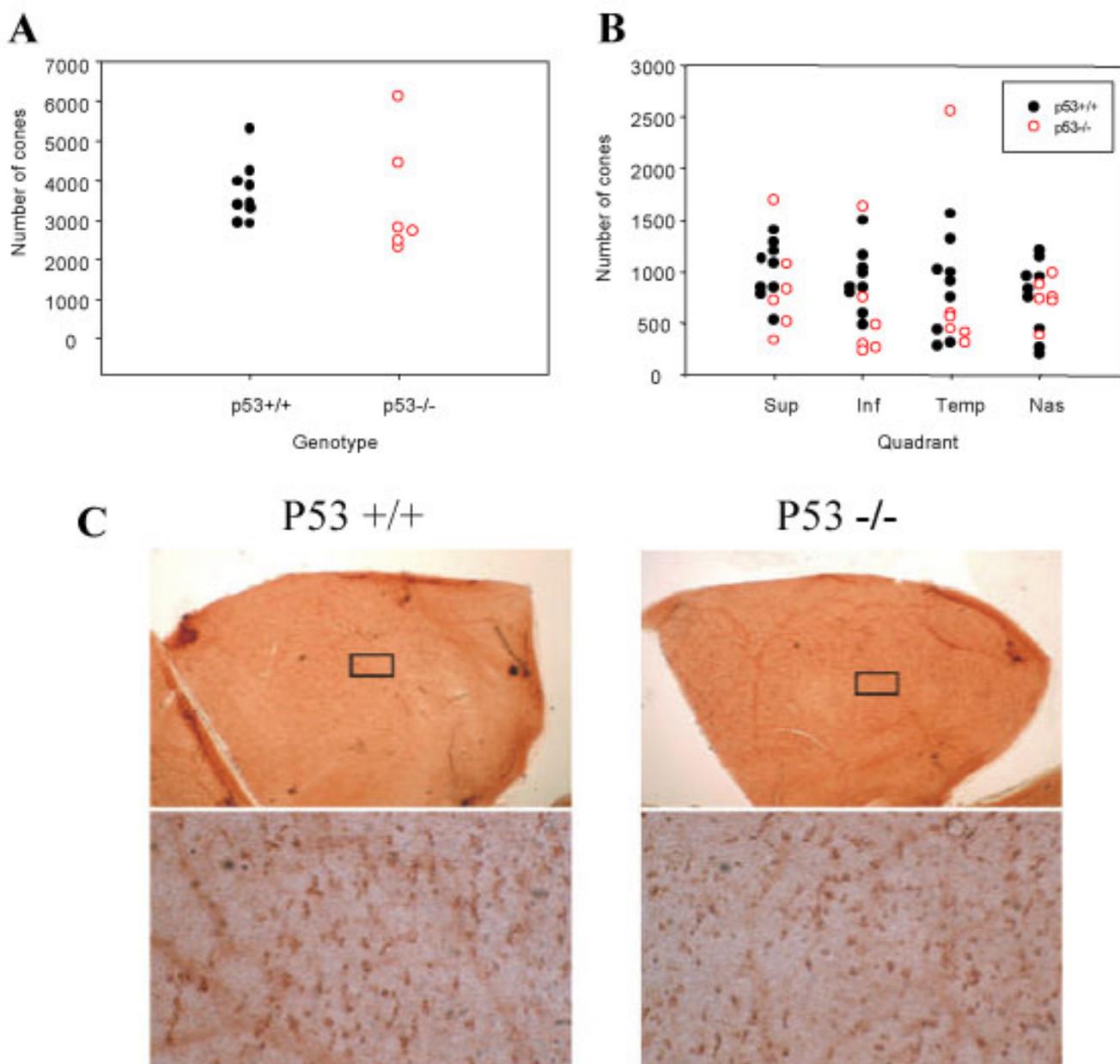


Figure 2. p53 independent cone cell degeneration. Twenty one 0.25 mm x 0.25 mm boxes per quadrant (eighty four per eye) were used to count PNA positive cones in whole mount retinas. Retinas from three month old mice of the indicated genotypes were stained and total counts (A), or the number of cones in each quadrant (B) were plotted. Nine *p53*^{+/+} and six *p53*^{-/-} eyes were analyzed. Typical PNA stained whole mounts are shown in C. Individual PNA positive cone cells are visible in the lower panels, which represent magnified views of the boxed region in the upper panels. Similar numbers of cones were observed in the presence or absence of p53

In the temporal and inferior quadrants, most of the *p53*^{-/-} retinas actually had lower numbers of cones (Figure 2B). The ANOVA analysis also suggested that, within or between genotypes, there was no significant difference in the number of cones between different quadrants.

DISCUSSION

Role of p53 in rod and cone death in the rd mouse: The death of cones is the final and most devastating effect of RP, but virtually nothing is known about the factors that mediate this event. Our study is the first that has used a genetic approach to address this issue. Despite tantalizing associations between *p53* and neuronal/retinal cell death [15,20-25], our data suggest that the loss of cones in the *rd* model is *p53* independent.

Two caveats should be noted. First, we cannot formally exclude that *p53* affects cone survival at times other than the 3-month time point analyzed here. However, later time points are difficult to assess due to the high mortality of *p53*^{-/-} animals. Moreover, significant effects on cone survival at earlier time points should, by definition, result in an increased number of cones at 3 months, especially since this time point is well within the period of dynamic cone loss. This was not the case. Delayed cell death was evident in rod photoreceptors of *p53* deficient *rds* mice [15] and following *Bcl2* expression in various models of retinal degeneration [12,14,18]. Second, since the rodent retina has only 3% cones, caution is required in extrapolating our data to the more cone rich human retina. Nevertheless, the rodent is the only animal model where genetic studies can be exploited to study cone survival. Similarities in cone structure and function suggest that the rodent model will serve as a valuable tool for identifying death pathways that may affect their survival in the human retina.

We also re-examined the role of *p53* in rod cell death. Previously, Hopp et al. [26] observed that at P14, there were slightly fewer apoptotic cells in the central *p53* deficient *rd* retina, but at P12 or P16 the amount of cell death was equivalent in the presence or absence of *p53*. In our study, which analyzed five rather than three time points, no difference was detected. Thus, *p53* neither blocks nor slows the rate of rod death in the *rd* retina. These findings are consistent with the observation that inactivation of *Bax*, a *p53* induced pro-apoptotic gene, does not protect rods in the *rd* retina [17].

In light of these findings other factors that regulate apoptosis may represent better targets for treatment of RP. Furthermore, since *p53* is mutated in a large proportion of human cancers, interfering with its function should only be contemplated where the anticipated benefits are substantial.

Gradients of cone survival in the rd retina: Previous studies have presented contrasting results regarding the gradient of cone survival in *rd* retinas. For example, using morphological criteria (heterochromatin staining) to identify cones in sections of C57BL/6J *rd* eyes of various ages, Carter-Dawson et al. [5] did not observe preferential cone survival in any quadrant. Using a similar approach, this group reported subsequently that in P60-P66 C3H/HeOuj *rd* mice, cones were more abundant in the inferior than superior hemisphere [7]. In 15% of animals the trend was reversed, but was less pronounced

[7]. Using a polyclonal antibody (CERN-906) that identifies both short and long wave cone opsins, Foster's group found that by P60, far more immunoreactive cells were present in the superior hemisphere of C57BL/6J *rd* retinas [6,30]. By P100-P120, zero cones were observed in the inferior hemisphere. Finally, Ogilvie et al. [8] analyzed cone survival qualitatively in an albino line of mixed genetic background by PNA labeling, and also reported that cones were more abundant in the superior hemisphere of retinas taken from mice aged 3 months and older. In agreement with the original findings of Carter-Dawson et al. [5], we found no significant difference in the number of PNA positive cones in any quadrant of 3 month old retinas from C57BL/6J *rd* mice (Figure 2B).

These differences are not straightforward to reconcile. It seems unlikely that genotype is the explanation, since different results have been obtained with the same strains of mice. As suggested previously [7], another possibility is the method of detection. Again, however, there is no simple correlation. A parallel study, using all three criteria, is required to resolve this issue. It would also be informative for different groups to analyze age- and strain-matched retinas from each other's mouse colonies by the same method to investigate the possibility of environmental effects and/or subtle genetic drift.

ACKNOWLEDGEMENTS

This work was supported by grants from the Retinitis Pigmentosa Foundation and National Cancer Institute of Canada.

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