Retinal development anomalies and cone photoreceptors degeneration upon Bmi1 deficiency

Andrea Barabino$^{1,2}$, Vicky Plamondon$^{1,2}$, Mohamed Abdouh$^1$, Wassim Chatoo$^1$, Anthony Flamier$^1$, Roy Hanna$^1$, Shufeng Zhou$^1$, Noboru Motoyama$^2$, Marc Hébert$^3$, Joëlle Lavoie$^5$, and Gilbert Bernier$^{1,4,5,*}$

$^1$ Stem Cell and Developmental Biology Laboratory, Hôpital Maisonneuve-Rosemont, 5415 Boul. l’Assomption, Montréal, Canada, H1T 2M4
$^3$ Department of Ophthalmology, Otorhinolaryngology and Cervico-Facial Surgery, Faculty of Medicine, Université Laval, Laval, Canada, G1V 0A6.
$^4$ Department of Neurosciences
$^5$ Department of Ophthalmology, Université de Montréal, Montréal, Canada, H3T 1J4

$^2$ Equal contribution

*Corresponding author
Tel: 514-252-3400 ext. 4648
E-mail: gbernier.hmr@ssss.gouv.qc.ca

Key words: Polycomb, Bmi1, retina, development, bipolar neuron, cone, photoreceptor, degeneration, mouse, human, embryonic stem cell
Running title: Bmi1 in cone photoreceptors survival
ABSTRACT

Retinal development occurs through the sequential but overlapping generation of six neuronal and one glial cell types. Of these, rod and cone photoreceptors represent the functional unit of light detection and phototransduction and are frequently affected in retinal degenerative diseases. During mouse development, the Polycomb group protein Bmi1 is expressed in immature retinal progenitors and differentiated retinal neurons, including cones. We show here that Bmi1 is required to prevent post-natal degeneration of cone photoreceptors and bipolar neurons, and that inactivation of Chk2 or p53 could improve but not overcome cone degeneration in Bmi1−/− mice. The retinal phenotype of Bmi1−/− mice was also characterized by loss of heterochromatin, activation of tandem-repeats, oxidative stress, and Rip3-associated necroptosis. In the human retina, BMI1 was preferentially expressed in cones at heterochromatic foci. BMI1 inactivation in human embryonic stem cells was compatible with retinal induction but impaired cone terminal differentiation. Despite this developmental arrest, BMI1-deficient cones recapitulated several anomalies observed in Bmi1−/− photoreceptors such as loss of heterochromatin, activation of tandem-repeats and p53 induction, revealing partly conserved biological functions between mouse and man.
INTRODUCTION

The distinct competence of retinal progenitor cells (RPCs) to generate in a sequential order the diverse class of neurons and a single glial cell type during retinal development is thought to be modulated by an intrinsic transcription factors molecular program and by extrinsic clues (Belecky-Adams et al., 1996; Cepko et al., 1996; Reh and Kljavin, 1989; Watanabe and Raff, 1990). Loss- and gain-of-function studies in model organisms have revealed that the transcription factors Pax6, Rax (also called Rx), Lhx2, Otx2, Sox2, Six6 and Six3 are involved in early eye patterning and retinal developmental processes (Bernier et al., 2000; Carl et al., 2002; Chow et al., 1999; Lagutin et al., 2003; Loosli et al., 1999; Marquardt et al., 2001; Mathers et al., 1997; Porter et al., 1997; Taranova et al., 2006). Later on, specific sets of transcription factors define retinal cell type identity, including photoreceptors (Swaroop et al., 2010). Photoreceptor progenitor and precursor cells express Otx2 and Crx, and conditional deletion of Otx2 in the developing mouse retina impairs photoreceptors fate (Nishida et al., 2003). In turn, Crx is required for terminal differentiation and maintenance of photoreceptors and is mutated in human retinal degenerative diseases (Chen et al., 1997; Freund et al., 1997; Freund et al., 1998; Furukawa et al., 1997; Furukawa et al., 1999; Swaroop, 1999). Photoreceptors exist in two types i.e. rods and cones. Rods are involved in low-intensity night vision and cones are involved in high-intensity color vision. During development, photoreceptors follow a S-cone default pathway, which is determined by Crx and Thrß2; Crx induces the expression of Opn1sw (encoding for S-Opsin) by default while Thrß2 suppresses it and induces the expression of Opn1mw (encoding for M-Opsin) (Ng et al., 2001; Yanagi et al., 2002). In turn, expression of Nrl, RORbeta and Notch1 inhibit cones formation, while both Nrl and RORbeta promote rods genesis at the expense of cones (Jadhav et al., 2006; Jia et al., 2009; Mears et al., 2001; Yaron et al., 2006).

Although the transcription factors dynamics controlling retinal development has been well described, the role of chromatin remodeling factors in retinal biology has been poorly explored (Hennig et al., 2013). Polycomb group proteins form large multimeric complexes that silence specific target genes by modifying chromatin organization (Valk-Lingbeek et al., 2004). The Polycomb group protein Bmi1 is a component of the Polycomb Repressive Complex 1 (PRC1), which promotes chromatin compaction and gene repression through its mono-ubiquitin ligase activity on histone H2A at lysine 119 (Buchwald et al., 2006; Cao et al., 2005; Li et al., 2006). Bmi1−/− mice show axial skeleton defects, reduced post-natal growth and lifespan, and progressive cerebellar degeneration (Jacobs et al., 1999; van der Lugt et al.,...
Most Bmi1 functions in normal development and stem cell maintenance have been attributed to transcriptional repression of the *Cdkn2a* (also called *Ink4a/Arf*) locus, encoding for p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} (Sherr, 2001; Sharpless et al., 2004; Valk-Lingbeek et al., 2004). p16\textsuperscript{Ink4a} is a cyclin-dependent kinase inhibitor that blocks the activity of Cdk4/6 by preventing its association to cyclin D, which results in Rb hypophosphorylation and cell cycle arrest or senescence. p19\textsuperscript{Arf} binds and inhibits the activity of the E3-ubiquitin ligase mouse double minute 2 (Mdm2), which prevents p53 targeting for proteosomal degradation (Sherr, 2001; Sharpless et al., 2004). More recently, activation of the DNA damage response protein checkpoint kinase 2 (Chk2) was found to contribute to several pathologies found in *Bmi1\textsuperscript{-/-}* mice (Liu et al., 2009). *Bmi1\textsuperscript{-/-}* mice also develop a progeroid phenotype in the CNS characterized by lens cataracts, cortical neurons apoptosis, p53 activation and oxidative damage accumulation (Chatoo et al., 2009). In the developing retina, it was found that Bmi1 is not required for the proliferation of the main RPC population, but for proliferation and post-natal maintenance of most immature RPCs located at the retinal ciliary margin. *Bmi1* overexpression in RPCs with short-term proliferating activity induces chromatin remodeling and conversion into long-term RPCs with stem cell characteristics (Chatoo et al., 2010). *Bmi1* thus distinguishes most immature progenitor/stem cells from the main RPC population during retinal development (Chatoo et al., 2010). Notably, *Bmi1* is also expressed in differentiated retinal neurons, including photoreceptors, raising the possibility that it may be important for their post-natal development or maintenance (Chatoo et al., 2009; Chatoo et al., 2010).

We report here that while retinal cell type genesis occurs relatively normally in *Bmi1\textsuperscript{-/-}* mice, cone bipolar neurons and cone photoreceptors rapidly degenerate during post-natal eye development through necroptosis. In the human retina, BMI1 was preferentially expressed in cones, and BMI1 inactivation in human embryonic stem cells impaired CRX expression and cone terminal differentiation. The cellular phenotype was also associated with chromatin compaction anomalies, activation of tandem-repeats and p53 induction, similarly as found in *Bmi1\textsuperscript{-/-}* mice. These findings revealed new and partly conserved biological functions for Bmi1 during cone photoreceptors development between mouse and man.
RESULTS

To investigate for a possible function of Bmi1 in retinal neurons differentiation, we performed immunohistochemistry (IHC) and immunofluorescence (IF) analyses on retinas from WT and Bmi1<sup>−/−</sup> littermates at post-natal day 30 (P30). This revealed that retinal organization and cell type genesis were possibly perturbed in Bmi1<sup>−/−</sup> mice (Figs. 1-2 and S1-S2). The distribution of Syntaxin, which labels amacrine neurons, was abnormal, resulting in its focal accumulation in the inner nuclear layer (Figs. 1A and S2). The distribution of S-Opsin, which labels the outer segment of cones, was also perturbed (Figs. 1A and S1). In contrast, the overall intensity and distribution of Rhodopsin, which labels the outer segment of rods, was apparently normal. The total number of nuclei in the outer nuclear layer, which is a measure of the total number of photoreceptors (mostly rods), was also comparable between the two genotypes (Figs. 1A-B and S2). We quantified the number of all major retinal cell types using specific antibodies (Haverkamp and Wassle, 2000; Marquardt, 2003; Marquardt et al., 2001). We found that the number of ganglion, amacrine and horizontal cells was comparable between both genotypes (Figs. 1G-H and S2). On the other hand, the number of S-cones as well as that of rod bipolar (P<sub>κ</sub>α<sup>−</sup>/Chx10<sup>+</sup>) and cone bipolar (Chx10<sup>+</sup>/P<sub>κ</sub>α<sup>−</sup>) neurons was highly reduced in Bmi1 mutants (Figs. 1B and 2A-C). Amongst the two subcategories of cone bipolar neurons, T2 ON and T8 OFF neurons (labeled by Recoverin) were also nearly absent (Figs. 2G and S2).

To test if the observed reduction in cone numbers in Bmi1<sup>−/−</sup> mice was the result of a developmental defect or secondary to degeneration, we compared P12 and P30 mice. On retinal flat mounts, we found that S-Opsin<sup>+</sup> and PNA<sup>+</sup> cone cells were equally abundant in the ventro-nasal region between WT and Bmi1<sup>−/−</sup> mice at P12, but not at P30, thus suggesting cone degeneration (Figs. 1C-F and S1). The apparent over-representation of S-Opsin in the ventro-nasal region, in contrast to the dorso-temporal region, is due to the dual nature of mouse cone photoreceptors, which frequently express both S-Opsin and M-Ospin (Fig. S4A) (Ortin-Martinez et al., 2014). These analyses also highlighted a significant reduction in cone numbers in Bmi1<sup>+/−</sup> mice, revealing a gene-dosage effect (Fig. 1C-D). We performed labeling with an antibody against activated caspase-3 to test for apoptosis. Positive cells were however not observed in both WT and Bmi1<sup>−/−</sup> retinas at P30, suggesting that apoptosis is not the main mechanism of retinal cell death in Bmi1<sup>−/−</sup> mice (data not shown). This is consistent with our previous findings, where differences in the number of caspase-3<sup>+</sup> cells were not observed between WT and Bmi1<sup>−/−</sup> retinas at P6 (Chatoo et al., 2010). To test for an altered retinal
differentiation program, we performed quantitative RT-PCR (qPCR) on retinal extracts. While expression of the Bmi1-repressed locus Cdkn2a (encoding for the p16Ink4a and p19Arf transcripts) was increased in Bmi1+/− retinas (Fig. 2I) (Molofsky et al., 2005), no difference in the expression level of several retinal determination genes was observed between both genotypes.

We also investigated Bmi1 expression in the WT mouse retina at P30. While Bmi1 was expressed in nearly all retinal cell types, it was most abundant in Chx10+ bipolar neurons (Fig. S5). In the outer nuclear layer, it was predominant in cones, not in rods, as further proved using the cone-only retina of Nrl-null mice (Fig. S3A) (Mears et al., 2001). To test whether the cone degeneration phenotype was cell autonomous, we performed dissociated retinal cultures from WT and Bmi1−/− mice at P1. Although the percentage of S-cones was comparable after 4 days in vitro (DIV) between both genotypes as revealed using Bmi1 and S-Opsin immunolabeling (Fig. S3B-C), it was highly reduced in Bmi1−/− cultures after 8 DIV and 12 DIV, suggesting cell-autonomous degeneration of cones (Fig. S3C). Taken together, these observations revealed predominant expression of Bmi1 in retinal neurons that degenerate in Bmi1−/− mice.

**Cone function is severely perturbed in Bmi1−/− mice**

To test the visual function, we performed electroretinogram (ERG) recordings of mouse retinal activity at P30. Examination of the ERG traces for the cone system revealed that cone activity was severely affected in Bmi1−/− mice when compared to WT (Fig. 3A-C). Notably, we observed a dose dependent effect as cone activity was also perturbed in Bmi1+/− mice. When tracing the Luminance Response Functions (LRFs), significant genotypes-dependent differences were observed between WT, Bmi1+/− and Bmi1−/− mice for all four ERG parameters in photopic condition, namely the a-wave amplitude (F2, 7 = 6.195, p = 0.028) (Fig. 3B and Table S1), the b-wave amplitude (F2, 7 = 18.970, p = 0.001) (Fig. 3C and Table S1), the a-wave implicit time (F2, 7 = 5.127, p = 0.043) (Table S1), and the b-wave implicit time (F2, 7 = 20.852, P = 0.001) (Table S1). These results suggest that cone photoreceptor activity, as measured with the a-wave, and the cone bipolar cell activity, as measured with the b-wave, were severely altered in Bmi1−/− mice. Also, rod system was affected in Bmi1−/− mice, as observed by the lower b-wave amplitude when compared to WT mice, but the difference was not significant for Bmi1+/− mice (Fig. 3D-F and Table S1). When analyzing the LRFs, only the b-wave amplitude was found to be significantly different between WT and Bmi1−/− mice (F2, 7
Loss of heterochromatin and necroptosis in $Bmi1^{-/-}$ cones

We next analyzed the retina of $Bmi1^{-/-}$ mice by transmission electron microscopy (TEM) to search for ultra-structural anomalies at P30. In WT mice, the electron-dense heterochromatin of rods was preeminent, located in the center of the nucleus, and generally organized as a single large chromocenter (Fig. 4A, grey arrows) (Solovei et al., 2009). In contrast, cone nuclei had 1-3 chromocenters that occupied a much-reduced nuclear surface (Fig. 4A, white arrows). Cone cell bodies were also located close to the junction with the inner segment in contrast to rod cell bodies, which were evenly distributed in the outer nuclear layer (Fig. 4A and S3A). In $Bmi1^{-/-}$ mice, the nucleus of rods appeared normal but the chromocenter was slightly reduced in size and showed less condensation (Fig. 4B). The cell body, inner segment and outer segment of rods were apparently normal. In contrast, the cell body and nucleus of cones were swollen and highly degenerative (Fig. 4A, white arrows). Accumulation of swollen mitochondria and fibrous material in the cell body of cones was also observed, suggesting necrotic cell death (also referred as necroptosis) (Fig. 4A, white and black arrows in the high magnification image) (Linkermann and Green, 2014).

To further investigate the observed chromatin phenotype, we analyzed mice at P15, when chromatin condensation of immature rods is not yet completed. Marked differences in rod’s heterochromatin condensation could be observed between WT and $Bmi1^{-/-}$ mice by TEM (Fig. 4B). By IF on retinal sections at P25, and using antibodies against “open” chromatin (H3K9ac), facultative heterochromatin (H3K27me3) and constitutive heterochromatin (H3K9me3), we found that the chromatin in the outer nuclear layer of $Bmi1^{-/-}$ mouse retinas was less condensed when compared to that of WT mice (Fig. 4C) (Fodor et al., 2010). The intergenic and pericentromeric constitutive heterochromatin contains numerous repetitive DNA sequences of retroviral origin that can be transcribed but generally silenced by heterochromatin formation (Fodor et al., 2010; Karimi et al., 2011). We thus compared the expression level of repetitive DNA sequences ($Iap1$, $Line$, $Sine$, $Major$ $satellite$ $repeats$ and $Minor$ $satellite$ $repeats$) between retinas from WT and $Bmi1^{-/-}$ mice by qPCR using RNA extracts first treated with DNaseI, since these transcripts are intron-less. We found increased expression of all tested repetitive sequences in $Bmi1^{-/-}$ retinas, consistently with the reduced heterochromatin compaction phenotype (Fig. 4D).
To investigate the mechanism of cell death, we measured the expression of genes known to mediate necroptosis (Murakami et al., 2012; Vandenabeele et al., 2010; Viringipurampeet et al., 2014). We found that the mRNA level of receptor-interacting protein kinase 3 (Ripk3) was significantly increased in Bmi1−/− retinas when compared to WT (Fig. 5A). Rip3 protein accumulation in Bmi1−/− and Bmi1+/− retinas was confirmed by Western blot analysis (Fig. 5B). In these preparations, we noticed that Rip3 accumulation could be readily observed upon Ponceau staining of the nitrocellulose membrane. Furthermore, protein accumulation was present at ~20kDa in Bmi1−/− retinal extracts, suggesting “programmed” proteolytic cleavage (Fig. 5B). To identify which retinal cell types were most affected, we performed IF analyses on sections. In both P12 and P30 Bmi1−/− retinas, we observed Rip3 immunolabeling in cones, but not in rods, and in neurons located in the inner nuclear layer, thus possibly corresponding to bipolar neurons (Fig. 5C-G). Rip3 immunolabeling was observed in the cell body and outer segment of cones at P12 (Fig. 5E-F), but was predominant in the outer segment of cones at P30 (Fig. 5C-D). Morphological anomalies of the cone outer segment were also easily visible in Bmi1−/− retinas at P30 (Fig. 5D). Taken together, these results revealed that necroptosis is the main mechanism of cone photoreceptors cell death in Bmi1−/− mice.

**Chk2 or p53 genetic deficiency can partially improve the Bmi1−/− retinal phenotype**

To investigate additional molecular mechanisms leading to cone degeneration, we generated double-null mutants for the Bmi1 and p16Ink4a/p19Arf, p19Arf or Chk2 alleles. As previously reported, the p16Ink4a/p19Arf or p19Arf mutant alleles did not rescue size or lifespan defects in Bmi1-null mice (Molofsky et al., 2005). In contrast, insertion of the Chk2 mutant allele improved size and lifespan in Bmi1-null mice, although Bmi1−/−/Chk2−/− mice remained smaller than normal (Fig. S6). Interestingly, the number of cones in Bmi1-null retinas at P30 was significantly improved by the Chk2 mutant allele but not by the p16Ink4a/p19Arf or p19Arf mutant alleles (Fig. 6A-B). Yet, morphology of the cone outer segment remained highly abnormal in Bmi1−/−/Chk2−/− mice (Fig. 6A). Hence, analysis of Bmi1−/−/Chk2−/− mice at P150 revealed severe depletion of S-cones, suggesting that Chk2 deletion only provided a transitory rescue (Fig. 6C-D).

In cortical neurons of Bmi1-null mice, stabilization of p53 leads to accumulation of ROS through repression of the Nqo1, Gsta1 and Sesn2 anti-oxidant genes (Chatoo et al., 2011; Chatoo et al., 2009). p53 also promotes neuronal cell death through activation of Apaf1, Fas and Lpo (Fortin et al., 2001). Bmi1 was also proposed to blocks ROS accumulation in
blood cells and thymus through direct transcriptional repression of the pro-oxidant genes Cyp24a1 and Duox2 (Liu et al., 2009). By qPCR analysis on retinal extracts, we found that the expression of anti-oxidant genes in Bmi1−/− mice was unchanged or increased (Nqo1), while that of pro-oxidant genes Cyp24a1 and Duox2 was increased (Fig. 7A). Notably, the expression of Apaf1 and Fas was increased in Bmi1−/−retinas, consistently with the observed accumulation of p53 by Western blot (Fig. 7B). Among 200 offspring, we obtained expected numbers of WT, Bmi1+/−/p53+/−, Bmi1+/+/p53−/−, Bmi1+/−/p53+/+, Bmi1+/−/p53+/+, and Bmi1+/−/p53+/− mice, but a single Bmi1−/−/p53−/− mouse (expected n = 12), for which size and viability were not improved. To evaluate the contribution of p53 to the cone degeneration phenotype of Bmi1−/− mice, we performed double staining with S-Opsin and Peanut Agglutinin (PNA)-which labels the outer segment of all cones. By confocal microscopy, we reconstructed retinal sections in 3D to calculate the number of cones and evaluate their morphology. Interestingly, while the number of S-Opsin/PNA-positive cells was reduced in Bmi1−/−/p53+/− retinas when compared to WT, it was improved in the unique Bmi1−/−/p53−/− retina sample (Fig. 7C-D). However, S-Opsin labeling in the Bmi1−/−/p53+/− retina remained fragmented and disorganized, suggesting improved survival but not morphology of S-cones (Fig. 7C). We also analyzed the samples using the MitoSoxRed reagent, which reacts with mitochondrial ROS on unfixed tissue. In Bmi1−/−/p53+/− retinal sections, we observed robust fluorescence when compared to WT, suggesting increased mitochondrial ROS. Notably, fluorescence was highly reduced in the Bmi1−/−/p53−/− retina but was not completely restored to WT levels (Fig. 7E). These results revealed partial contribution of Chk2 and p53 to the cone degeneration phenotype of Bmi1−/− mice.

**BMI1 is enriched at heterochromatic foci in human cones**

We previously reported on BMI1 expression in the human retina and down-regulation during ageing (Abdouh et al., 2012). Here we investigated BMI1 localization in human photoreceptors. Using confocal IF analyses on adult human retinas, we observed punctuate BMI1 immunolabeling in the nucleus of photoreceptors (Fig. 8A-C). In some cells that co-labeled with S-Opsin or M-Ospin, BMI1 immunolabeling was even more robust and present as multiple foci/nucleus (Fig. 8A). To characterize BMI1 immunolocalization pattern on the chromatin of cones, we performed dual immunolabeling with antibodies directed against distinct histone modifications. While BMI1 did not co-localize with H3K9ac, it co-localized with H3K27me3 and H3K9me3, suggesting distribution at both facultative and constitutive heterochromatin in human cones (Fig. 8B).
**BMI1 is required for human cone differentiation and chromatin integrity**

To investigate BMI1 function during human cone development, we used a protocol allowing the differentiation of ~70% of human embryonic stem (hES) cells into cones (Zhou et al., 2015). This method results in the generation of immature S-cones expressing CRX (CRX), cone Arrestin (ARR3) and S-Opsin (OPN1SW) within 21 days (Fig. 9A, D and E). Importantly, in vitro generated S-cones expressed BMI1, which also co-localized with H3K9me3 (Fig. 9A-B). We infected hES cells with a lentivirus expressing a small hairpin RNA against BMI1 (shBMI1) or scramble sequence (shScramble) and performed hygromycin selection for 10 days (Abdouh et al., 2009). The hES cells were next differentiated into S-cones for 21 days. By Western blot analysis, BMI1 expression was hardly visible in naive hES cells, in contrast to in vitro differentiated cones (Fig. 9C). Histone H2Aub is the target of BMI1/RING1a/b biochemical activity (Buchwald et al., 2006; Cao et al., 2005; Li et al., 2006; Wang et al., 2004). Consistently, H2Aub was reduced in differentiated cones upon BMI1 knockdown (Fig. 9C). To confirm cone differentiation, we analyzed cells for S-Opsin and CRX expression (Chen et al., 1997; Freund et al., 1997; Freund et al., 1998; Furukawa et al., 1997; Furukawa et al., 1999). In control cells, S-Opsin and CRX expression was observed in greater than 70% of cells, as visualized by IF and Western blot (Fig. 9A and D). In contrast, S-Opsin and CRX expression was highly reduced in differentiated cones upon BMI1 knockdown (Fig. 9A and D). When using a hypomorphic construct against BMI1 that reduces BMI1 levels by ~50% (shBMI1 50%) (Abdouh et al., 2009), CRX expression in differentiated cones was reduced by ~25% when compared to control cells, suggesting a modest gene-dosage effect (Fig. 9D). Considering the role of p53 activation in the context of Bmi1 deficiency, we investigated p53 expression (Chatoo et al., 2009). Using a pan-antibody against p53 that recognizes all family members i.e. p73, p63 and p53, we observed predominant expression of p73 in shScramble and shBMI1 50% cones (Fig. 9D) (Jacobs et al., 2006). In contrast, p73 was lost in shBMI1 95% cones and associated with induction of p63 and p53 (Fig. 9D). It was previously proposed that one main function of Polycomb group proteins in mouse ES cells is to prevent differentiation through repression of lineage-specific homeobox genes such as PAX, SOX and LHX families (Boyer et al., 2006). To further characterize the cone differentiation phenotype, we analyzed the cells by qPCR. We found that while the expression of cone-specific genes (ARR3 and OPN1SW) and retinal homeobox genes (RAX and SIX6) was reduced by 50-75% in shBMI1 cells, expression of SOX1 was increased by ~6 folds when compared to control cells (Fig. 9E). SOX1 and SOX2 are enriched
in neural stem cells and retinal progenitors but are not expressed in photoreceptors, thus providing a possible explanation for the defective terminal differentiation of cones upon BMI1 knockdown (Avilion et al., 2003; Ellis et al., 2004; Graham et al., 2003; Taranova et al., 2006; Yan et al., 2005).

To investigate the effect of BMI1 knockdown on the chromatin of human cones, we performed IF analyses. BMI1 knockdown resulted in increased H3K9ac levels, but reduced H3K27me3 and H3K9me3 levels when compared to control cells (Fig. 9F). BMI1 knockdown was also associated with the formation of γH2Ax foci, a histone modification characteristic of DNA double-strand breaks and genomic instability (Fig. 9G) (Chagraoui et al., 2011; Facchino et al., 2010; Ismail et al., 2010; Rogakou et al., 1998). Consistently with the chromatin relaxation phenotype, we observed induction of repeat-DNA sequences in BMI1 knockdown cones, with a modest gene-dosage effect when using the hypomorphic shBMI1 construct (Fig. 9H). These experiments revealed that BMI1 is required for terminal differentiation, heterochromatin compaction, repeat-DNA silencing and genomic stability in human cones.
DISCUSSION

We showed here that cone photoreceptors and bipolar neurons are normally generated but then undergo rapid degeneration in $Bmi1^{-/-}$ mice, and this through Rip3-associated necroptosis. Selective retinal cell degeneration in $Bmi1^{-/-}$ mice also correlated with predominant Bmi1 expression in bipolar neurons and cone photoreceptors. Cone number but not morphology in $Bmi1^{-/-}$ mice was partially rescued by either $Chk2$ or $p53$ deletion, implicating these additional pathways in neurodegeneration. BMI1 was expressed in human cones, where it localized at heterochromatic foci. BMI1 inactivation in hES cells severely perturbed differentiation of cones, in contrast to the situation found in $Bmi1^{-/-}$ mice. However, BMI1-deficient human cones also presented common features with the $Bmi1^{-/-}$ mouse retinal phenotype, thus revealing partially conserved functions across species.

In previous work, it was proposed that $Bmi1$ inactivation could prevent rod and cone photoreceptors degeneration in $Rd1$ mice, a model of retinitis pigmentosa (Zencak et al., 2013). $Rd1$ mice carry a mutation in $Pde6b$, which is only expressed in rods. Thus, cone degeneration in $Rd1$ mice is thought to be secondary to the loss of trophic support provided by rods. Since rod photoreceptors apoptosis in $Rd1$ mice is preceded by cell cycle re-entry and cyclin dependent kinase (CDKs) activation, it was suggested that loss of $Bmi1$ provided neuroprotection by blocking CDKs activation and thus cell cycle re-entry of rods (Marigo, 2007; Sancho-Pelluz et al., 2008; Zencak et al., 2013). Here we found that $Bmi1$ deficiency was associated with the activation of several cell cycle inhibitors in the retina such as $p16^{Ink4a}$, $p19^{Arf}$, $Chk2$ and $p53$, consistently with Bmi1 general function at inhibiting the $p16^{Ink4a}$/CDK6/Rb and $p19^{Arf}$/p53/p21$^{Cip1}$ pathways (Sauvageau and Sauvageau, 2010). Thus, and taken in the context of the $Rd1$ mutation, the proposed model is likely valid, but only in rods. Also, whether acute Bmi1 inhibition in $Rd1$ mice can prevent rod degeneration remains to be demonstrated, as this would be more relevant to a clinical context. Interestingly, the outer segment of cones is abnormal in $Rd1/Bmi1^{-/-}$ mice, as shown using S-Opsin immunolabeling, suggesting rod-independent cone degeneration (Zencak et al., 2013). This is consistent with our overall findings suggesting cell-autonomous degeneration of cones in $Bmi1^{-/-}$ mice.

Our genetic studies further revealed that $Chk2$ or $p53$ deletion could improve initial cone number in $Bmi1^{-/-}$ mice but not the outer segment morphology and progressive degeneration.
This is consistent with previous work showing that in Bmi1−/− mice, Chk2 inactivation improved several pathologies and lifespan, and that p53 inactivation prevented cortical neuron cell death (Chatoo et al., 2009; Liu et al., 2009). However, Bmi1 and p53 functions in the mouse retina, and especially in cones, are apparently not identical as in cortical neurons. For example, while p53-dependent apoptotic cell death was predominant in Bmi1−/− cortical neurons, it was not in Bmi1−/− cones, where we observed Rip3-associated necroptosis. At the molecular level, expression of the phase II anti-oxidant genes Nqo1 and Gst1α was also not reduced in Bmi1−/− retinas, in contrast to Bmi1−/− cortices. Since these are direct p53 targets, it suggests that p53 activity is distinct between retina and brain in the context of Bmi1 deficiency.

In recent years, necroptosis was revealed as an alternative mechanism of cell death in many pathological contexts (Linkermann and Green, 2014; Vandenabeele et al., 2010). In the retina, it was found that cones, not rods, are especially vulnerable to necroptosis. For example, mutation in the cone-specific gene PDE6C results in Achromaptosis, a disease characterized by cone degeneration (Chang et al., 2009). Using a zebrafish mutant for pde6c, it was shown that cone degeneration is mediated by rip1 and rip3, and that inhibition of necroptosis with necrostatin-1 can delay the disease process. Interestingly, necroptosis also operates in cones even when cone cell death is non cell-autonomous, such as in rd10 mice carrying a mutation in the rod-specific gene pde6b (Murakami et al., 2012). Here again, treatment of rd10 mice with necrostatin-1 greatly improved the cone degeneration phenotype, bringing hope for a possible pharmaceutical treatment of cone degenerative diseases.

Using directed differentiation of hES cells as model of retinal development, we demonstrated that BMI1 is required for the terminal differentiation of human cones. In contrast, expression of retinal homeobox genes and of S-Opsin was not altered in Bmi1−/− mouse retinas, revealing possible inter-species differences. These differences may be however explained by compensation mechanisms operating during development in Bmi1−/− mice but not in BMI1 knockdown cells or by the highly distinct experimental systems. For example, acute Bmi1 inactivation in embryonic mouse cortical progenitors was shown to induce massive apoptosis, in sharp contrast to the situation observed in Bmi1−/− mice (Fasano et al., 2007). Taken into a broader context, it is interesting to note that mice conditional deficient for Ezh2, the catalytic subunit of the PRC2 which tri-methylates histone H3 at lysine 27, show reduced RPC proliferation and increased apoptosis, but not post-natal degeneration of retinal neurons.
Likewise, mice deficient for G9a, which di-methylates histone H3 at lysine 9, show increased RPC apoptosis and persistent cell proliferation (Katoh et al., 2012). In both cases, inefficient repression of non-retinal genes such as Six1 (Ezh2 knockout) or RPC genes such as Hes1, Chx10 and Lhx2 (G9a knockout) perturbs the terminal differentiation of retinal cells. These two situations are thus similar to what we have observed in BMI1 deficient cones, where expression of SOX1 was abnormally upregulated. At the level of the chromatin, it was demonstrated that rod photoreceptors of mice deficient for all 3 linker histone H1 genes have reduced chromatin condensation and increased nuclear diameter (Popova et al., 2013), a phenotype also similar to the one found in Bmil"/-" mouse photoreceptors and BMI1-deficient human cones. BMI1 functions at preventing heterochromatin loss and tandem-repeats expression in photoreceptors also represent novel findings. Since constitutive heterochromatin is the most instable portion of the mammalian genome (Bhaskara et al., 2010; Kappes et al., 2011; Larson et al., 2012; Peng and Karpen, 2009; Rowe et al., 2010), this could explain part of the genomic instability phenotype observed in BMI1 deficient cells (Chagraoui et al., 2011; Facchino et al., 2010; Ismail et al., 2010).

In conclusion, we demonstrated that Bmi1 expression in the retina is not required for retinal cell type genesis but important to prevent bipolar neurons and cone photoreceptors degeneration during post-natal development. Retinal cell death in Bmil"/-" mice was mediated by the activity of Chk2 and p53 on one hand, and by Rip3-associated necroptosis on the other hand. Whether necroptosis is directly repressed by Bmi1 or indirectly mediated by the activity of p53 or Chk2 remains to be elucidated. Using an in vitro model of human cone development, we further demonstrated that BMI1 is required for the terminal differentiation of cones and maintenance of their genomic integrity. Further experiments, through BMI1 inactivation in terminally differentiated cones, should address whether BMI1 is important to prevent human cone photoreceptor degeneration and/or cell type identity maintenance.
MATERIALS AND METHODS

Ethic statement
The Animal Care Committee of the Maisonneuve-Rosemont Hospital Research Centre approved the use of the animals in this study. Post-mortem human eyes were provided by the Banque d’yeux du Québec du Centre Michel-Mathieu (http://www.maisonneuverosemont.org/pages/h/hopital/HMRCentreMichelMathieu.aspx) and were used with approbation of the Comité d’Éthique à la Recherche de l’Hôpital Maisonneuve-Rosemont. Human embryonic stem cells were used in accordance to Canadian Institute Health Research (CIHR) guidelines and approved by the Comité de Surveillance de la Recherche sur les Cellules Souches (CSRCs) of the CIHR.

Animals
Mice knockout for p53, p16^Ink4a or p16^Ink4a/p19^Arf were obtained from the Jackson Laboratory, Bar Harbor, Ma, USA. Wild type mice of the C57BL/6 genetic background were obtained from Charles River, St-Constant, Canada.

Cell cultures
The hES cell line H9 (WiCell) was cultured on BD matrigel-coated plate (BD bioscience) with a daily change of mTeSR medium according to the manufacturer instruction (Stemcell Technologies) (Thomson et al., 1998). The H9 hES cell line was first established on mouse embryonic fibroblasts (MEFs) and then cultured on Matrigel in mTeSR media. For S-cones derivation, undifferentiated hES cell colonies expanded at near confluence were next cultured in DMEM-F12 media supplemented with 2% B27, 1% N2, 1% NEAM (Life technologies), 10 ng/ml IGF-1, 10 ng/ml FGF2 (Peprotech), 10mg/ml Heparin (Sigma) and 30 ng/ml Coco (R&D Systems) for 21 days.

ERG recording and analysis
Electroretinograms (ERGs) were recorded on mice (WT: n = 4; Bmi1^+/--; n = 3; Bmi1^+/--; n = 3) in photopic and scotopic conditions to assess the cone and rod systems activity respectively according to the procedure previously described (Lavoie et al., 2014). Detailed information is available in Supplementary Materials and Methods.
**Immunohistochemistry and immunofluorescence**

Tissues were fixed by immersion for 1 h at room temperature in 4% paraformaldehyde (PFA)/3% sucrose in PBS, pH 7.4. Samples were washed three times in PBS, cryoprotected in PBS/30% sucrose, and frozen in CRYOMATRIX embedding medium (CEM) (Thermo Shandon, Pittsburgh, PA) or in Tissue-Tek® optimum cutting temperature (O.C.T.) compound (Sakura Finetek, USA). Otherwise, tissues were fixed in 10% buffered formalin and embedded in paraffin according to standard protocols. 5 to 12 µm thick sections were mounted on Super-Frost glass slides (Fisher Scientific) and processed for immunofluorescence or immunohistochemistry staining. Detailed information on the experimental procedure and antibody list are available in Supplementary Materials and Methods.

**Retinal dissection and eyes orientation**

Eyes were labeled in the dorsal pole of the cornea by puncturing with a needle tip. Eyes were extracted, fixed in 4% PFA o/n and stored in PBS. The retinas were marked by a small incision following the respective mark on the cornea. Retinas were then dissected as flattened whole-mounts by four radial cuts and processed for IF using standard procedure as describe above. Retinas were mounted between two cover slips that were attached to a slide by scotch tape, allowing flip-flopping the retinas for analysis by microscope. For the preparation of the blocks for cryosections, eyes were marked as described above, and oriented in blocks as to maintain the eye polarity.

**Quantifications of retinal cell types**

To collect sections from ventral retina, blocks were trimmed up to the optic nerve and sections collected. For quantification, three successive images were taken on the nasal side from a set distance from the optic nerve. At least three different mice were used for each genotype (1 eye/animal), except for the p53−/−/Bmi1−/− mouse where only two eyes were available. Positive cells were counted manually using imagJ software. For IF analysis, single analysis of Green channel (488) and Red channel (568) were done to prevent filter overlap. To assess the general distribution of cones, whole mount were photographed with a 10x objective using a microscope Zeiss (Observer.Z1) equipped with computer-driven motorized stage (VEXTA stepping motor), and individual frames were tiled to reconstruct the whole-mounts (about 36 images/retina) using AxioVision 4.8 software. For quantification of IF
images, photos were taken with a confocal microscope equipped with a 60x objective. 3 images were taken/sample, where n = 4 eyes/condition, at the same distance from the optic nerve in the naso-ventral portion of the retina. Photoreceptors labeled with PNA or with S-Opsin and PNA were counted manually using imagJ software. Quantifications of IHC experiments were performed in the same way but using images taken with a microscope Zeiss (Observer.Z1) equipped with a 25x objective.

**Quantitative RT-PCR**
All primers were designed to flank individual exons and tested by PCR in RT+ and RT-control extracts. Total RNA was isolated using TRizol reagent (Invitrogen). Reverse transcription (RT) was performed using 1 µg of total RNA and the MML-V reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed using the Platinum SYBRGreen SuperMix (Invitrogen) and a real-time PCR apparatus (ABI Prism 7000). GAPDH was used as an internal standard for data calibration. The \(2^{-\Delta \Delta Ct}\) formula was used for the calculation of differential gene expression. All experiments were performed at least in triplicates. Primer sequences are available in Supplementary Materials and Methods. Primer sets for repetitive sequences were as described in (Zhu et al., 2011).

**Lentiviral infection**
The shRNA-expressing lentiviral plasmids were cotransfected with plasmids pCMVdR8.9 and pHCMVG into 293FT packaging cells using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Viral containing media were collected, filtered, and concentrated by ultracentrifugation. Viral titers were measured by serial dilution on 293T cells followed by microscopic analysis 48 h later. For viral transduction, lentiviral vectors were added to dissociated cells before plating. Hygromycin selection (150 µg/ml) was added 48 h later.

**Western blot**
The experimental procedure and antibody list are available in Supplementary Materials and Methods.

**Statistical analyses**
Statistical differences were analyzed using Student’s \(t\)-test for unpaired samples with equal SD using two-tailed P.value. For ERG experiments analysis of variance were made by one-way ANOVA followed by the Bonferroni's multiple comparisons test. For PRs quantification
of flat mount retinas the analysis of variance was performed by two way ANOVA followed by Tukey’s multiple comparison test with 95% of confidence. Data are presented as mean ± standard deviation (error bars). Values are representative of at least 3 experiments. The criterion for significance ($P$ value) was set as mentioned in the figures.
Figure 1. S-cones degenerate during post-natal eye development in Bmi1<sup>−/−</sup> mice

(A) Analyses of WT and Bmi1<sup>−/−</sup> retinas at P30. Abnormal distribution of Syntaxin (arrowheads in A’’) and cone photoreceptor outer segments breaks in Bmi1<sup>−/−</sup> mice.
(arrowheads in A***') as visualized by IHC. Cone photoreceptor outer segments breaks but normal looking rod photoreceptors Bmi1+/− mice, as visualized by IF. (B) Quantifications of the total number of photoreceptors (upper graft) and of S-cones (lower graft). (C and E) Representative microscopy images from retinal flat-mount of WT, Bmi1+/− and Bmi1−/− mice at P30 (C) and of WT and Bmi1−/− at P12 (E). Images were taken in the ventro-nasal part of the retina. (D and F) Quantification of S-cone photoreceptors (S-Opsin+) and total cone photoreceptors (PNA+) at P30 (D) and P12 (F). Retinal pigment epithelium (RPE); outer nuclear layer (ONL); inner nuclear layer (INL); ganglion cell layer (GCL). Scale bars: 40μm. All values are mean ± SEM. (*) P ≤ 0.05; (**) ≤ 0.01; (***) ≤ 0.001; (****) ≤ 0.0001; Student t-test (B, F), two-way ANOVA (D).
Figure 2. Bipolar neurons degenerate during post-natal eye development in Bmi1−/− mice
(A and D) Representative images of WT and Bmi1−/− retinas at P30 (A) and P12 (D) labeled with antibodies against Pkcα and Chx10. (B and E) Cropped images indicated by the respective dashed rectangles indicated in (A and D). White full arrows: cone bipolar cells (Chx10+/Pkcα−). White-edged arrows: rod bipolar cells (Chx10+/Pkcα+). Note the decrease in Chx10-positive cells in Bmi1−/− retinas at P30. (C and F) Quantifications of data acquired in (A) and (D). (G) Quantification of T2 OFF and T8 ON cone bipolar cells (Recoverin+), amacrine cells (Pax6+) and horizontal cells (Calbindin+) in the INL. (H) Quantification of ganglion cells (Pax6+) in the GCL. (I) Gene expression analysis of WT and Bmi1−/− retinas at...
P30, and where p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} were used as positive controls. Retinal pigment epithelium (RPE); outer nuclear layer (ONL); inner nuclear layer (INL); ganglion cell layer (GCL). Scale bars: 40μm. All values are mean ±SEM. (*) P ≤ 0.05; (**) ≤ 0.01; Student t-test.
Figure 3. Cone and rod function is severely perturbed in Bmi1−/− mice

ERG recorded in photopic (A-C) and scotopic (D-F) condition. (A and D) ERG waveforms for WT, Bmi1+/− and Bmi1−/− mice. Luminance response function curve of a-wave (B and E) and b-wave (C and F) representing the dynamic electrical response from the cone (B and C) and rod (E and F) system. Note the significant decrease in the retinal responses of Bmi1+/− mice and Bmi1−/− mice in photopic condition for both the a-wave and b-wave amplitude at the Vmax compared to WT mice and the decrease of the b-wave amplitude at the Vmax in scotopic condition in Bmi1−/− mice compared to WT and Bmi1+/− mice. All values are mean ± SEM. (*) P ≤ 0.05; (**) ≤ 0.01; (*** ≤ 0.001; One-way ANOVA.
Figure 4. Cone necrosis, loss of heterochromatin and activation of tandem repeats in Bmi1−/− mouse retinas

(A) TEM on WT and Bmi1−/− retinas at P30. White arrows: cone photoreceptors; grey arrows: rod photoreceptors characterized by electron-dense heterochromatin organized as a single chromocenter; black arrows: degenerative cone cell bodies; white-edged arrows: swollen mitochondria in cones. (B) TEM analysis of WT and Bmi1−/− rod photoreceptors at P15. (C) Analysis of histone modifications in WT and Bmi1−/− retinas by IF on sections at P25. (D) Quantitative PCR analysis of repetitive-DNA sequences expression in WT and Bmi1−/− retinas at P30. Bmi1 is use as negative control and values are expressed as fold of WT. Outer nuclear layer (ONL); inner segment (IS); outer segment (OS) and retinal pigment epithelium (RPE). Scale bars: (A) 10µm (left, center and top-right images) and 2µm (bottom-right image); (B) 5µm; (C) 25µm. All values are mean ±SEM. (*) P ≤ 0.05; (**) ≤ 0.01; Student t-test.
Figure 5. Cone degeneration in Bmi1\(^{-/-}\) mice operates through necroptosis

(A) RT-qPCR analysis of Bmi1\(^{-/-}\) and WT retinal extracts at P30; Bmi1 and P16Ink4a were used as internal controls. Values are expressed as fold of WT. (B) WB analyses of retinal extracts from WT, Bmi1\(^{+/+}\) and Bmi1\(^{-/-}\) mice. Note the gene-dosage dependent increase in Rip3 expression and the decrease in S-Opsin expression in Bmi1\(^{-/-}\) mice. On the right: Ponceau-red staining of the membrane. Histone H3 was used for protein normalization. (C-F) IF analyses on WT and Bmi1\(^{-/-}\) retinas at P30 (C and D) and P12 (E and F). White-edged arrows: accumulation of Rip3 in S-cone outer segments; gray arrows: Rip3 positive cells in the INL. White arrows: S-Opsin positive cells with a nuclear expression of Rip3 in Bmi1\(^{-/-}\) mice at P12 not present at P30. (G) Control with secondary antibody only. Outer nuclear layer (ONL); inner nuclear layer (INL); inner segment (IS); outer segment (OS). Scale bars: 40\(\mu\)m. All values are mean ±SEM. (*) P ≤ 0.05; (**) ≤ 0.01; (***) ≤ 0.001; Student t-test.
Figure 6. Chk2 genetic deficiency improves the Bmi1⁻⁻ cone degeneration phenotype

(A) IHC analyses of WT, Bmi1⁻⁻ and double-mutant retinas at P30. (B) Quantification of the number of S-Opsin positive photoreceptors per section for the five genotypes at P30 (n = 4 mice for each genotype). (C) IHC analyses of retinas at P150. (D) Quantification of the number of S-Opsin positive cells per field at P30 and P150. Scale bars: 40μm. Quantifications are made from 25x images. All values are mean ±SEM. (*) P ≤ 0.05; Student t-test.
Figure 7. p53 genetic deficiency improves the Bmi1−/− cone degeneration phenotype

(A) Quantitative RT-PCR analysis of WT and Bmi1−/− retinal extracts at P30. Bmi1 was used as negative control and all values are expressed as fold of WT. (B) Western blot analysis of mouse retinas at P30 showing p53 accumulation in Bmi1-mutants. (C) 3D reconstruction by confocal microscopy analyses of WT, Bmi1−/−/p53+/− and Bmi1−/−/p53−/− retinas at P30. White box: detail of a S-Opsin in the outer segment in the dashed box. Note the lower immunoreactivity and the fragmentation of the S-Opsin in Bmi1+/−/p53−/− mice and a rescue in the quantity of S-Opsin in the Bmi1−/−/p53−/− even if it is still fragmented. (D) Quantification of
S-Opsin positive cells per section in WT, \textit{Bmi1}^{+/−}/\textit{p53}^{+/−} and \textit{Bmi1}^{+/−}/\textit{p53}^{−/−} mice. (E) IF analyses of WT and \textit{Bmi1}^{−/−} retinas at P30 stained with MitoSoxRed. Note the partial rescue in \textit{Bmi1}^{−/−}/\textit{p53}^{−/−} mice when compared to WT. Retinal pigment epithelium (RPE); Outer nuclear layer (ONL); inner nuclear layer (INL); outer segment (OS). Scale bar: 50μm (C) and 20μm (E). All values are mean ±SEM. (*) \( P \leq 0.05; (** \leq 0.01; \) Student t-test.
Figure 8. BMI1 is enriched at heterochromatic nuclear foci in human cones

(A-C) Confocal IF analysis of BMI1 expression in the adult human retina. (A) BMI1 is expressed in the nucleus of M-Ospin and S-Opsin positive cones. (B) BMI1 is enriched at heterochromatic nuclear foci as shown by co-localization with H3K9me3 and H3K27me3 in cones. (C) Negative control with only the secondary antibody. Note the strong autofluorescence present in the IS and OS. Inner segment (IS), outer segment (OS), outer nuclear layer (ONL); inner nuclear layer (INL); and ganglion cell layer (GCL). Scale bars: 20μm.
Figure 9. BMI1 is required for terminal differentiation, heterochromatin compaction and genomic stability of human cones

(A and B) IF analysis of hES cells differentiated for 21 days toward the cone photoreceptor cell lineage. (A) In controls (shSrcamble), BMI1 is expressed in the nucleus of S-Opsin
positive cells. In shBMI1 cells, BMI1, S-Opsin and CRX expression were highly reduced when compared to controls. (B) Co-localization of BMI1 and H3K9me3 in cones (arrows). (C and D) WB analyses of hES cells and in vitro differentiated cones (+CI 21 days) infected with shScramble or shBMI1 viruses. Note: BMI1 knockdown affects H2Aub levels only in cones; BMI1 knockdown in human cones was accompanied by induction of p53. Quantification of CRX levels normalized to TUBULIN; note the dose-dependent reduction in CRX expression in BMI1 knockdown cells. (E and H) Quantitative RT-PCR analyses of in vitro differentiated cones for retinal markers (E) and repeat-DNA sequences (H). BMI1 was used as a negative control and values are expressed as fold of Scramble. (F and G) IF analyses of hES cells-derived cones (day 21). Scale bars: 20μm (A, F, G) and 2μm (B). All values are mean ±SEM. (*) P ≤ 0.05; (**) ≤ 0.01; Student t-test.
ACKNOWLEDGEMENT

We thank M. Van Lohuizen for the Bmi1+/− mice and A. Swaroop for the Nrl−/− mouse eyes. This work was supported by grants from the Natural Science and Engineering Research Council of Canada (NSERC), Antoine-Turmel Foundation for Macular Degeneration Research, and Foundation Fighting Blindness Canada. V.P. was supported by a fellowship from NSERC. A.F. was supported by Fellowships from Montreal University Molecular Biology Program and Réseau Vision du Québec.
REFERENCES


