RESEARCH ARTICLE

Loss of Bmi1 causes anomalies in retinal development and degeneration of cone photoreceptors

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ABSTRACT

Retinal development occurs through the sequential but overlapping generation of six types of neuronal cells and one glial cell type. 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 postnatal 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 heterochromatotic foci. Bmi1 inactivation in human embryonic stem cell lines were coupled 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 induction of p53, revealing partly conserved biological functions between mouse and man.

KEY WORDS: Polycomb, Bmi1, Retina, Retinal development, Bipolar neuron, Cone, Photoreceptor, Degeneration, Mouse, Human, Embryonic stem cell

INTRODUCTION

The distinct competence of retinal progenitor cells (RPCs) to generate in a sequential order the diverse classes of neurons and a single glial cell type during retinal development is thought to be modulated by an intrinsic transcription factor molecular program and by extrinsic cues (Belecky-Adams et al., 1996; Cepko et al., 1996; Reh and Kljavin, 1989; Watanabe and Rafi, 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, 1998; Furukawa et al., 1997, 1999; Swaroop et al., 1999). Photoreceptors exist as two cell types – rods and cones. Rods are involved in low-intensity night vision and cones are involved in high-intensity color vision. During development, photoreceptors follow an S-cone (cones containing S-opsin) default pathway, which is determined by cone-rod homeobox (Crx) and thyroid receptor β2 (Thrβ2, encoded by Thrβ); Crx induces expression of Opn1sw (encoding S-opsin) by default, whereas Thrβ2 suppresses it and induces expression of Opn1lw (encoding M-opsin) (Ng et al., 2001; Yanagi et al., 2002). In turn, expression of neural retina leucine zipper (Nrl), RAR-related orphan receptor β (Rorb) and Notch1 inhibit cone formation, whereas both Nrl and RORβ promote rod 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 factor 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 postnatal growth and lifespan, and progressive cerebellar degeneration (Jacobs et al., 1999; van der Lugt et al., 2004). Most Bmi1 functions in normal development and stem cell maintenance have been attributed to transcriptional repression of the Cdkn2a (also called the INK4a/ARF) locus, encoding p16INK4a and p19ARF (Sherr, 2001; Sharpless et al., 2004; Valk-Lingbeek et al., 2004). p16INK4a is a cyclin-dependent kinase inhibitor that blocks the activity of Cdk4/6 by preventing its association with cyclin D, which results in Rb hypophosphorylation and cell cycle arrest or senescence. p19ARF binds and inhibits the activity of the E3-ubiquitin ligase mouse double minute 2 (Mdm2), which prevents targeting of p53 for protosomal 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−/− mice (Liu et al., 2009). Bmi1−/− mice also develop a progeroid phenotype in the
CNS characterized by lens cataracts, cortical neurons apoptosis, p53 activation and accumulation of oxidative damage (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 postnatal 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 might be important for their postnatal development or maintenance (Chatoo et al., 2009, 2010).

We report here that whereas retinal cell type genesis occurs relatively normally in Bmi1−/− mice, cone bipolar neurons and cone photoreceptors rapidly degenerate during postnatal eye development through necrosis. 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 induction of p53, as found in Bmi1−/− mice. These findings reveal new and partly conserved biological functions for Bmi1 during cone photoreceptor development between mouse and man.

RESULTS

To investigate a possible function of Bmi1 in retinal neurons differentiation, we performed immunohistochemistry (IHC) and immunofluorescence (IF) analyses on retinas from wild-type (WT) and Bmi1−/− littermates at postnatal day 30 (P30). This revealed that retinal organization and cell type genesis were possibly perturbed in Bmi1−/− mice (Figs 1,2 and Figs S1,S2). The distribution of syntaxin, which labels amacrine neurons, was abnormal, resulting in its focal accumulation in the inner nuclear layer (Fig. 1A, Fig. S2). The distribution of S-opsin, which labels the outer segment of cones, was also perturbed (Fig. 1A, Fig. S1). By contrast, the overall intensity and distribution of rhodopsin, which labels the rod outer segment, 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 (Fig. 1A,B, Fig. S2). We quantified the number of all major retinal cell types using specific antibodies (Haverkamp and Wässle, 2000; Marquardt, 2003; Marquardt et al., 2001). We found that the number of ganglion, amacrine and horizontal cells was comparable between both genotypes (Fig. 1G,H, Fig. S2). However, the number of S-cones as well as rod bipolar (PKCζ+/−/Chx10+) and cone bipolar (Chx10+/−/PKCζ−) neurons was highly reduced in Bmi1 mutants (Fig. 1B, Fig. 2A-C). Amongst the two subcategories of cone bipolar neurons, T2 ON and T8 OFF neurons (labeled by recoverin) were also nearly absent (Fig. 2G, Fig. S2).

To test whether the observed reduction in cone numbers in Bmi1−/− 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 and PNA cone cells were equally abundant in the ventro-nasal region of WT and Bmi1−/− mice at P12, but not at P30, thus suggesting cone degeneration (Fig. 1C-F, Fig. 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-opsin (Fig. S4A) (Ortín-Martínez et al., 2014). These analyses also highlighted a significant reduction in cone numbers in Bmi1−/− 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 either WT or Bmi1−/− retinas at P30, suggesting that apoptosis is not the main mechanism of retinal cell death in Bmi1−/− mice (data not shown). This is consistent with our previous findings, where differences in the number of caspase-3+ cells were not observed between WT and Bmi1−/− retinas at P6 (Chatoo et al., 2010). To test for an altered retinal differentiation program, we performed quantitative RT-PCR (qPCR) on retinal extracts. Although expression of the Bmi1-repressed locus Cdkn2a (encoding 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 the two genotypes.

We also investigated Bmi1 expression in the WT mouse retina at P30. Although 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 shown 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 with 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 genotype-dependent differences were observed between WT, Bmi1+/− and Bmi1−/− mice for all four ERG parameters in the photopic condition, namely the a-wave amplitude (F2,7=6.195, P=0.028; Fig. 3B, Table S1), the b-wave amplitude (F2,7=18.970, P=0.001; Fig. 3C, 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, the rod system was affected in Bmi1−/− mice, as observed by the lower b-wave amplitude when compared with WT mice, but the difference was not significant for Bmi1+/− mice (Fig. 3D-F, Table S1). When analyzing the LRFs, only the b-wave amplitude was found to be significantly different between WT and Bmi1−/− mice (F2,7=10.440, P=0.008; Fig. 3F, Table S1), suggesting that rod bipolar cell activity was affected in Bmi1−/− mice, but at a lower level than in photopic conditions (Fig. 3C).

Loss of heterochromatin and necroptosis in Bmi1−/− cones

We next analyzed the retina of Bmi1−/− mice by transmission electron microscopy (TEM) to search for ultrastructural anomalies at P30. In WT mice, the electron-dense heterochromatin of rods was pre-eminent, located in the center of the nucleus and...
generally organized as a single large chromocenter (Fig. 4A, gray arrows) (Solovei et al., 2009). By 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, Fig. S3A). In Bmi1−/− mice, the nucleus of rods appeared normal but the chromocenter was slightly reduced in size

Fig. 1. S-cones degenerate during post natal eye development in Bmi1−/− mice. (A) Analyses of WT and Bmi1−/− retinas at P30. Abnormal distribution of syntaxin (arrowheads in A″) and cone photoreceptor outer segments breaks in Bmi1−/− mice (arrowheads in A‴) as visualized by IHC. Cone photoreceptor outer segments break but rod photoreceptors appear normal in Bmi1−/− mice, as visualized by IF. (B) Quantification of total number of photoreceptors (top) and S-cones (bottom). (C,E) Representative microscopy images from retinal flat-mount of WT, Bmi1+/− and Bmi1−/− mice at P30 (C) and WT and Bmi1−/− at P12 (E). Images were taken in the ventro-nasal part of the retina. (D,F) Quantification of S-cone photoreceptors (S-opsin+) and total cone photoreceptors (PNA+) at P30 (D) and P12 (F). RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 40 μm. All values are means±s.e.m. *P≤0.05; ***P≤0.001; ****P≤0.0001; Student’s t-test (B,F), two-way ANOVA (D).
and showed less condensation (Fig. 4B). The cell body, inner segment and outer segment of rods were apparently normal. By contrast, the cell bodies and nuclei 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 known 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 heterochromatin condensation could be observed between WT and Bmi1−/− mice by transmission electron microscopy (TEM) (Fig. 4B). Using retinal sections at P25 and 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 with 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 are 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, because these transcripts are intron-less. We found increased expression of all tested repetitive sequences in \( Bmi1^{-/-} \) retinas, consistent 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; Viringipurampeer et al., 2014). We found that the mRNA level of receptor-interacting protein kinase 3 (\( Ripk3 \)) was significantly increased in \( Bmi1^{-/-} \) retinas when compared with 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 Red staining of the nitrocellulose membrane. Furthermore, protein accumulation was present at \( \sim 20 \) kDa in \( Bmi1^{-/-} \) retinal extracts, suggesting ‘programmed’ proteolytic cleavage (Fig. 5B). To
identify which retinal cell types were most affected, we performed immunofluorescence (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 P30 (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 clearly visible in Bmi1−/− retinas at P30 (Fig. 5D). Taken together, these results reveal 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). By 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). However, 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 deletion of Chk2 only provided a transitory rescue (Fig. 6C,D).

In cortical neurons of Bmi1 null mice, stabilization of p53 leads to accumulation of reactive oxygen species (ROS) through repression of the Nqo1, Gstα1 and Sesn2 antioxidant genes (Chatoo et al., 2011, 2009). p53 also promotes neuronal cell death through activation of Apaf1, Fas and Lpo (Fortin et al., 2001) and Bmi1 was proposed to block 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 antioxidant genes in Bmi1−/−/p53−/− mice was unchanged or increased (Nqo1), whereas that of Cyp24a1 and Duox2 was increased (Fig. 7A). Notably, the expression of Apaf1 and Fas was increased in Bmi1−/−/p53−/− retinas, consistent 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 did not improve. 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 segments of all cones in wild-type (WT) and Bmi1−/− retinas at P30.
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+ cells was reduced in Bmi1−/−/p53+/- retinas when compared with 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 samples using the MitoSoxRed reagent, which reacts with mitochondrial ROS on unfixed tissue. In Bmi1−/−/p53+/- retinal sections, we observed robust fluorescence when compared with 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 the partial contribution of Chk2 and p53 to the cone degeneration phenotype of Bmi1−/− mice.

BMI1 is required for human cone differentiation and chromatin integrity

To investigate the function of BMI1 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,E). Importantly, in vitro generated S-cones expressed BMI1, which also colocalized with H3K9me3, suggesting distribution at both facultative and constitutive heterochromatin in human cones (Fig. 8B).

BMI1 is enriched at heterochromatic foci in human cones

We previously reported BMI1 expression in the human retina and its downregulation during aging (Abdouh et al., 2012). Here, we investigated localization of BMI1 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-opsin, BMI1 immunolabeling was even more robust and present as multiple foci per nucleus (Fig. 8A). To characterize the BMI1 immunolocalization pattern on the chromatin of cones, we performed dual immunolabeling with antibodies directed against distinct histone modifications. Although BMI1 did not colocalize with H3K9ac, it colocalized with H3K27me3 and H3K9me3, suggesting distribution at both facultative and constitutive heterochromatin in human cones (Fig. 8B).

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Fig. 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) Western blot 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. Ponceau-red staining of the membrane is shown on the right. Histone H3 was used for protein normalization. (C-F) IF analyses on WT and Bmi1−/− retinas at P30 (C,D) and P12 (E,F). White-edged arrows, accumulation of Rip3 in S-cone outer segments; gray arrows, Rip3+ cells in the INL; white arrows, S-opsin+ cells with nuclear expression of Rip3 in Bmi1−/− mice at P12 not present at P30. (G) Control with secondary antibody only. INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment; IS, inner segment. Scale bars: 40 μm. All values are means±s.e.m. **P≤0.01; ***P≤0.001; Student’s t-test.
then differentiated into S-cones for 21 days. Upon 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 and RING1a/b biochemical activity (Buchwald et al., 2006; Cao et al., 2005; Li et al., 2006; Wang et al., 2004). Consistently, histone H2Aub was reduced in differentiated cones upon knockdown of BMI1 (Fig. 9C). To confirm cone differentiation, we analyzed cells for expression of S-opsin and CRX (Chen et al., 1997; Freund et al., 1997, 1998; Furukawa et al., 1997, 1999). In control cells, S-opsin and CRX expression was observed in more than 70% of cells, as visualized by IF and western blot (Fig. 9A and D). By contrast, S-opsin and CRX expression was highly reduced in differentiated cones upon BMI1 knockdown (Fig. 9A,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 with levels in control cells, suggesting a modest gene-dosage effect (Fig. 9D). To further characterize the cone differentiation phenotype, we analyzed the hES 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-fold when compared with 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 knockdown of BMI1 (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 with 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). Consistent 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, silencing of repeat DNA 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 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 deletion of either Chk2 or p53, 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 photoreceptor 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. As apoptosis of rod photoreceptors in Rd1 mice is preceded by cell cycle re-entry and activation of cyclin dependent kinases (CDKs), it was suggested that loss of Bmi1 provides neuroprotection by blocking CDK 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 p16INK4a, p19ARF, Chk2 and p53, consistent with the general function of Bmi1 in inhibiting the p16INK4a/CDK6/Rb and p19ARF/p53/p21Cip1 pathways (Sauvageau and Sauvageau, 2010). Thus, taken in the context of the Rd1...
mutation, the proposed model is likely to be 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 (Chattoo et al., 2009; Liu et al., 2009). However, the function of Bmi1 and p53 in the mouse retina, and especially in cones, are apparently not identical as in cortical neurons. For example, although 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 antioxidant genes Nqo1 and Gsta1 was also reduced in Bmi1−/− retinas, in contrast to Bmi1−/− cortices. Because these are direct p53 targets, this suggests that p53 activity is distinct between the 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 achromatopsia, 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. By contrast, expression of retinal homeobox genes and of Opn1sw was not altered in Bmi1−/− mouse retinas, revealing possible inter-species differences. These differences might, however, be 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 in a broader context, it is interesting to note that mice conditionally 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 (Zhang et al., 2015). Likewise, mice deficient for G9a (Ehmt2), 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.
Fig. 9. **BMI1 is required for terminal differentiation, heterochromatin compaction and genomic stability of human cones.** (A,B) IF analysis of hES cells differentiated for 21 days toward the cone photoreceptor cell lineage. (A) In controls (shScramble), BMI1 is expressed in the nucleus of S-opsin+ cells. In shBMI1 cells, BMI1, S-opsin and CRX expression were highly reduced when compared with controls. (B) Colocalization of BMI1 and H3K9me3 in cones (arrows). (C,D) Western blot analyses of hES cells and in vitro differentiated cones (+CI 21 days) infected with shScramble or shBMI1 viruses. Note that BMI1 knockdown affects histone 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,H) Quantitative RT-PCR analyses of in vitro differentiated cones for retinal markers (E) and repeat DNA sequences (H). BMI1 was used as negative control and values are expressed as fold of Scramble value. (F,G) IF analyses of hES cell-derived cones (day 21). Scale bars: 20 μm in A,F,G; 2 μm in B. All values are means±s.e.m. *P≤0.05; **P≤0.01; Student’s t-test.
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 three linker histone H1 genes have reduced chromatin condensation and increased nuclear diameter (Popova et al., 2013), a phenotype similar to that found in Bmi1<sup>−/−</sup> mouse photoreceptors and BMI1-deficient human cones. The results suggesting that BMI1 functions to prevent heterochromatin loss and expression of tandem repeats in photoreceptors are also 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 Karnen, 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 is important to prevent bipolar neuron and cone photoreceptor degeneration during post natal development. Retinal cell death in Bmi1<sup>−/−</sup> mice was mediated by the activity of Chk2 and p53 on the one hand, and by Rip3-associated necroptosis on the other. 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 inactivation of BMI1 in terminally differentiated cones, should address whether BMI1 is important to prevent human cone photoreceptor degeneration and/or maintenance of cell type identity.

**MATERIALS AND METHODS**

**Mice and human tissues**

Mice knockout for p53, p16<sup>INK4a</sup> or p16<sup>INK4a</sup>/p19<sup>ARF</sup> were obtained from the Jackson Laboratory. Wild-type mice from the C57BL/6 genetic background were obtained from Charles River, Saint-Constant, Canada. Fresh human eyes were provided by the eye bank of Maisonneuve-Rosemont Hospital.

The Animal Care Committee of the Maisonneuve-Rosemont Hospital Research Centre approved the use of the animals in this study. Post mortem was approved by the Banque d’yeux du Québec du Centre Michel-Mathieu (http://www.maisonneuve-rosemont.org/pages/hospital/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 with Canadian Institute Health Research (CIHR) guidelines and approved by the Comité de Surveillance de la Recherche sur les Cellules Souches (CSRCs) of the CIHR.

**Cell culture**

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’s instructions (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 medium. For derivation of S-cones, undifferentiated hES cell colonies expanded at near confluence were cultured in DMEM-F12 medium supplemented with 2% B27, 1% N2, 1% NEAM (Life Technologies), 10 ng/ml IGF-1, 10 ng/ml FGF2 (Peprotech), 10 mg/ml heparin (Sigma) and 30 ng/ml Coco (R&D Systems) for 21 days. Retinal cells were cultured from eyes of WT and knockout mice as described in detail in supplementary Materials and Methods.

**ERG recording and analysis**

Electroretinograms (ERGs) were recorded on mice (WT, n=4; Bmi1<sup>−/−</sup>, n=3; Bmi1<sup>−/−</sup>, n=3) in photopic and scotopic conditions to assess the cone and rod systems activity, respectively, according to a previously described procedure (Lavoie et al., 2014). Detailed information is available in supplementary Materials and Methods.

**Immunohistochemistry and immunofluorescence**

Tissues were fixed by immersion in 4% paraformaldehyde (PFA) 3% sucrose in PBS, pH 7.4 for 1 h at room temperature. Samples were washed three times in PBS, cryoprotected in PBS/30% sucrose and frozen in Cryomatrix embedding medium (CEM) (Thermo Shandon) or in Tissue-Tek optimum cutting temperature (O.C.T.) compound (Sakura Finetek). 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 antibodies used is available in supplementary Materials and Methods.

**Retinal dissection and eye orientation**

Eyes were labeled in the dorsal pole of the cornea by puncturing with a needle tip. Eyes were extracted, fixed in 4% PFA overnight 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 procedures, as describe above. Retinas were mounted between two coverslips that were attached to a slide by scotch tape, allowing flip-flopping of the retinas for analysis by microscope. For preparation of the blocks for cryosectioning, eyes were marked as described above, and oriented in blocks to maintain the eye polarity.

**Quantification of retinal cell types**

To collect sections from the 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 per animal), except for the p53<sup>Δ/Δ</sup>/Bmi1<sup>−/−</sup> mouse where only two eyes were available. Positive cells were counted manually using ImageJ software (NIH). 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 mounts were photographed with a 10× objective using a Zeiss microscope (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 60× objective. Three images were taken per sample, where n=4 eyes per 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 ImageJ software. Quantifications of IHC experiments were performed in the same way but using images taken with a Zeiss microscope (Observer.Z1) equipped with a 25× objective.

**Quantitative RT-PCR**

All primers were designed to flank individual exons and tested by PCR in RT<sup>+</sup> and RT<sup>−</sup> control extracts. Total RNA was isolated using TRizol reagent (Invitrogen). Reverse transcription (RT) was performed using 1 μg total RNA and the MML-V reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed using the Platinum SYBR Green SuperMix (Invitrogen) and a real-time PCR apparatus (ABI Prism 7000). GAPDH was used as an internal standard for data calibration. The 2<sup>−ΔΔCt</sup> formula was used for the calculation of differential gene expression. All experiments were performed at least in triplicate. Primer sequences are available in supplementary Materials and Methods. Primer sets for repetitive experiments were performed as described previously (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. Virus-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 started 48 h later.

Western blot
Western blots of WT and Bmi1 mutant mouse retinae were carried out using standard procedures with antibodies listed in supplementary Materials and Methods.

Statistical analyses
Statistical differences were analyzed using the Student’s r-test for unpaired samples with equal s.d. using two-tailed P-value. For ERG experiments, analysis of variance were made by one-way ANOVA followed by Bonferroni’s multiple comparisons test. For photoreceptor quantification in flat mount retinas, the analysis of variance was performed by two-way ANOVA followed by Tukey’s multiple comparison test with 95% confidence. Data are presented as means±s.d. Values are representative of at least three experiments. The criterion for significance (P-value) was set as reported in the figures.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Performed the experiments: A.B., V.P., M.A., W.C., A.F., R.H., S.Z. and J.L.


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