**Introduction**

Ultraviolet (UV) radiation from sunlight is the main cause of skin and ocular neoplasia. Brahma (BRM) is part of the SWI/SNF chromatin remodeling complex. It provides energy for rearrangement of chromatin structure. Previously we have found that human skin tumours have a hotspot mutation in BRM and that protein levels are substantially reduced. Brm−/− mice have enhanced susceptibility to photocarcinogenesis. In these experiments, Brm−/− mice, with both or a single Trp53 allele were exposed to UV for 2 or 25 weeks. In wild type mice the central cornea and stroma became atrophic with increasing time of exposure while the peripheral regions became hyperplastic, presumably as a reparative process. Brm−/−, Trp53+/−, and particularly the Brm−/− Trp53+/− mice had an exaggerated hyperplastic regeneration response in the corneal epithelium and stroma so that the central epithelial atrophy or stromal loss was reduced. UV induced hyperplasia of the epidermis and corneal epithelium, with an increase in the number of dividing cells as determined by Ki-67 expression. This response was considerably greater in both the Brm−/− Trp53+/− and Brm−/− mice indicating that Brm protects from UV-induced enhancement of cell division, even with loss of one Trp53 allele. Cell division was disorganized in Brm−/− mice. Rather than being restricted to the basement membrane region, dividing cells were also present in the suprabasal regions of both tissues. Brm appears to be a tumour suppressor gene that protects from skin and ocular photocarcinogenesis. These studies indicate that Brm protects from UV-induced hyperplastic growth in both cutaneous and corneal keratinocytes, which may contribute to the ability of Brm to protect from photocarcinogenesis.

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**Abstract**

Ultraviolet radiation (UV) from sunlight is the primary cause of skin and ocular neoplasia. Brahma (BRM) is part of the SWI/SNF chromatin remodeling complex. It provides energy for rearrangement of chromatin structure. Previously we have found that human skin tumours have a hotspot mutation in BRM and that protein levels are substantially reduced. Brm−/− mice have enhanced susceptibility to photocarcinogenesis. In these experiments, Brm−/− mice, with both or a single Trp53 allele were exposed to UV for 2 or 25 weeks. In wild type mice the central cornea and stroma became atrophic with increasing time of exposure while the peripheral regions became hyperplastic, presumably as a reparative process. Brm−/−, Trp53+/−, and particularly the Brm−/− Trp53+/− mice had an exaggerated hyperplastic regeneration response in the corneal epithelium and stroma so that the central epithelial atrophy or stromal loss was reduced. UV induced hyperplasia of the epidermis and corneal epithelium, with an increase in the number of dividing cells as determined by Ki-67 expression. This response was considerably greater in both the Brm−/− Trp53+/− and Brm−/− mice indicating that Brm protects from UV-induced enhancement of cell division, even with loss of one Trp53 allele. Cell division was disorganized in Brm−/− mice. Rather than being restricted to the basement membrane region, dividing cells were also present in the suprabasal regions of both tissues. Brm appears to be a tumour suppressor gene that protects from skin and ocular photocarcinogenesis. These studies indicate that Brm protects from UV-induced hyperplastic growth in both cutaneous and corneal keratinocytes, which may contribute to the ability of Brm to protect from photocarcinogenesis.
In this study we have examined whether Brm loss gives UV irradiated keratinocytes or corneal epithelial cells a growth advantage. We studied mice with both or only a single Trp53 allele. One of the important molecular mechanisms for protection from UV carcinogenesis is inhibition of UV-induced cell division. This provides cells more time to repair damaged DNA, reducing the incidence of UV mutagenesis, and reduces uncontrolled growth of cells. Trp53 functions in this process in part by regulating cell growth and apoptosis [11]. Therefore whether Brm loss would also affect UV-induced division of cells with only a single Trp53 allele is of interest. In mice that commenced the irradiation regime with either one or both Trp53 alleles, Brm protected from UV-induced proliferation of both epidermal keratinocytes and corneal epithelial cells.

**Materials and Methods**

**Mice**

Brm+/− and Trp53+/− mice were on a C57BL/6 background and were bred and housed at the University of Sydney animal house [7]. This study was carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes by the National Health and Medical Research Council of Australia. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Sydney (Permit Number: K14/1-2011/3/5456). All efforts were made to minimize suffering. Mice were supplied with standard chow and water ad libitum. All mice were female and 6 weeks of age at commencement of experiments. Each mouse was ear marked for identification and tested for Brm and Trp53 gene status by PCR in order to establish the genotype of each mouse. Examples and technical details of genotype determination are shown in Figure S1 in File S1. The Brm+/− mice used in our studies have been shown to lack functional BRM protein [12]. The Trp53+/− mice we used in these studies have been shown to express about half of the protein levels found in wild-type cells [13].

**UV irradiation**

A custom built bank of fluorescent tubes consisting of 4 UVA tubes (Philips, CLEO 80w-R, Netherlands) and 2 UVB tubes (Oliphant FL40SE, Oliphant-UV, Adelaide, S.A.) was used for irradiation. Monitoring of spectral intensity was as previously described [7]. Irradiated and un-irradiated groups of mice were shaved weekly on their dorsal trunk. The irradiation source consisted of 0.6% UVC (280–290 nm), 8.6% UVB (290–320 nm) and 90.8% UVA (320–400 nm). The UV dose is reported as the UVB component only but contained the appropriate amount of the other wavebands. An incremental irradiation protocol was used to avoid sunburn while maintaining a damaging dose as the skin adapted to the repeat exposures [14]. Week 1, 250 mJ/cm² Monday and Friday; week 2, 250 mJ/cm² Monday, Wednesday and Friday; week 3, 300 mJ/cm² Monday, Tuesday, Thursday and Friday; week 4, 400 mJ/cm² on the same days, weeks 5–25, 500 mJ/cm² on the same days.

**Tissue sampling, preparation and measurement of epidermal or corneal epithelium thickness**

Eyes and irradiated dorsal trunk skin were removed from euthanized mice 24 h after the final irradiation. Where possible both eyes were removed but in some cases one eye was damaged during removal and was excluded from the study. The tissues were fixed in 10% neutral formalin (skin for 24 h, eyes for 48 h) before transfer into 70% ethanol, and left for at least 24 h before processing. Tissues were embedded in paraffin and sections (skin 5 μm, eye 4 μm) cut onto coated glass slides (Superfrost Plus, Lobm Scientific Pty Ltd. Sydney, Australia). Slides were dried overnight at 45°C. Hematoxylin and eosin stained sections (Survgpath Leica Biosystems, Richmond IL, USA) were scanned using the Aperio Scanscope XT (Aperio Technologies, Sandigo, USA). ImagePro viewing software was used for the measurement of epidermal and corneal epithelial thickness in 10 and 5 (respectively) randomly selected 200x magnification fields. Counts in the corneal epithelium were performed between limbus to limbus in transverse tissue sections. The observer was masked during analysis.

**Immunohistochemistry**

Sections from the paraffin blocks were incubated at 60°C for 30 min, then de-waxed by immersion in xylene, then through a series of ethanol baths, 100%, 95% and 70%. Antigen retrieval was performed by microwaving tissues in citrate buffer (pH 6) for 12 min. Endogenous peroxidase was blocked by incubating sections in 0.3% H2O2 in methanol for 10 min at room temperature and this was followed by blocking for non-specific protein binding by incubation for 20 min in 10% normal goat serum in tris-buffered saline (TBS). All antibodies were diluted in TBS. Rabbit polyclonal antibody to murine Ki-67 (15580, Abcam, Cambridge, MA, USA) was used at 1:1000 dilution was added to the sections and incubated at room temperature for 60 min followed by rinsing in TBS-Tween 20 (0.05% Tween 20; Sigma, St Louis, MO, USA) wash buffer thrice over 2 min. Biotinylated goat anti-rabbit IgG (Vectorstain Elite ABC kit [rabbit IgG] in TBS) was added for 30 min at room temperature followed by rinsing thrice over 2 min with TBS-Tween 20. The chromagen (Impact Nova red solution, Vector Laboratories Cat no SK-4905, Burlingame, CA, USA) was then added for 11 min to visualize the immunoreactivity. The slides were then rinsed in water for 2–5 min, dehydrated through ethanol to 100% ethanol, and cleared in xylene x 2 for 5 min. Specificity controls included omitting the primary antibody and a control rabbit IgG (Dako, Glostrup, Denmark). Digital images were taken of the stained sections using an Olympus BX51 microscope (Olympus, Melbourne, Australia). The number of positive cells was counted from digital images and the length of each section determined using Image J64 (Wayne Rasband, National Institutes of Health, USA) for expression of data as positive cells per mm tissue length. For skin, at least 15 digital photomicrographic images for each mouse were assessed and a mean of these used as the value for that mouse. For the eyes, at least 5 random images per eye were assessed. The images were analysed in a blinded manner.

**Data Analysis**

Data was analysed using one-way ANOVA with Holm-Sidak’s multiple comparison test (Prism 6; Graphpad Software Inc., San Diego, CA, USA). A P value of <0.05 was regarded as significant and data for individual mice is shown in the figures. Data descriptions in the text are means and SEM or differences in means between data sets.

**Results**

**Brm** protects epidermal keratinocytes from UV-induced proliferation

**Epidermal thickness and Ki-67+ cells in mice with both Trp53 alleles**. Brm+/− and wild type mice with both Trp53 alleles intact were irradiated for 2 or 25 weeks and then examined for epidermal thickness and Ki-67+ within the epidermis (Fig. 1,
Unirradiated controls were age matched to the 2 and 25-week groups and treated identically, including shaving, except for irradiation. In the absence of UV there were no significant differences in epidermal thickness between \(Brm^{+/+}\) and wild type mice at either time. Two weeks of irradiation was not sufficient to cause epidermal hyperplasia in the wild type mice but significantly increased epidermal thickness in the \(Brm^{-/-}\) mice from a mean (SEM) of 30.44 (1.39) to 51.15 (1.37) \(\mu\)m. The difference between the 2 week irradiated \(Brm^{-/-}\) and wild type mice was statistically significant. By 25 weeks of UV, epidermal hyperplasia became evident in the wild type mice with a significant increase from 23.85 (2.44) to 34.55 (3.29). Even with this extended UV irradiation, the response in the \(Brm^{-/-}\) mice was significantly greater than in the controls. This difference was maintained at 25 weeks of irradiation with a significant increase in epidermal thickness from a mean (SEM) of 30.44 (1.39) to 51.15 (1.37) \(\mu\)m.

Ki-67 expression confirmed that UV increased the frequency of cell divisions in \(Brm^{-/-}\) murine epidermis to a greater extent than in controls (Fig. 2B). Unirradiated \(Brm^{-/-}\) epidermis had significantly greater numbers of dividing cells at the 2-week time point (increase of 14.7 cells/mm epidermis), but the genotypes were not different by 25 weeks. After 2 weeks of irradiation, the number of Ki-67+ cells was significantly increased in both wild type (increase of 14.5 cells/mm) and \(Brm^{-/-}\) (increase of 31.5) mice. The response in the \(Brm^{-/-}\) mice was statistically significantly greater than in the controls. This difference was maintained at 25 weeks of irradiation with a significant increase in both mouse genotypes but with the response in the \(Brm^{-/-}\) mice (increase of 51.3 cells/mm) being significantly greater than the controls (increase of 14.0).

**Epidermal thickness and Ki-67+ cells in mice with loss of a single Trp53 allele.** The function of p53 is frequently lost in human skin tumours, and we have previously determined the effect of \(Brm\) loss on photocarcinogenesis in mice with loss of a single \(Trp53\) allele. Therefore these experiments included mice with a single \(Trp53\) allele (\(Trp53^{+/+}\), Fig. 3A). There were no significant effects of \(Brm\) loss on epidermal thickness in un-irradiated mice at either time. Two weeks of UV did not induce significant epidermal hyperplasia in either \(Brm^{+/+}\) \(Trp53^{+/+}\) or \(Brm^{-/-}\) \(Trp53^{+/+}\) mice. However 25 weeks of UV caused a significant increase in both strains. The epidermal thickness after 25 weeks of UV in the \(Brm^{-/-}\) \(Trp53^{+/-}\) mice (85.08±12.79 \(\mu\)m) was significantly greater than the \(Brm^{+/+}\) \(Trp53^{+/-}\) mice (57.70±4.76 \(\mu\)m).

Ki-67 expression in the epidermis of unirradiated \(Brm^{-/-}\) \(Trp53^{+/-}\) mice was not significantly different to the \(Brm^{+/+}\) \(Trp53^{+/-}\) group at either the 2 or 25-week time points. UV for 2 weeks significantly increased the number of Ki-67+ cells in \(Brm^{-/-}\) \(Trp53^{+/-}\) mice (increase of 54.7 cells/mm) but not in the \(Brm^{+/+}\) \(Trp53^{+/-}\) mice and there was a significant difference between the genotypes. By 25 weeks of irradiation both genotypes developed significant increases, but the number of Ki-67+ cells was significantly higher in \(Brm^{-/-}\) \(Trp53^{+/-}\) (increase of 51.6 cells/mm) compared to \(Brm^{+/+}\) \(Trp53^{+/-}\) mice (increase of 39.8) (Fig. 3B). Therefore even with loss of a single \(Trp53\) allele,
additional loss of Brm increased the number of keratinocytes that proliferated in response to UV.

**Supra-basal dividing cells in epidermis.** In the 25 week irradiated groups there was a large number of Ki-67+ cells more than 2 cells above the epidermal base. All 4 irradiated genotypes had significantly higher numbers of supra-basal Ki-67+ cells than the corresponding unirradiated genotype (Fig. 4). The irradiated Brm−/− Trp53+/+ group was significantly higher than the Brm+/+ Trp53+/+ group. This indicates that Brm loss enabled cell division to occur in more differentiated cells further from the basement membrane.

**Brm protects corneal epithelial cells from UV-induced proliferation**

We also examined the cornea as this is the other external surface directly exposed to UV. In addition to the 2 and 25 week irradiation groups that were analysed, a small number of wild type (Brm+/+ Trp53+/+) mice were irradiated for different times enabling the development of eye pathology to be documented. A detailed description can be found in the supporting information (File S1) and is summarized here. No histopathological changes to the cornea were observed after 2 weeks of UV. At 5 weeks the epithelium displayed minor changes centrally that led to central atrophy and parakeratosis. Eventually central ulceration occurred in a third of the eyes examined while peripheral regions of corneal epithelium were hyperplastic often containing metaplastic foci.

Histological changes resulting from 25 weeks of UV exposure were compared between the genotypes (Table 1). Loss of Brm (Brm−/− Trp53+/+) increased the number of eyes showing no discernable damage with a corresponding decrease in the number of eyes with atrophy or regions of ulceration. Hyperplasia and dysplasia were increased. Loss of Brm increased regeneration or corneal epithelial division in response to UV. Damage was obvious
in all eyes of mice with loss of a single Trp53 allele (Brm−/−, Trip53+/−), but with higher levels of metaplasia, hyperplasia and dysplasia compared to the wild type mice. There was decreased atrophy but a similar level of ulceration to the wild type mice occurred. Hence, similarly to Brm2/2, the Trip53+/2 mice had increased regeneration of epithelial cells so that atrophy was present in less of the eyes examined. The Brm2/2 group contained greater numbers of eyes with hyperplasia or dysplasia but displayed similar levels of atrophy to the Brm−/− or Trip53+/− mice. The pattern of ocular change was consistent with Brm loss on either a Trip53+/+ or Trip53+/− background increasing UV-induced proliferation. Consequently these findings were investigated in more detail.

**Corneal thickness and Ki-67+ cells in mice with both Trp53 alleles**

There were no significant differences in corneal thickness between the unirradiated genotypes at either time point, or after 2 weeks exposure (Fig. 5A). However, after 25 weeks of UV there was a significantly increased corneal thickness in Brm−/− (increase of 14.2 μm above unirradiated group) but not Brm+/+ mice (increase of 1.7). This statistically significant difference (Fig. 5A) between the irradiated genotypes was consistent with the histopathological observations of reduced atrophy and increased hyperplasia in the Brm−/− mice.

Ki-67+ cell numbers confirmed that Brm regulates corneal epithelial cell division induced by UV (Fig. 5B, 6). There were no differences between the un-irradiated genotypes at either time point. Similarly to corneal thickness, Ki-67+ cell numbers were not altered in either genotype by exposure to 2 weeks of UV. Irradiation for 25 weeks significantly increased the number of Ki-67+ cells in both genotypes with the response in Brm−/− mice (increase of 20.2 cells/mm above the un-irradiated group) significantly greater than in Brm+/+ mice (increase of 9.0). This is consistent with the histopathology assessment (Table 1) that loss of Brm increased proliferation/regeneration of epithelial cells after 25 weeks of UV.
Corneal thickness and Ki-67+ cells in mice with loss of a single Trp53 allele

There were no significant differences between genotypes for any un-irradiated or UV irradiated groups for either corneal thickness (Fig. 7A) or Ki-67+ cells (Fig. 7B) in mice with loss of a single Trp53 allele. UV exposure for 25 but not 2 weeks caused a significant increase in both parameters for both genotypes (increased corneal thickness of 21.8 µm for Brm+/+ Trp53+/− and 26.22 µm for Brm−/− Trp53+/− mice, increased Ki-67+ cells/mm of 26.2 for the Brm+/+ Trp53+/− and 29.5 for the Brm−/− Trp53+/− mice).

Supra-basal dividing cells in corneal epithelium

Many of the Ki-67+ cells in the 25 week irradiated groups were more than 2 cells above the base of the corneal epithelium. Therefore these supra-basal Ki-67+ cells were counted (Fig. 8). For mice with both Trp53 alleles intact, the UV irradiated Brm−/− but not the Brm+/+ group was significantly higher than the corresponding un-irradiated genotype (Fig. 8). Loss of a single Trp53 allele resulted in both the Brm+/+ Trp53+/− and the Brm−/− Trp53+/− irradiated groups being significantly higher than the un-irradiated controls. This indicates that Brm loss enabled cell division to occur in a more disorganized manner higher in the corneal epithelium.

Brm protects corneal stromal cells from UV-induced proliferation

Analysis of the skin sections for UV-induced histopathological changes in the dermis did not reveal any obvious differences in cell content or structure between the genotypes. The expected changes

|                | Brm+/+ | Brm−/− | Brm+/+ | Brm−/− |
|----------------|--------|--------|--------|--------|
| No change      | 0%     | 25%    | 0%     | 0%     |
| Atrophy        | 80%    | 38%    | 43%    | 38%    |
| Ulcer          | 33%    | 0%     | 29%    | 25%    |
| Metaplasia     | 40%    | 25%    | 71%    | 50%    |
| Hyperplasia    | 20%    | 38%    | 50%    | 63%    |
| Dysplasia      | 0%     | 13%    | 7%     | 25%    |
| Resident Stroma: 25% or more | 13% | 13% | 14% | 63% |
| Resident Stroma: Minimum | 53% | 88% | 86% | 38% |
| Resident Stroma: None (complete necrosis) | 33% | 0% | 0% | 0% |
| Vascular proliferation beyond expected reparative FV1 | 0% | 0% | 0% | 50% |
| Reparative Fibrovascular tissue (FV)2 | 60% | 75% | 21% | 25% |
| FV entered &/or breached central zone | 40% | 25% | 79% | 75% |
| Excessive with large luminal vessels3 | 0% | 13% | 57% | 50% |
| Excessive vascular proliferation3 | 0% | 13% | 50% | 50% |

Table 1. Histopathological assessment of eyes in mice irradiated with UV for 25 weeks.
in response to UV were not noticeably different between the genotypes.

Histopathological examination of corneal stroma in the 2 week UV group showed scattered loss of cells but no other noticeable damage. There were no significant differences between the unirradiated Brm+/+Trp53+/+ and Brm−−/−Trp53+/+ groups, or between the unirradiated Brm+/+Trp53+/+ and Brm−−/−Trp53+/− groups (Fig. 9). UV statistically significantly reduced the number of stromal cells in Brm−−/− (UV-induced reduction of 21.3 cells) but not Brm+/+ mice (non-significant decrease of 11.9 cells) with both Trp53 alleles intact although there was no significant difference between these genotypes. The irradiated Brm−−/−Trp53+/− group had significantly fewer stromal cells (significant UV-induced reduction of 33.0 cells) than the Brm+/+Trp53+/− mice (non-significant decrease of 5.5 cells). This therefore suggested that in the stroma, Brm protected from 2 weeks of UV-induced damage. Eye damage was noticed in the corneal stroma at an earlier time than in the corneal epithelium.

The time course for histopathologically observed damage to the corneal stroma in wildtype mice is described in supporting information (File S1). In summary, initially there was loss of cells and oedema. With time this resulted into centrally positioned collagenolytic clefts and voids. Simultaneously fibrovascular proliferative twigs formed and extended presumably by migration towards the centrally damaged zones. The proliferative fibrovascular tissue often had a sprinkling of inflammatory reactive cells (Fig. 10).

Stroma in the different genotypes exposed to UV for 25 weeks were compared (Fig. 11; Table 1) and appeared to show a reparative program to match that of corneal epithelium. While the UV exposed Brm−−/− mice contained fewer stromal cells at 2 weeks as described above, both Brm−−/− and Trp53+/− mice

**Figure 5. Brm protects corneal epithelial cells from UV-induced proliferation.** Groups of Brm wildtype (+/+, closed circles) or knockout (−/−, closed triangles) mice on a Trp53 wildtype background were exposed to UV radiation for 2 or 25 weeks. Unirradiated controls were age matched to the 2 or 25 week irradiated groups. Eyes were removed and (A) corneal epithelial thickness, (B) Ki-67+ cells in the corneal epithelium were determined. Each point represents a single mouse. Where both eyes were available for a mouse the mean of the two eyes is used. The numerical P values centred between wildtype and knockout groups indicate statistical comparison between these genotypes for each treatment (n.s. - not significant; ANOVA). Significance of UV effect compared to relevant unirradiated group shown as *(P<0.05) or **(P<0.001). Lack of indication of statistical difference indicates not significant (ANOVA). Mean shown as horizontal line.

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had an increased regenerative response by 25 weeks. While 33% of wild type mice had complete necrosis of the stroma by 25 weeks, all mice from the other 3 genotypes had at least minimal stroma retained and more excessive vascular proliferation. Furthermore, 50% of eyes from the \( \text{Brm}^{2/-} \) and \( \text{Trp53}^{+/2} \) group had greater than expected vascular proliferation.

The stroma of \( \text{Brm}^{2/-} \) and \( \text{Trp53}^{+/2} \) mice was the least damaged of the other genotypes. The original caudal stroma remained present and this genotype contained the largest number of eyes with more than 25% of the stroma remaining. Reparative vascular proliferation was excessive (beyond the wild type) in 50% of the \( \text{Brm}^{2/-} \) and \( \text{Trp53}^{+/2} \) mice, which was greater than any other genotype. In one eye there was a more aberrant vascular formation nearby to which were a few megalocytic cells. In one eye the neo-angiogenesis was most proliferative. Megalocytes were sprinkled in these eyes and also observed in the eye with a haemangioma.

In summary, the pattern in the stroma was that both \( \text{Brm}^{2/-} \) and \( \text{Trp53}^{+/2} \) mice, and particularly the combined knockout had increased reparative processes, which included the fibrovascular response resulting in reduced loss of stromal cells. This is consistent with both of these genes being protective from proliferative responses that occur as a result of UV radiation. Thus in the absence of either \( \text{Brm} \) or \( \text{Trp53} \), an increased proliferation/reparative process lead to lower levels of central necrosis and increased fibrovascular responses.

**Discussion**

Our recent studies indicate that \( \text{Brm} \) is a tumour suppressor gene that protects from UV-induced skin and ocular carcinogenesis. \( \text{Brm} \) knockout mice have increased sensitivity to both skin and ocular photocarcinogenesis [7]. Unexpectedly the \( \text{Brm}^{2/-} \) mice were resistant to UV-induced immunosuppression, which would be expected to protect from photocarcinogenesis indicating that different mechanisms are involved. In human skin cancers, the \( \text{BRM} \) gene contains a hotspot mutation that was predicted to affect function [5], and protein expression is decreased compared to normal skin [6]. In the present study we show that \( \text{Brm} \) protects from UV-induced proliferation of keratinocytes and ocular epithelial cells even when one \( \text{Trp53} \) allele is knocked out. Additionally \( \text{Brm} \) reduces the proliferative response of stromal cells in the UV-irradiated eye. The greater reparative response in the stroma of UV-irradiated \( \text{Brm}^{2/-} \) mice may contribute to the higher proliferation of epithelial cells and provide greater support to development of ocular neoplasia. These events may partially explain the functional role of \( \text{Brm} \) in protecting from skin and ocular cancer.

The Ki-67 antigen, a high molecular weight non-histone protein, is generally accepted to be a reliable marker of many types of proliferating cells [15] [16]. It is a nuclear structure expressed during all phases of the cell cycle (G1, S, G2 and mitosis) but not by cells that are failing to undergo division. While Ki-67 redistributes throughout the nucleus and cytoplasm during cell division, it appears to be required for progression through the cell cycle [17] [16] [18]. The number of Ki-67 expressing keratino-
cytes and corneal epithelial cells was higher in irradiated Brm \(-/-\) mice than in irradiated Brm+/+ controls. This was observed with as little as 2 weeks of UV in keratinocytes. UV-induced cell proliferation remained elevated after 25 weeks of UV in both the skin and eye, which is at about the time that skin and ocular tumours commenced to appear in our photocarcinogenesis study using mice of these genotypes [7]. Furthermore, in both keratinocytes and corneal epithelial cells, UV-induced proliferation was more disorganized in the Brm \(-/-\) mice, with substantial numbers of proliferating cells in the suprabasal region. In unirradiated, and even UV irradiated wild type mice, proliferation was largely restricted to basal cells. Examination of epidermal and corneal thickness gave consistent data to assessment of Ki-67+ cells showing that Brm protects from UV-induced hyperplasia of the respective tissues. Therefore Brm protects from UV-induced proliferation during the entire time course of photocarcinogenesis without any indication of adaptive mechanisms capable or compensating for loss of Brm.

Similar findings were observed in mice with both wild type Trp53 alleles intact and also in mice with loss of a single Trp53 allele. This suggests that even with partial loss of p53 function, Brm protects from UV-induced cellular proliferation.

Thickening of the corneal stroma was also evident in UV irradiated Brm \(-/-\) mice. Initially, after 2 weeks of UV, there was increased UV-induced damage in the Brm \(-/-\) mice as indicated by the more severe loss of central corneal stromal cells. With increasing time, the central corneal stromal cells were also lost from Brm+/+ mice as previously reported for 129 mice [19], and the damage to this region of the eye became more pronounced with increasing time of irradiation. Either this loss, or chronic UV for 25 weeks then caused an enhanced reparative or proliferative fibrovascular response arising from peripheral zones. The Brm \(-/-\) mice, with either both or a single Trp53 allele had an
even greater peripheral reparative response resulting in protection of the central resident stroma. Thus Brm also appears to protect the stroma from UV-induced hyperproliferation or excessive regeneration. The enhanced proliferative response in UV-irradiated stroma of the eye may contribute to the enhanced ocular photocarcinogenesis in these mice. This stromal response may even contribute to the hyperproliferation observed in corneal epithelial cells by the production of growth factors or by some other cell to cell mechanism.

Brm loss has also been shown to enable hyperplasia in prostate cells, which was associated with the cell cycle regulator E2F1 [20]. The liver cells of Brm−/− mice proliferate at an enhanced rate indicative that this gene regulates hepatic cellular proliferation consistent with this previous report that Brm regulates cell cycle machinery have been reported to enable Brm−/− murine fibroblasts to maintain relatively normal control of the cell cycle under normal growth conditions. However the steps are less tightly regulated so that the stress of serum starvation alters re-entry into the cell cycle upon serum stimulation [21].

Following irradiation with 1 mJ/cm² UV from a Stratalinker 2400 (Stratagene), Brm−/− MEFs proliferated at a faster rate than MEFs from wild type mice [12]. In these experiments the UV exposure substantially decreased proliferation compared to unirradiated cells, with the inhibitory effect less substantial in the Brm−/− MEFs. This differs from our data, which found UV to enhance, rather than inhibit proliferation compared to unirradiated cells. There could be many reasons for this discrepancy. We performed chronic irradiation of whole mice as opposed to a single UV exposure to cultured cells. We examined different cell types that are on the surface of the body and therefore usually exposed to UV from sunlight. We also used a different UV irradiation source. The Stratalinker was used to induce DNA damage as it is used for UV crosslinking of DNA and RNA. It has a maximal output at 254 nm, which is within the UVC range. Our UV source emitted 90% UVA with a peak irradiance at 360 nm and did not emit any wavelengths as low as 254 nm. Consequently, the biological effects of these two UV sources are very different, and up to 500 fold different UV doses were used in our studies. Despite these differences our data is fundamentally consistent with this previous report that Brm regulates cell proliferation after chronic UV exposure.
proliferation and that Brm knockout gives cells a proliferative advantage following exposure to UV. However our studies contrast with the finding of decreased survival resulting from knockout of the Brm analogue psa-4 in the nematode Caenorhabditis elegans (C. elegans) exposed to UVB [22]. The reason for this is not obvious, but it may be due to differences in sensitivity to UVB between C. elegans and mammalian keratinocytes. C. elegans were killed by the very low dose of 12–16 mJ/cm² UVB, while even doses of 4,000 mJ/cm² UVB have little effect on viability of human keratinocytes [23].

Cell division prior to repair of DNA damage can lead to a mutation. Therefore the increased cell division seen in these studies in Brm−/− mice would reduce the time available for repair of UV damaged DNA. Additionally, cell division in the UV-irradiated Brm−/− mice occurred at higher levels in both the epidermis and corneal epithelium. These more superficial suprabasal positioned cells would be exposed to higher amounts of UV than the more protected cells at the basement membrane and therefore could be particularly susceptible to UV-induced mutagenesis. These events could contribute to the augmented photocarcinogenesis observed in our previous study [24]. Chromatin remodeling by SWI/SNF is important for efficient repair of damaged DNA [3]. Downregulation of BRM has been shown to decrease repair of DNA damaged by the genotoxic agent cisplatin in human cancer cells [25]. Additionally, Brm−/− mice responding to serum stimulation following serum starvation display genetic instability with frequent loss of chromosome stability [21]. It is not known whether loss of Brm also inhibits repair of UV-induced genetic damage. However the combination of DNA repair defects and UV-induced hyperproliferation of keratinocytes and corneal epithelia cells would be expected to enable an increased frequency of UV-induced DNA damage becoming fixed into mutations. These molecular events are likely to be responsible for the enhanced photocarcinogenesis that we

Figure 10. Photomicrographs representing the histological features of the eyes from 25 week unirradiated and UV irradiated Trp53+/+Brm+/+ mice. Sections stained with H&E. Cranial synechia containing proliferating iris fibrovacular tissue, with melanin containing cells. The UV irradiated tissue had oedema which was marked particularly under the epithelium. doi:10.1371/journal.pone.0107931.g010
have observed in \( Brm^{-/-} \) mice. These events would also give a growth advantage to those tumour cells that have lost \( Brm \) function so that they would outgrow tumour cells with normal \( Brm \) function.

Previously we have found that \( Brm \) protects from UV-induced skin and ocular carcinogenesis. To make these experiments relevant to human skin and ocular cancer, we used a UV spectrum that simulated sunlight and chronic exposure with doses that did not cause excessive sunburn damage. This therefore mimics the exposure of humans to sunlight during their normal daily activities. Our goal was to examine the molecular events by which \( Brm \) protects from photocarcinogenesis. We had previously shown that \( Brm^{-/-} \) does not affect UV induction of apoptotic sunburn cells [7] and therefore examined markers of cell proliferation. The absence of \( Brm \) increased UV-induced keratinocyte and corneal epithelial cell proliferation. This occurred in mice that started the experiments with both or only a single \( Trp53 \) allele in order to mimic the loss of this important tumour suppressor gene in human UV carcinogenesis. \( Brm \) protection from UV-induced cellular hyperproliferation could contribute to its ability to protect from photocarcinogenesis.

Figure 11. Photomicrographs representing the histological features of the eyes from 25 week unirradiated and UV irradiated \( Trp53^{+/+} Brm^{-/-} \) mice. Sections stained with H&E. The UV irradiated tissue had odema which was marked particularly under the epithelium. The unirradiated photomicrograph does not have additional labels as they would be similar to the unirradiated \( Trp53^{+/+} Brm^{+/+} \) figure.

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Supporting Information

File S1 Genotyping of individual mice. Examples of PCR gels with the expected PCR products obtained during genotyping of individual mice. Each mouse was genotyped. The allele being detected is shown as heading to each panel with the expected band size. The genotype of the mouse is shown below each lane. HyperLadder II DNA Ladder (Bioline (Aust) Pty Ltd, Sydney, Australia) was used (M). Genomic DNA isolated from mouse tails PCR-amplified with specific primers. The PCR products were resolved on 1.5% AMRESCO agarose (Astral Scientific Pty Ltd, Sydney, Australia) and then visualized by SYBR Safe DNA Gel (Invitrogen, Carlsbad, CA, USA) staining.

Author Contributions
Conceived and designed the experiments: GMH JGL NDG. Performed the experiments: NH NP CRH AF. Analyzed the data: GMH NH JGL. Contributed reagents/materials/analysis tools: GMH NDG CRH JGL. Contributed to the writing of the manuscript: GMH NH JGL NDG CRH.

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