Highlights

- Chronic exposure to below-threshold doses of UV-B radiation induce cataract in mice
- Both wild-type and *Epha2* heterozygous mice develop UV-B dose-dependent cataract
- Recovery time after UV-B exposure and *Epha2* genotype influence cataract severity
- Lens epithelial cell proliferation underlies UV-B induced cataract in mice
- Partial *Epha2* deficiency favours against UV-B induced ongoing damage to the lens

***Epha2* genotype influences ultraviolet radiation induced cataract in mice**

Short title: UV-B and *EPHA2* interaction in cataract

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Abstract

Age-related cataract is the major cause of blindness worldwide. Both genetic and environmental factors contribute to the disease. Genetic variation in the *Ephrin type-A receptor 2* (*EPHA2*) gene is associated with the risk of age-related cataract in multiple populations, and exposure to ultraviolet-B (UV-B) radiation is a well-established risk factor for the disease. *Epha2* knockout and UV-B radiation independently lead to cataract in mice, and UV-B radiation reportedly alters *EPHA2* expression in cultured cells. We hypothesised that an interaction between UV-B radiation exposure and *Epha2* signalling may influence cataract development. To test this hypothesis, 5-week-old *Epha2*^{+/+} and *Epha2*^{+/-} mice (n=8 per group) were exposed to repeated below-threshold doses of UV-B radiation (0.0125 to 0.05 J/cm²), before development of *Epha2*-mediated cataract. Cataract development was monitored after termination of exposure and at least one month later. Histological analysis of exposed and unexposed lenses was performed to assess pathological changes, and gene expression analysis to investigate the mechanism underlying cataract. Both *Epha2*^{+/+} and *Epha2*^{+/-} mice developed UV-B dose-dependent anterior polar cataract; cataract severity in both genotypes of mice exposed to either 0.025 or 0.05 J/cm² UV-B was significantly higher than that in matched unexposed mice ($p<0.05$). Histological analysis of lenses of both genotypes of mice exposed to 0.025 or 0.05 J/cm² UV-B radiation consistently revealed disruption of the lens architecture. A month after the exposure, cataract severity increased in *Epha2*^{+/+} mice treated with the highest dose of UV-B radiation ($p=0.03$) but remained unchanged in *Epha2*^{+/-} mice. Gene expression analysis of lenses of both genotypes of mice showed significant upregulation of the cell proliferation marker *Mki67* in *Epha2*^{+/+} ($p=0.036$) but not in *Epha2*^{+/-} mice exposed to the highest dose of UV-B radiation compared to matched unexposed mice. In conclusion, this study suggests that repeated exposure to doses of UV-B radiation lower than the single minimum dose required for

inducing cataract leads to cataract in wild-type and *Epha2* heterozygous knockout mice. Furthermore, this study indicates, for the first time, a potentially favourable effect of partial *Epha2* deficiency against UV radiation-induced damage in the lens.

1.1 Introduction

Cataract presents rarely at birth (congenital cataract), or during early childhood, but is more common after the age of ~45 years (age-related cataract) (Shiels and Hejtmancik, 2007). Age-related cataract accounts for more than 40% of cases of blindness worldwide, mostly in developing countries (Abraham et al., 2006, Lee and Afshari, 2017). A complex interaction of genetic and environmental factors is thought to contribute to the disease risk (Abraham et al., 2006). Ultraviolet (UV) radiation exposure, mainly from sunlight, is one of the well-established risk factors for developing age-related cataract (West and Valmadrid, 1995, Abraham et al., 2010, McCarty et al., 2000).

UV radiation reaching the earth comprises UV-A (400-315 nm), UV-B (315-280 nm) and UV-C (280-100 nm) (Michael, 2000). All UV-C and most UV-B is absorbed by the ozone layer, while some UV-B and all of the UV-A spectrum reach ground level (Michael, 2000, Lucas, 2011, Modenese et al., 2018). A large proportion of UV-B reaching the human eye is absorbed by the cornea and the lens absorbs the remainder (McCarty and Taylor, 2002), whereas a small amount of UV-A entering the eye is absorbed by the cornea and some by the lens, the majority reaches the retina (Lucas, 2011). Exposure to UV-B has been associated with age-related cortical cataract development in humans but UV-A has not been considered cataractogenic (West et al., 1998, McCarty et al., 2000, Taylor et al., 1988). However, some studies support a role of UV-A in cataract development in humans (Roberts, 2011). Regardless of whether UV-A or UV-B contributes to cataract, population-based studies in countries such as the U.S.A., Australia, France and Iceland have associated day-to-day UV radiation exposure with increased risk of cataract development (Cruickshanks et al., 1992, McCarty et al., 2000, Delcourt et al., 2000, Katoh et al., 2001). Exposure to UV radiation can also lead to cataract in animals. However, due to differences in corneal thickness and general anatomy of the eye, the amount of UV radiation transmitted to the lens is different in animals

compared to humans (Dillon et al., 1999). Despite these differences, animals have been used as models to understand the effect of UV radiation exposure on the ocular lens and on cataract development. Rodent models have been used to investigate the doses of UV radiation sufficient to cause cataract and mechanisms of damage to the crystalline lens (Galichanin et al., 2012, Soderberg, 1990, Michael et al., 1998b, Michael et al., 2000). In rodents, similar to humans, UV-B is more cataractogenic than UV-A (Wegener, 1994).

The majority of the studies in rodents have demonstrated damage to the ocular lens caused by a single exposure to an *above-threshold* dose of UV-B (Michael, 2000, Galichanin et al., 2010, Ayala et al., 2000b, Galichanin et al., 2012), with threshold dose being the minimum dose sufficient to cause cataract. These studies are useful indicators of the effect of exposure to relatively higher doses of UV radiation on the ocular lens experienced by certain individuals due to their professions such as welders, radiation workers and farmers. Most humans experience repeated exposure to lower levels of UV radiation from the atmosphere. To date, only a few studies have investigated the effects of repeated below-threshold doses of UV-B radiation on the lens (Galichanin et al., 2014, Wegener, 1994) that are relevant to the human population.

Genetic factors also influence age-related cataract development in humans. Mutations or common variants in genes including *Galactose-1-phosphate uridylyltransferase (GALT)*, *Heat shock factor 4 (HSF4)*, *Crystallin alpha A (CRYAA)*, *Potassium voltage-gated channel subfamily A member regulatory beta subunit 1 (KCNAB1)*, *Superoxide dismutase 1 (SOD1)*, *Xeroderma pigmentosum complementation group D (XPD)* and *Idoleamine 2,3 dioxygenase (IDO)* have been linked with age-related cataract in certain populations but these findings are yet to be replicated in other populations (Shi et al., 2008, Bhagyalaxmi et al., 2009, Karas et al., 2003, Zhang et al., 2011, Mamata et al., 2011, Padma et al., 2011, Liao et al., 2014). Only genetic variation in the *Ephrin type-A receptor 2 (EPHA2)* gene has been reproducibly

associated with age-related cataract in humans in multiple populations (Jun et al., 2009, Shiels et al., 2008, Zhang et al., 2009). Variants in this gene are associated with the risk of cortical, nuclear and posterior subcapsular cataract in different populations (Jun et al., 2009, Sundaresan et al., 2012, Shiels et al., 2008). Additionally, highly penetrant mutations in the *EPHA2* gene cause congenital cataract (Kaul et al., 2010, Dave et al., 2013, Zhang et al., 2009, Shentu et al., 2013, Shiels et al., 2008). *EPHA2* belongs to the Eph receptor tyrosine kinase family (Pasquale, 2008). These molecules interact with Ephrin ligands to bring about cell signalling. This signalling plays an important role in cell adhesion and repulsion, cell survival, cell migration and epithelial to mesenchymal transformation. *Epha2* is expressed in epithelia during development and throughout life and is involved in maintaining epithelial homeostasis. It is upregulated in various types of tumors and is a target for drug development. Apart from its involvement in cataract in humans, knockout of the orthologous *Epha2* gene in mice leads to development of age-related cortical cataract (Jun et al., 2009, Shi et al., 2012, Cheng et al., 2013). Thus *EPHA2* is an important gene in cataract, and *Epha2* signalling is important for maintaining transparency of the mammalian lens.

Interestingly, UV radiation treatment has been reported to increase *Epha2* expression levels in human and mouse melanocytes, keratinocytes and embryonic fibroblast (MEF) cells (Zhang et al., 2008) indicating that UV radiation interacts with the *EPHA2* gene and possibly *Epha2* signalling. Thus we hypothesised that an interaction between UV exposure and *Epha2* signalling may influence cataract development. In the present study, we tested this hypothesis using the *Epha2* knockout mouse model in the context of repeated exposures to below-threshold doses of UV-B radiation, relevant to the human population.

1.2 Materials and Methods

1.2.1 Ethics statement

All experimental procedures in animals and post-procedural animal welfare conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) and were approved by the Animal Welfare Committee, Flinders University, Adelaide, Australia (Approval number 796/11). All eye examinations were performed under anaesthesia.

1.2.2 Animals

Epha2 knockout mice (B6; 129P2-*Epha2*^{Gt(KST085)Byg}; KST085) on C57BL/6J background were purchased from the Mutant Mouse Regional Resource Center, University of Missouri, U.S.A. The mice were generated using the secretory trap method (Leighton et al., 2001, Mitchell et al., 2001). *Epha2*^{+/+} and *Epha2*^{+/-} mice used for this study were obtained from crosses between *Epha2*^{+/-} × *Epha2*^{+/-}, *Epha2*^{+/-} × *Epha2*^{+/+}, *Epha2*^{+/+} × *Epha2*^{+/+} and *Epha2*^{-/-} × *Epha2*^{+/+} mice.

1.2.3 Genotyping

The 129P2 strain, used for generation of the *Epha2* knockout mouse, carries a deletion mutation in the *Beaded filament structural protein 2* (*Bfsp2*) that is known to cause anterior polar cataract (Sandilands et al., 2004, Alizadeh et al., 2004). The cataract-causing allele was eliminated by polymerase chain reaction (PCR)-based *Bfsp2* genotyping in the first two generations and selecting non-mutation carrier mice as breeders for colony maintenance.

For genotyping the *Bfsp2* mutation, genomic DNA from mouse tail tip or ear notch was extracted in lysis buffer (0.1 M Tris-HCl pH 8.3, 0.2 M NaCl, 0.005 M EDTA, 0.2% SDS,

100 µg/ml Proteinase K), precipitated using isopropanol and resuspended in MilliQ water. Genotyping was performed as previously described (Alizadeh et al., 2004) using gene-specific primers (Table 1) and genomic DNA template. PCR was performed in the presence of Hot Star Taq *Plus* DNA polymerase (Qiagen Pty Ltd, VIC, Australia). The enzyme was activated at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 68°C for 30 seconds and extension at 72°C for 30 seconds; a post-extension cycle at 72°C for 1 minute was followed by incubation at 4°C for 5 minutes. The products were analysed by agarose gel electrophoresis and difference in size of the PCR product, 905 bp for wild-type allele and 386 bp for mutant allele, distinguished between the three genotypes. All mice used in the present work were wild-type for *Bfsp2*.

To determine the status of *Epha2* knockout in offspring, *Epha2* genotyping was performed using primers M30828F, M30828WTR and M30828MutR (Table 1) and PCR conditions described above except annealing was performed at 61°C. PCR products were analysed by agarose gel electrophoresis. A PCR product of 200 bp and 400 bp corresponding to the wild-type and mutant allele, respectively, distinguished between *Epha2*^{+/+}, *Epha2*^{+/-} and *Epha2*^{-/-} mice.

Table 1: Sequences of primers used in this study

Gene	Primer Name	Sequence 5'>3'
Genotyping		
<i>Bfsp2</i>	CP49fwd	TGGGGTTGGGCTAGAAATCTCAGA
	CP49WTR	TACTCAAAGGCAGTAGTCCCTGC
	CP49MutR	AGCCCCTACGACCTGATTTTGTGAG
<i>Epha2</i>	M30828F	CACCTCACTCAGTATAGTGCTCCA
	M30828WTR	CGTCTTCCCAGTCATACTCTACTT

	M30828MutR	CCATACAGTCCTCTTCACATCCAT
Gene expression analysis		
<i>Mki67</i>	Mki67-F	AGAGCTAACTTGCGCTGACTG
	Mki67-R	TCTTGAGGCTCGCCTTGATG
<i>Acta2</i>	mActa2-F	CCTTCGTGACTACTGCCGAG
	mActa2-R	GTCAGCAATGCCTGGGTACAT
<i>Epha2</i>	mEpha2Ex5Fwd	GAGCGTGCGCTATTCAGAAC
	mEpha2Ex6Rev	GTACTTCCACACACGGCTCT
<i>B2m</i>	mB2mqpcrFwd	ATCACCCCCACTGAGACTG
	mB2mqpcrRev	TGC TAT TTC TTC TGC GTG C

1.2.4 UV-B exposure

The UV-B exposure station used for exposing animals was designed and built in-house (Department of Biomedical Engineering, Flinders Medical Centre, Adelaide, Australia). Two 20 W UV-B broadband (290-315 nm) fluorescent lamps (Philips TL 20 W/12 RS SLV) mounted under the top shelf of a two-shelf trolley served as the UV-B radiation source. The UV-B lamps were covered with a 0.25 mm thick polytetrafluoroethylene (PTFE, Teflon) layer that attenuated 48% of UV-B to minimise corneal damage upon exposure. The UV-B radiation dose was recorded from a fixed distance (43 cm) using a UVX digital radiometer attached to the UVX series sensor (UVP Inc, CA, U.S.A.). The recorded output was logged on a 0 to 2.5 V Tiny Talk data logger (Gemini Data Loggers Ltd, West Sussex, U.K.) for calculating the duration of exposure. Exposure time and the administered dose were controlled with a timer.

For UV-B exposure, mice were individually placed in a polycarbonate animal holder and restrained with a wire mesh cover. The same mesh was placed on the UVX sensor for estimation of ‘actual’ administered UV-B radiation dose. Animals were exposed to UV-B radiation from a distance of 43 cm, placed on bottom shelf of the trolley.

The Maximum Tolerable Dose (MTD_{2.3:16}), i.e. the threshold dose of UV-B for avoidance of cataract in C57BL/6J mice, is 0.29 J/cm² (Meyer et al., 2008, Soderberg et al., 2002). The doses of UV-B radiation administered in this study (0.0125 - 0.05 J/cm²) were lower than the threshold dose (below-threshold) and the lowest administered in rodent models to date (Michael and Brismar, 2001) except the lowest daily dose administered by Galichanin et.al (Galichanin et al., 2014). Five-week-old *Epha2*^{+/+} and *Epha2*^{+/-} mice (n=8 per group) were exposed to 0.0125, 0.025 or 0.05 J/cm² of UV-B radiation twice a week, every third and fourth day, for a total of seven times. The duration of each treatment to achieve the desired dose ranged from 6-11, 13-23 and 27-46 minutes for 0.0125, 0.025 or 0.05 J/cm² UV-B, respectively. For an intended dose of 0.0125 J/cm², the actual administered dose ranged from 0.0112-0.0137 J/cm², for 0.025 J/cm² from 0.022-0.0268 J/cm², and for 0.05 J/cm² from 0.045-0.055 J/cm². To avoid gender bias, equal numbers of males and females were included in each group, except *Epha2*^{+/+} mice exposed to 0.025 J/cm² UV-B (6 males and 2 females). Similar size groups of age- and gender- matched unexposed *Epha2*^{+/+} and *Epha2*^{+/-} mice were used as controls. Cataract development was monitored 24-48 hours and 4-7 weeks after the last exposure.

1.2.5 Ophthalmic examination

Cataract grading was performed in anesthetized mice. For ophthalmic examination, mice were mildly sedated by isoflurane inhalation and then anesthetized with an intraperitoneal injection of 75 mg/kg ketamine (Lyppard Australia Pty. Ltd., SA, Australia) and 0.01 mg medetomidine (Troy Laboratories Pty. Ltd., NSW, Australia). Mydriasis was induced by topical administration of 1% tropicamide (Bausch & Lomb Pty. Ltd., NSW, Australia). Examination was performed on a photo-slit lamp biomicroscope (Topcon Medical Systems Inc., NJ, U.S.A.) in a masked fashion and cataract graded in each eye of an animal. Cortical

cataract was graded according to the Lens Opacities Classification System (LOCS) III (Chylack et al., 1993, Sparrow et al., 1986). As a standard system for grading anterior polar cataract was unavailable, we developed a grading system based on the size of anterior polar lens opacity for measuring severity of this form of cataract. Anterior polar cataract was graded on a 4-point scale from 0-3, with 0 representing no cataract, 1 mild, 2 moderate and 3 severe cataract (Fig 1). Both eyes of all mice were examined within 5-10 minutes after anesthesia. The observations were recorded by digital photography at 40× magnification using the EZ Capture software (Topcon Medical Systems Inc., NJ, U.S.A.). Three images of each eye, with 1) diffuse illumination, 2) cross-section illumination of the lens, and 3) retroillumination were captured, all at maximum flash intensity. For diffuse illumination imaging, the slit height was set at 5 with widest slit width, and the light angle was set at 35°. This enabled documentation of any cortical lens opacity. For capturing cross-section illumination of the lens, the slit height and slit width were set at 2 mm each, and the light angle was set at 45°. This showed any anterior opacity in the lens. Retroillumination image was captured with the slit height and slit width set at 5 mm each. This revealed any total lens opacity and corneal aberrations. After the first examination, mice were revived with an intraperitoneal injection of 0.05 mg atipamizole (Troy Laboratories Pty. Ltd., NSW, Australia).

1.2.6 Tissue collection and histological analysis

For tissue collection, mice were euthanized and whole eyes enucleated under an ophthalmic microscope, fixed in 10% neutral buffered formalin for 24-72 hours and paraffin embedded. Alternatively, ocular lens was dissected and snap frozen for later use. For histology, 7 µm thick serial sections were stained with hematoxylin and eosin and imaged on a Brightfield Olympus BX50 microscope equipped with QImaging RTV 5 megapixel Digital Camera

(QImaging, BC, Canada) and QCapture software (QImaging, BC, Canada) using a 20× objective.

1.2.7 Gene expression analysis

Total RNA from mouse lens was extracted using the RNeasy Mini kit (Qiagen Pty Ltd, VIC, Australia) as per the manufacturer's protocol with Proteinase K (400 µg) digestion for 10 minutes. For gene expression analysis in UV-exposed and unexposed mice, lenses of two mice of the same gender within a group were pooled for RNA extraction. Genomic DNA was removed by Ambion™ DNase I (RNase-free; Thermo Fisher Scientific Australia Pty Ltd, VIC, Australia) treatment as recommended. From 1 µg total RNA, cDNA was synthesized using Superscript III reverse transcriptase and random hexamers (Invitrogen, Life Technologies Corporation, CA, USA). Relative expression of *Mki67*, *Acta2* and *Epha2* genes in UV-B treated and untreated lenses was analysed by semi-quantitative RT-PCR; expression was normalised to *B2m* expression. Messenger RNA of each gene was amplified from cDNA template using mouse-specific primers (Table 1) and Hot Star Taq *Plus* DNA polymerase. PCR conditions were, enzyme activation at 95°C for 3 minutes, followed by cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds and elongation at 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes. Annealing temperature for amplification of *Mki67* mRNA was 65°C, for *Acta2* and *Epha2* 60°C, and for *B2m* 62°C. *Mki67*, *Acta2*, *Epha2* and *B2m* mRNA were amplified for 39, 25, 32 and 40 cycles, respectively. *Mki67* mRNA was amplified in the presence of Q solution (Qiagen Pty Ltd, VIC, Australia). Amplified products were analysed by agarose gel electrophoresis and band intensities quantified using the ImageJ software. For *Epha2* gene expression analysis in different genotypes of mice, both lenses of an animal were pooled for RNA extraction and 0.74 µg total RNA used for cDNA synthesis; *B2m* expression served as the housekeeping control.

1.2.8 Statistical analysis

Statistical analyses of ophthalmic data were performed using IBM Statistical Package for the Social Science (SPSS) Statistics 19 and Stata 14.1 (StataCorp, College Station, Texas, USA). The primary endpoints were average anterior cortical cataract and average anterior polar cataract grades in both eyes of an animal. To determine agreement of cataract grades between the two eyes of each animal Intraclass Correlation Coefficient (ICC) was calculated according to the two-way random-effects model using the *icc* function from the *irr* package in R (Team, 2013).

The effect of UV-B radiation on cataract severity in a genotype at a time-point was analysed using Mann-Whitney *U* test. The difference in cataract severity between genotypes upon UV-B exposure at each time-point was also analysed by Mann-Whitney *U* test. The difference in cataract severity between the two time-points in a genotype was analysed using Wilcoxon Signed Ranks test. No adjustment for multiple comparisons was made as the study was not powered to detect a specific effect. As the data were not normally distributed, adopting a conservative approach, the results at each time-point are presented as median and interquartile range. The results of comparison between time-points are presented as mean and standard error of the mean. Gene expression data was analysed by Student's *t*-test using the GraphPad Prism 7 software. A *p* value less than or equal to 0.05 (two-tailed) was deemed to be statistically significant.

1.3 Results

In this study, we determined the cumulative effect of repeated exposures to below-threshold doses of UV-B radiation on cataract development in wild-type and *Epha2* heterozygous knockout mice. Multiple below-threshold doses of UV-B radiation were administered

through whole-body exposure in wakeful mice to mimic repeated exposure of humans to UV radiation from sunlight. The *Epha2*^{-/-} mice on C57BL/6J background develop severe bilateral cortical cataract by 4-5 months of age and *Epha2*^{+/-} mice at a later age (to be published elsewhere). To avoid confounding effects of genetically induced cataract, *Epha2*^{+/-} mice at a younger age with clear lenses, were used for the study. Expression levels of *Epha2* between lenses of *Epha2*^{+/-} and *Epha2*^{+/+} mice were compared by semi-quantitative RT-PCR; the expression level of *Epha2* gene in wild-type mouse lens was too low for analysis by real-time RT-PCR. *Epha2*^{+/-} mice generally expressed lower levels of *Epha2* mRNA in the lens than wild-type mice (Fig 2). Thus instead of using *Epha2*^{-/-} mice that completely lack *Epha2* expression in the lens, we used *Epha2*^{+/-} mice to test our hypothesis. Both wild-type and *Epha2*^{+/-} mice were repeatedly exposed to 0.0125, 0.025 or 0.05 J/cm² of UV-B radiation and cataract development monitored as described in Section 1.2.5.

The corneas of mice exposed to 0.05 J/cm² and 0.025 J/cm² doses of UV-B radiation showed slight punctate epithelial erosion at the first ophthalmic examination that did not interfere with grading of cataract (not shown). Corneal epithelial erosion had resolved at the second eye examination, indicating repair of the original epitheliopathy. Mice exposed to 0.0125 J/cm² UV-B did not develop any observable corneal damage.

1.3.1 Dosage effect of UV-B radiation on cataract development

After termination of UV-B exposure and 4-7 weeks later, depending upon the dose of UV-B administered, wild-type and *Epha2*^{+/-} mice developed mild anterior cortical cataract with or without anterior polar cataract. Each type of cataract, cortical and polar, was separately graded and analysed at each time-point. Both unexposed mice of each genotype, and mice exposed to 0.0125, 0.025 and 0.05 J/cm² UV-B radiation developed mild cortical cataract after the final exposure and 4-7 weeks later (Table 2). The difference in cortical cataract

severity between unexposed and exposed mice of each genotype at both the time-points was not significant. Thus, mild cortical cataract was considered to be temporarily induced due to anaesthesia rather than UV-B treatment and was not further analysed. In contrast, anterior polar cataract was present in UV-exposed mice of both genotypes at both time-points and was absent in all except one unexposed mouse (Fig 3 and 4). Furthermore, the severity of anterior polar cataract correlated with the dose of UV-B radiation exposure in mice of each genotype. Thus, anterior polar cataract was considered to be UV-B induced and was analysed in detail. The ICC of 0.85 (95% CI = 0.80 - 0.89) indicated that cataract grades between the two eyes of each animal were highly consistent.

Table 2: Comparison of anterior cortical cataract severity in *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to different doses of UV-B radiation.

Dose of UV-B J/cm ² (Group size)	<i>Epha2</i> ^{+/+}		<i>Epha2</i> ^{+/-}		<i>p</i> value (<i>Epha2</i> ^{+/+} versus <i>Epha2</i> ^{+/-})
	Median (IQR)	<i>p</i> value [†]	Median (IQR)	<i>p</i> value [†]	
24-48 hours after final exposure					
0 (n=9)	1.5 (1.5-2)	-	2 (1.5-2.13)	-	0.48
0.0125 (n=8)	2 (1.25-2)	0.88	2 (1.75-2)	0.96	0.38
0.025 (n=8)	2 (1.5-2.25)	0.39	2 (1.88-2)	0.82	0.57
0.05 (n=8)	2 (1.88-2)	0.24	2 (1.63-2)	0.96	0.85
4-7 weeks after final exposure					
0 (n=9)	2 (1.5-2)	-	1.5 (1.13-2)	-	-
0.0125 (n=8)	2 (1.63-2.13)	0.45	1.63 (1.5-2)	0.49	0.36
0.025 (n=8)	2 (1.63-2.13)	0.51	1.63 (1.5-1.75)	0.67	0.10
0.05 (n=8)	1.75 (1.5-2.13)	0.76	1.88 (1.5-2)	0.31	0.95

Median grade of cataract in each group at the two time-points is shown. *p* values of comparison between unexposed and exposed group of each genotype, and comparison between the two genotypes exposed to a dose of UV-B radiation are indicated. Comparisons were performed using Mann-Whitney *U* test. IQR, inter-quartile range.

[†] *p* values represent difference between a given dose (0.0125, 0.025 or 0.05) versus no dose (0) at a time-point.

At the first time-point (24-48 hours after the last exposure), mild (grade 1) anterior polar cataract was observed in only one of the eight *Epha2*^{+/+} mice and none of the *Epha2*^{+/-} mice exposed to 0.0125 J/cm² UV-B radiation (Fig 3 and 4). Thus, there was no significant difference in cataract severity between unexposed and mice exposed to the lowest dose of UV-B radiation (Table 3). Of the mice exposed to 0.025 J/cm² UV-B, three out of eight *Epha2*^{+/+} and five out of eight *Epha2*^{+/-} mice displayed anterior polar cataract at the first time-point (*Epha2*^{+/+}, median (IQR) = 0 (0-1.25); *Epha2*^{+/-}, median (IQR) = 0.75 (0-1.75); Table 3 and Fig 3 and 4). The exposed wild-type mice compared to unexposed mice showed only a borderline significant increase in cataract severity ($p = 0.051$, Table 3). However, the exposed *Epha2*^{+/-} mice displayed significantly greater increase in cataract severity than their unexposed counterparts ($p = 0.01$, Table 3). Similarly, five out of eight *Epha2*^{+/+} mice and all *Epha2*^{+/-} mice exposed to 0.05 J/cm² UV-B radiation exhibited anterior polar cataract (*Epha2*^{+/+}, median (IQR) = 0.5 (0-1.88); *Epha2*^{+/-}, median (IQR) 1.5 (1.25-2); Table 3 and Fig 3 and 4) compared to no cataract in unexposed mice of each genotype. The difference in cataract severity between exposed and unexposed mice of each genotype was highly significant (*Epha2*^{+/+} $p = 0.007$, *Epha2*^{+/-} $p < 0.001$; Table 3).

Furthermore, comparison of cataract severity between mice exposed to the lowest and the two higher doses of UV-B showed that in *Epha2*^{+/+} mice exposed to 0.05 J/cm² anterior polar cataract severity was significantly greater than in those exposed to 0.0125 J/cm² UV-B ($p = 0.046$, Table 3). *Epha2*^{+/-} mice exposed to both 0.025 and 0.05 J/cm² UV-B developed more severe anterior polar cataract than genotype matched mice exposed to 0.0125 J/cm² UV-B (0.025 J/cm² $p = 0.011$, 0.05 J/cm² $p < 0.001$; Table 3).

Table 3: Comparison of anterior polar cataract severity in *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to different doses of UV-B radiation.

Dose of UV-B J/cm ² (Group size)	<i>Epha2</i> ^{+/+}			<i>Epha2</i> ^{+/-}			<i>p</i> value (<i>Epha2</i> ^{+/+} versus <i>Epha2</i> ^{+/-})
	Median (IQR)	<i>p</i> value [†]	<i>p</i> value [‡]	Median (IQR)	<i>p</i> value [†]	<i>p</i> value [‡]	
24-48 hours after final exposure							
0 (n=9)	0 (0-0)	-	-	0 (0-0)	-	-	-
0.0125 (n=8)	0 (0-0)	0.29	-	0 (0-0)	1	-	0.31
0.025 (n=8)	0 (0-1.25)	0.05*	0.24	0.75 (0-1.75)	0.01**	0.011*	0.46
0.05 (n=8)	0.5 (0-1.88)	0.007**	0.046*	1.5 (1.25-2)	<0.001***	<0.001***	0.20
4-7 weeks after final exposure							
0 (n=9)	0 (0-0)	-	-	0 (0-0)	-	-	0.29
0.0125 (n=8)	0 (0-0)	0.29	-	0 (0-0)	0.32	-	0.31
0.025 (n=8)	0.13 (0-1.88)	0.02*	0.13	0.25 (0-1)	0.13	0.027*	0.69
0.05 (n=8)	2 (1.88-2)	0.001***	0.006**	1.63 (1.38-1.88)	0.002**	0.001***	0.09

Median grade of cataract in each exposed and unexposed group at the indicated time-points is shown. p values of comparisons between unexposed and exposed groups, between exposed groups of each genotype, and between genotypes are shown. Significance set at $p<0.05$. IQR, inter-quartile range.

† p values represent difference between a given dose (0.0125, 0.025 or 0.05) versus no dose (0) at a time-point.

‡ p values represent difference between a given dose (0.025 or 0.05) versus the lowest dose (0.0125) at a time-point.

*, $p\leq 0.05$; **, $p\leq 0.01$; ***, $p\leq 0.001$.

At the second time-point (4-7 weeks after the last exposure), similar to the first time-point, only one of the eight *Epha2*^{+/+} mice and none of the *Epha2*^{+/-} mice exposed to 0.0125 J/cm² UV-B exhibited anterior polar cataract (Fig 4). The lenses of unexposed *Epha2*^{+/+} and *Epha2*^{+/-} mice at this time-point were clear except one *Epha2*^{+/-} mouse developed mild anterior polar cataract (grade 1, Fig 4). Thus, once again, no difference in cataract severity was observed between unexposed and mice exposed to the lowest dose of UV-B radiation (Table 3). Of the mice exposed to 0.025 J/cm² UV-B radiation, four out of eight mice of each genotype displayed the presence of anterior polar cataract at the second time-point (*Epha2*^{+/+}, median (IQR) = 0.13 (0-1.88); *Epha2*^{+/-}, median (IQR) = 0.25 (0-1); Table 3 and Fig 3 and 4). There was a significant difference in cataract severity between the exposed and unexposed wild-type mice at this time-point ($p = 0.02$, Table 3); a similar difference was not observed between the exposed and unexposed *Epha2*^{+/-} mice ($p = 0.13$, Table 3). Of the mice exposed to 0.05 J/cm² UV-B, seven out of eight mice of each genotype exhibited anterior polar cataract at the second time-point (*Epha2*^{+/+}, median (IQR) = 2 (1.88-2); *Epha2*^{+/-}, median (IQR) = 1.63 (1.38-1.88); Table 3 and Fig 3 and 4). The severity of cataract in the exposed mice of each genotype was significantly greater than that in the respective unexposed mice (*Epha2*^{+/+} $p < 0.001$, *Epha2*^{+/-} $p = 0.002$, Table 3).

As seen at the first time-point, comparison of anterior polar cataract severity between mice exposed to the lowest and the two higher doses of UV-B at the second time-point, showed a significant difference in severity between *Epha2*^{+/+} mice exposed to 0.05 and 0.0125 J/cm² UV-B radiation ($p = 0.006$, Table 3). The *Epha2*^{+/-} mice exposed to both 0.025 and 0.05 J/cm² UV-B radiation exhibited significantly more severe cataract than those exposed to 0.0125 J/cm² UV-B (0.025 J/cm² $p = 0.027$, 0.05 J/cm² $p = 0.001$; Table 3).

In sixty percent of mice in the study, the second ophthalmic examination was performed four weeks after the last exposure and in the remaining 5-7 weeks after the last exposure. The

majority of the latter mice were either untreated or treated with the lowest dose of UV-B (0.0125 J/cm²); only a few mice were treated with 0.025 or 0.05 J/cm² UV-B. Hence variation in the timing of ophthalmic examination is unlikely to be the cause of significant differences in anterior polar cataract severity observed between groups at the second time-point.

1.3.2 Effect of genotype and termination time on cataract development in response to UV-B exposure

To determine the effect of *Epha2* genotype on susceptibility to anterior polar cataract development following UV-B radiation exposure, cataract severity between *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to a particular dose of UV-B radiation was compared at each time-point. No significant difference in severity was observed between the two genotypes of mice exposed to any dose of UV-B radiation at either time-point (Table 3).

Next, to determine the effect of time after termination of UV-B exposure on cataract severity, anterior polar cataract severity in exposed mice of each genotype was compared between time-points. No difference in cataract severity between the two time-points was observed in mice of both the genotypes exposed to 0.0125 and 0.025 J/cm² UV-B radiation (Fig 5 and data not shown). Wild-type mice exposed to 0.05 J/cm² UV-B radiation displayed significantly greater cataract severity 4-7 weeks compared to 24-48 hours after termination of exposure ($p = 0.03$, Fig 5). However, *Epha2*^{+/-} mice exposed to this dose exhibited no significant difference in cataract severity between the two time points (Fig 5). This suggests that after termination of UV-B exposure cataract severity continues to increase in *Epha2*^{+/+} but not in *Epha2*^{+/-} mice or increases in *Epha2*^{+/+} mice at a faster rate than in *Epha2*^{+/-} mice.

1.3.3 Structural changes in the lens in response to UV-B exposure

To determine any structural changes in the lens due to UV-B radiation exposure, histological analysis was performed on lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.025 and 0.05 J/cm² UV-B radiation. Lenses from mice of both genotypes exposed to 0.025 J/cm² UV-B radiation showed the presence of vacuoles (white spaces) at the epithelial-fiber cell interface and occasional disruption of the lens epithelium (Fig 6, panels b and e) that were absent in lenses of respective unexposed mice (Fig 6, panels a and d). The lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.05 J/cm² UV-B radiation exhibited epithelial plaques invaginating into the lens fiber cell mass and disrupting the anterior lens cortex (Fig 6, panels c and f). Similar plaques were absent in unexposed mice (Fig 6, panels a and d). The equatorial and posterior regions of lenses of exposed and unexposed *Epha2*^{+/+} and *Epha2*^{+/-} mice were histologically normal (data not shown). The observed histological changes are consistent with the presence of anterior polar cataract in UV-B exposed *Epha2*^{+/+} and *Epha2*^{+/-} mice and suggestive of epithelial hyperplasia, particularly in mice exposed to the highest dose.

1.3.4 Mechanism of cataract development in response to UV-B exposure

UV-B radiation exposure reportedly leads to up-regulation of *EPHA2* expression in pigmented and non-pigmented cells (Zhang et al., 2008). To determine if UV-B exposure similarly influences *Epha2* expression in the lens, *Epha2* mRNA levels were compared between lenses of unexposed mice and mice exposed to 0.025 and 0.05 J/cm² UV-B, of each genotype. In both *Epha2*^{+/+} and *Epha2*^{+/-} mice, similar levels of *Epha2* mRNA were observed in unexposed lenses and lenses exposed to the two doses of UV-B radiation (Fig 7A, third panel and 7B, bottom panel) indicating that *Epha2* expression levels in the lens were unchanged 4-7 weeks after exposure.

Furthermore, to determine the mechanism underlying UV-B radiation induced anterior polar

cataract in mice, involvement of cell proliferation and epithelial-to-mesenchymal transition (EMT) was investigated. For this, expression levels of *Mki67* mRNA, a marker of cell proliferation, and *Acta2* mRNA, a marker of EMT, were compared between exposed and unexposed lenses of each genotype. Once again, due to low level expression of *Mki67* in mouse lens, expression levels were compared by semi-quantitative RT-PCR. Notably, *Acta2* mRNA was expressed at similar levels in the lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.025 and 0.05 J/cm² UV-B radiation and lenses of genotype-matched unexposed mice (Fig 7A, second panel and 7B, middle panel). In contrast, *Mki67* mRNA was expressed at significantly higher levels in the lenses of *Epha2*^{+/+} mice exposed to 0.05 J/ cm² UV-B radiation compared to unexposed mice (Fig 7A, top panel and 7B, top panel; *p* = 0.036). A similar up-regulation of *Mki67* transcript was not observed in lenses of *Epha2*^{+/-} mice exposed to the same dose of UV-B radiation (Fig 7A, first panel and 7B, top panel). However, a marked increase in *Mki67* expression levels could be seen in the lenses of *Epha2*^{+/-} mice exposed to 0.025 J/cm² UV-B radiation compared to unexposed mice (Fig 7A, first row). A similar upregulation was not obvious in lenses of *Epha2*^{+/+} mice exposed to this dose (Fig 7A, first row). Due to small available sample size, expression levels between unexposed and lenses exposed to 0.025 J/cm² of UV-B were not statistically analysed. Taken together, these data suggest that in the present mouse model, cell proliferation underlies anterior polar cataract development in response to UV-B radiation.

1.4 Discussion

A complex interaction between environmental stress factors and genetic factors results in the development of age-related cataract. Here we studied interaction of an environmental factor, UV radiation, with a known genetic factor associated with human age-related cataract risk, *EPHA2* gene variation, on cataract development, in a mouse model. The interaction was

investigated in mice carrying a single functional copy of the *Epha2* gene instead of mice completely lacking functional *Epha2*. This study design mimics the human scenario where, unless mutated or deleted, *EPHA2* gene is functional. The mice completely lacking functional *Epha2* are not appropriate to study the interaction due to complete lack of expression of the gene. It is well accepted that UV-B radiation from sunlight reaching the lens contributes to age-related cataract (Abraham et al., 2006, Cruickshanks et al., 1992, Hayashi et al., 2003). Therefore, in this study, we investigated the effect of UV-B radiation on cataract development. The cumulative effects of repeated exposures to various below-threshold doses of UV-B radiation on cataract formation were investigated to recapitulate repeated exposure of humans to UV radiation from sunlight. In the majority of the reported studies, UV exposure was performed in anesthetized animals in order to better control the dose delivered to the lens (Ayala et al., 2000a, Michael et al., 1998a, Ayala et al., 2000b), and the contralateral eye served as unexposed control. In the present study, we exposed wakeful mice in an attempt to recapitulate UV exposure in humans. It is difficult to estimate the minimum UV-B radiation exposure sufficient to cause cataract in humans primarily because of the difficulty in quantifying daily UV-B exposure of an individual. We observed dose-dependent differences in anterior polar cataract development after repeated exposures to below-threshold doses of UV-B radiation in both *Epha2*^{+/+} and *Epha2*^{+/-} mice. Similar to the systems developed for grading posterior polar cataract on the basis of lens opacity and its effect on vision in humans (Schroeder, 2005, Singh D. et al., 1993), in the present study, we developed a system for grading anterior polar cataract in mice. Notably, grading by this system is according to the size of lens opacity rather than severity of associated visual impairment. Hence, in the present study, severe anterior polar opacity (grade 3) is generally far less severe than that graded by other systems.

The variation in anterior polar cataract severity observed in both genotypes of mice exposed to a particular dose of UV-B radiation, apart from individual variation, can be attributed to small unavoidable variability in the administered dose (see Section 1.2.4). Similar variation in cataract severity due to unavoidable variability in actual administered dose has been reported in other studies (Meyer et al., 2005). The reported $MTD_{2,3;16}$ for C57Bl/6J mice is 0.29 J/cm^2 (Meyer et al., 2005). In the present study, cumulative doses of 0.35, 0.175 and 0.0875 J/cm^2 resulting from simple additive effect of multiple exposures to 0.05, 0.025 and 0.0125 J/cm^2 of UV-B, respectively, were administered. We found that both, the cumulative dose above (0.35 J/cm^2) and below (0.175 J/cm^2) the maximum tolerable dose led to anterior polar cataract, which is consistent with the cataract phenotype reported in rodents exposed to UV radiation (Galichanin et al., 2010, Ayala et al., 2000a, Wegener, 1994, Meyer et al., 2005). No significant effect of multiple exposures to 0.0125 J/cm^2 of UV-B radiation in this study suggests that either the dose was too low to initiate any damage in the lens or the damage was rapidly repaired. Histological analysis of lenses of exposed mice further supported the dose-dependent anterior polar cataract severity observed in response to different doses of UV-B radiation. The observed epithelial hyperplasia in lenses of mice exposed to 0.05 J/cm^2 UV-B radiation is consistent with previous reports of aberrant cell division of mitotically quiescent lens epithelial cells causing multi-layering under the lens capsule in response to UV-B radiation (Wegener, 1994). More importantly, it correlates with upregulation of the cell proliferation marker, *Mki67*, in these lenses (Fig 7). The lack of change in *Acta2* expression in UV-exposed lenses suggests that the anterior polar cataract induced by repeated exposure to below-threshold doses of UV-B radiation does not involve EMT.

In this study, generally, the *Epha2* genotype was not found to have an influence on the magnitude of anterior polar cataract severity in response to any particular dose of UV-B

radiation at either time-point. A future study with larger group sizes is warranted to discern the effect of allelic deficiency of *Epha2* on UV-B induced cataract at a time-point. Interestingly, the period after the final exposure to 0.05 J/cm² UV-B radiation had differing effects on cataract progression between the two genotypes. Wild-type mice had significantly more severe cataract at the second time-point compared to the first time-point whereas *Epha2*^{+/-} mice had similar cataract severity at both the time-points despite showing a trend for higher severity than wild-type mice at the first time-point (Fig 5). Rat lenses exposed to a single dose of 0.5 J/cm² of UV-B radiation exhibit similar forward light scattering, 1 week and 32 weeks later (Michael et al., 1996, Meyer et al., 2005). However, the interval between repeated UV-B exposures affects the degree of opacification of rat lens; an interval of 0 or 6 hours between two exposures leads to same degree of opacification whereas an interval of 24 and 48 hours results in increased lenticular damage possibly due to impairment of repair processes (Michael, 2000). In the present study, cataract development at each time-point reflects accumulated damage to the lens from repeated exposures to UV-B radiation. Aggravation of cataract severity in *Epha2*^{+/+} mice at the second time-point after exposure to the highest dose of UV-B suggests that lenticular damage continues even after stopping repeated exposures to a below-threshold dose of UV-B radiation. Absence of similar aggravation of severity in *Epha2*^{+/-} mice suggests less continuing damage to the lens and hence a favourable effect of *Epha2* deficiency in the long-term. The difference in exacerbation of cataract severity over time between the two genotypes of mice correlates with significant increase in lens epithelial cell proliferation in wild-type mice indicated by upregulation of *Mki67* expression (Fig 7). A trend for higher cataract severity in *Epha2*^{+/-} mice at the first time-point indicates that time after termination of UV-B exposure may affect the influence of *Epha2* deficiency on cataract severity. A larger study including *Epha2*^{-/-} mice is warranted to investigate this effect.

Apoptosis or necrosis is thought to be the main cause of lenticular damage in rodent lens induced by a single above-threshold dose of UV radiation (Ayala et al., 2007, Galichanin et al., 2012). However, repeated exposures to UV radiation leads to lens epithelial hyperplasia in rodents (Wegener, 1994). Repeated exposure to below-threshold doses of UV radiation also led to lens epithelial hyperplasia, in the present study. Thus, taken together, these studies suggest that the mechanism of cataract development in response to UV radiation depends upon the exposure regimen. Interestingly, UV-B exposure leads to an increase in *Epha2* receptor expression in cultured human and murine cells and in turn to cell apoptosis, whereas increased *EPHA2* expression in cancer reportedly leads to increased cell proliferation (Zhang et al., 2008). The opposing effect of UV-B exposure on lens epithelial cell fate noted in this study to that reported by Zhang et al. is most likely due to the differences in exposure regimes and time of repair after termination of exposure between the two studies; in the present study cell fate was analysed 4-7 weeks after reported exposure to UV-B compared to 24 hours after a single exposure by Zhang et al. The difference in the model systems used in the two studies may also contribute to the opposite results. Nevertheless, the lens epithelial cell hyperplasia due to increased cell proliferation observed in lenses of mice exposed to 0.05 J/cm² UV-B in this study was not accompanied by an increase in *Epha2* expression (Fig 7). Hence, a different mechanism may underlie the observed lens epithelial hyperplasia. Future research is warranted to determine the mechanism of hyperplasia underlying the cataract induced by repeated exposure to below-threshold doses of UV-B radiation in mice.

1.5 Conclusion

In conclusion, to the best of our knowledge, this is the first study to demonstrate the effect of repeated exposures to below-threshold doses of UV-B radiation on cataract development in an *Epha2* knockout mouse model. The findings demonstrate a dose-dependent effect of UV-

B radiation on anterior polar cataract development, and indicate a likely favourable effect of *Epha2* deficiency against progression of UV radiation-induced cataract over time in mice.

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Fig 1: Anterior polar cataract grading system used in this study.

Representative images of cross-section illumination of the lens showing no cataract, and grades 1-3 anterior polar cataract in mice as indicated. UV-B exposure of mice resulted in anterior polar cataract (see Section 1.2.4). Each image from left to right shows a cross-section of the cornea (thick bright arc), anterior chamber (dark region) and lens (narrow dim arc). Iris (brown pigment) is visible at the upper and/or lower end of the lens in each image. Anterior polar cataract (bright spot) in the lens is indicated by an arrow. Note the increasing size of cataract from grade 1 to 3. This grading system was used to monitor anterior polar cataract development following UV-B exposure.

Fig 2: *Epha2* expression in wild-type and *Epha2* knockout mouse lenses.

Expression levels of *Epha2* mRNA were analysed in lenses of two-month-old *Epha2*^{+/+}, *Epha2*^{+/-} and *Epha2*^{-/-} lenses by semi-quantitative RT-PCR. Expression was analysed in lenses of three animals per genotype; *B2m* mRNA was amplified as housekeeping control. The amplified *Epha2* and *B2m* specific products of the expected sizes (265 bp and 193 bp, respectively) can be seen as indicated. Numbers above each panel indicate independent animals and correspond between panels. Numbers on the left refer to size of molecular standards (M). RT⁻, no reverse transcription control; C⁻, no template control.

Fig 3: UV-B induced anterior polar cataract in *Epha2*^{+/+} and *Epha2*^{+/-} mice.

Five-week-old *Epha2*^{+/+} (WT) and *Epha2*^{+/-} (HET) mice were repeatedly exposed to 0.0125, 0.025 or 0.05 J/cm² of UV-B radiation and ophthalmic examination performed 24-48 hours and 4-7 weeks after the last exposure, as indicated. Unexposed mice of each genotype served as controls.

Anterior polar cataract was absent in unexposed mice (panels a, e, i, m) and in *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.0125 J/cm² UV-B radiation (panels b, f, j, n). Distinct anterior polar cataract could be seen in *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.025 (arrow, panels c, g, k, o) and 0.05 J/cm² (arrow, panels d, h, l, p) UV-B radiation at both the time-points. Slit-lamp microscopy images of eyes of representative animals from each experimental group are shown.

Fig 4: Distribution of anterior polar cataract grading data in unexposed and UV-B exposed *Epha2*^{+/+} and *Epha2*^{+/-} mice.

Scatter plots showing the distribution of average anterior polar cataract grades in unexposed and UV-B exposed *Epha2*^{+/+} (WT, left panels) and *Epha2*^{+/-} (HET, right panels) mice 24-48 hours (top panels) and 4-7 weeks (bottom panels) after the final exposure (n=8 per group). X-axis, UV-B dose in J/cm²; y-axis, average anterior polar cataract grade in the two eyes. Each circle represents average cataract grade in an animal. Note the UV-B dose-dependent increase in the numbers of animals with cataract and increase in cataract severity in each panel. Also note the increase in the number of animals with cataract and increase in cataract severity at the second compared to the first time-point in WT (left panels) and HET (right panels) mice exposed to 0.05 J/cm² of UV-B radiation. No cataract was observed in unexposed *Epha2*^{+/+} and *Epha2*^{+/-} mice at either time-point except mild anterior polar cataract in one *Epha2*^{+/-} mouse at the second time-point (bottom right panel).

Fig 5: Comparison of anterior polar cataract severity in *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to the two highest doses of UV-B radiation.

The plot shows average anterior polar cataract grades in *Epha2*^{+/+} (WT) and *Epha2*^{+/-} (HET) mice 24-48 hours and 4-7 weeks after termination of repeated exposure to 0.025 or 0.05 J/cm² UV-B radiation (n=8 per group). X-axis, UV-B dose in J/cm² and time; y-axis, average anterior polar cataract grade in both eyes of animals in a group. No significant difference in cataract severity was observed between *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to either dose of UV-B at either time-point. In *Epha2*^{+/+} mice exposed to 0.05 J/cm² UV-B, cataract severity had significantly increased at the second time-point compared to the first-time point (Wilcoxon Signed Rank test; $p = 0.03$). A similar increase in severity between time-points was not noted in *Epha2*^{+/-} mice exposed to 0.05 J/cm² of UV-B radiation. Error bars indicate standard error of the mean.

Fig 6: Histological analysis of unexposed and UV-B exposed mouse lenses.

Lenses of *Epha2*^{+/+} (WT) and *Epha2*^{+/-} (HET) mice exposed to 0.025 or 0.05 J/cm² UV-B radiation and unexposed (untreated) mice were analysed by hematoxylin and eosin staining after the second ophthalmic examination. Sections from the pupillary region of each lens are shown. The dose of UV-B radiation and *Epha2* genotype are as indicated. Hematoxylin stained nuclei (blue) and eosin stained cytoplasm (pink) of lens epithelial cells and of lens fiber cells can be seen. Cornea is visible in some sections (panels a, b and d). Unexposed mice of both genotypes showed normal lens architecture (panels a and d). Vacuoles and occasional disruption of the lens epithelium could be seen at the epithelial-fiber cell interface (arrow and inset, panels b and e) in the lenses of each genotype of mice exposed to 0.025 J/cm² UV-B radiation. Lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.05 J/cm² UV-B showed aberrant lens epithelial cell growth (arrow, panels c and f) and disruption of the underlying fiber cell arrangement. A relatively thinner lens capsule in some

sections is an artefact due to folded section. Representative images of lenses of three mice per group are shown. Scale-bar 50 μm .

Fig 7. Relative expression of *Epha2* gene and markers of cell proliferation and EMT in UV-B exposed and unexposed mouse lenses.

A. mRNA levels of *Epha2* gene, cell proliferation marker *Mki67* and EMT marker *Acta2* in lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice exposed to 0.05 or 0.025 J/cm² UV-B radiation and unexposed (0.0) mice were analysed by semi-quantitative RT-PCR. Analysis was performed 4-7 weeks after termination of exposure. *B2m* mRNA expression was used as the housekeeping control. Each lane represents amplification in pooled lenses of two mice of the same gender; lanes 1 and 2 indicate two independent pools. The amplified *Mki67*, *Acta2*, *Epha2* and *B2m* specific products of the expected sizes (490 bp, 338 bp, 265 bp and 193 bp, respectively) can be seen as indicated. Numbers on the left refer to size of molecular standards (M). RT-, no reverse transcription control. **B.** Relative quantification of *Mki67* (top), *Acta2* (middle) and *Epha2* (bottom) specific amplification products between 0.05 J/cm² UV-B radiation exposed and unexposed (0.0) lenses of *Epha2*^{+/+} and *Epha2*^{+/-} mice, shown in A. Mean expression of each gene was normalized to *B2m* mRNA levels. Data was analysed using two-tailed Student's *t*-test and significant *p* value indicated. Error bars represent standard deviation of the mean.

Grade 0

Grade 1

Grade 2

Grade 3

24-48 hours after UV-B treatment

4-7 weeks after UV-B treatment

A

B