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1 A novel role for CRIM1 in the corneal response to UV and pterygium development

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25 ABSTRACT

Pterygium is a pathological proliferative condition of the ocular surface, characterized by formation of a highly vascularized, fibrous tissue arising from the limbus that invades the central cornea leading to visual disturbance and, if untreated, blindness. Whilst chronic ultraviolet (UV) light exposure plays a major role in its pathogenesis, higher susceptibility to pterygium is observed in some families, suggesting a genetic component.

In this study, a Northern Irish family affected by pterygium but reporting little direct exposure to UV was identified carrying a missense variant in *CRIM1* NM_016441.2: c.1235 A>C (H412P) through whole-exome sequencing and subsequent analysis. *CRIM1* is expressed in the developing eye, adult cornea and conjunctiva, having a role in cell differentiation and migration but also in angiogenesis, all processes involved in pterygium formation. We demonstrate elevated *CRIM1* expression in pterygium tissue from additional individual Northern Irish patients compared to unaffected conjunctival controls.

UV irradiation of HCE-S cells resulted in an increase in ERK phosphorylation and *CRIM1* expression,
the latter further elevated by the addition of the MEK1/2 inhibitor, U0126. Conversely, siRNA
knockdown of *CRIM1* led to decreased UV-induced ERK phosphorylation and increased *BCL2*expression.

42 Transient expression of the mutant H412P *CRIM1* in corneal epithelial HCE-S cells showed that,
43 unlike wild-type *CRIM1*, it was unable to reduce the cell proliferation, increased ERK
44 phosphorylation and apoptosis induced through a decrease of *BCL2* expression levels.

45 We propose here a series of intracellular events where *CRIM1* regulation of the ERK pathway 46 prevents UV-induced cell proliferation and may play an important role in the in the pathogenesis of 47 pterygium.

- 49 Keywords: CRIM1; pterygium; UV; proliferation; ERK; apoptosis; variant
- 50
- 51 Abbreviations: BCL2, B-cell lymphoma 2; BMP, Bone Morphogenetic Proteins; CRIM1, Cysteine Rich Motoneuron protein1; EMT, Epithelial
- 52 mesenchymal transition; ERK (I), Extracellular signal-regulated kinases (Inhibitor); HCE-S, Human Corneal Epithelial cells; IC, Impression
- 53 Cytology; IGV, Integrative Genomic Viewer; MAF, Minor allele frequency; MAPK, Mitogen-activated protein kinases; MTT, 3-(4,5-
- 54 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGS, Next Generation sequencing; NI, Northern Irish; PolyPhen, Polymorphism
- 55 Phenotyping; SIFT, Sorting Intolerant From Tolerant; TiGER, Tissue-specific Gene Expression and Regulation; TGF-β (I), Transforming Growth
- 56 Factor- β (Induced); **TUNEL**, Terminal deoxynucleotidyl transferase dUTP nick end labelling; **VEGFA**, Vascular Endothelial Growth FactorA;
- 57 VW(F)-C, Von Willebrand (Factor) C; WES, Whole Exome Sequencing.

1. INTRODUCTION

60 Pterygium (OMIM 178000) is a triangular shaped proliferative fibrovascular growth arising from the corneal-scleral limbus that invades the cornea centripetally^{1,2}. The corneal invasion, through 61 an active process of cell proliferation, matrix remodelling, angiogenesis and inflammation^{1,3}, 62 results in astigmatism, irritation, tearing, and, if the visual axis is impinged upon, blindness in the 63 most severe cases⁴. The only effective treatment for pterygium is surgical excision which, together 64 with various adjuvant therapies, still presents a 12% recurrence rate⁵. A small conjunctival lesion, 65 pinguecula, is considered to be related to pterygium with a similar aetiology⁶, limbal localisation⁷ 66 and histology⁸. 67

The main trigger for pterygium has long been attributed to UV radiation⁹⁻¹¹, with epidemiologic 68 studies showing an average prevalence of 22% (as high as 40% in some Chinese populations¹² or 69 70 45% in provincial Indonesia¹³) in the "pterygium belt", an equatorial zone between latitudes 40°N and 40°S, compared to only 2% outside this area⁴. The human cornea represents a shield to protect 71 72 the anterior eye from UV light and its anisotropic properties ensure that UV transmittance is 73 reduced with a lower wavelength: while UVB (290-320nm) is completely absorbed by corneal 74 epithelium, UVA (320-400nm) is absorbed only by 20% by the epithelial layer with the rest reaching the underlying stroma¹⁴. Focussing of incident light upon the nasal limbus has been 75 76 proposed as the mechanism for the more frequent occurrence of pterygia at this location¹⁵.

In pterygium, while UVB mediates oxidative DNA damage^{16,17} and induction of cytokines and
 growth factors¹⁸, both UVB¹⁹ and UVA²⁰ are responsible for activation of ERK intracellular
 pathway.

80 Irritation and chronic inflammation caused by sand, dust and wind²¹, as well as viral 81 infections^{22,23}, have been suggested as additional environmental triggers for pterygium.

A genetic contribution to the incidence of pterygium was suggested in an Australian study²⁴,
 which revealed that 38% of patients admitted to hospital for excision of pterygia reported relatives

84 with the disease, and in studies where different ethnic groups living at the same geographical 85 location display differing prevalence of pterygium^{12,25}.

High incidence of pterygium has been reported in a number of multigenerational families^{26,27} and
 in monozygotic twins^{28,29}, where the most commonly reported mode of inheritance is autosomal
 dominant with reduced penetrance^{4,30,31}.

The lack of large families presenting with multiple affected individuals, compounded by the lateonset of the disease, together with incomplete penetrance, has hampered identification of causative genes for pterygium.

Candidate gene association studies, which derive from hypotheses in which the cause of pterygium is already assumed, have identified an increased predisposition to pterygium in individuals carrying germline mutations in genes related to: oxidative stress^{16,32}, carcinogenesis³³ or angiogenesis³⁴; even though these analyses have been found subject to false-positive associations³⁵. Next-generation sequencing techniques, such as Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS), provide a largely hypothesis-free approach to identifying causative genes in smaller families with fewer affected members³⁶.

99 In this study, using a WES approach, combined with bioinformatic and functional analysis, we 100 identified a missense variant, p.His412Pro, in the CRIM1 (cysteine rich motor neuron protein 1) 101 gene, found in pterygium affected members of large multigenerational Northern Irish family with 102 a documented small exposure to sunlight. CRIM1 response to UV exposure revealed, for the first 103 time, a central role within an intracellular mechanism involving ERK phosphorylation and 104 ultimately leading to cellular proliferation or apoptosis. Introduction of the H412P variant in 105 CRIM1 resulted in an impairment of the whole pathway, demonstrating its possible involvement 106 in the NI family's pterygium pathogenesis.

107

109 2. MATERIALS AND METHODS

110 2.1 Patient clinical examination

- Clinical examinations were performed at Cathedral Eye Clinic, Belfast, UK (pterygium family
 members, pterygium and control individual samples from Northern Ireland).
- 113 A total of 24 patients from three consecutive generations of a Caucasian Northern Irish family 114 affected by pterygium were investigated: three with pterygium, two with pinguecula and one
- 115 unaffected family member participated to the WES study. Additional Northern Irish and Bolivian
- 116 individuals (pterygium affected and unaffected controls) were recruited for sequence analysis of
- 117 *CRIM1* and *CRIM1* expression analysis.

Following informed consent, collection of blood, tissues and a completed questionnaire was obtained from each participating individual under ethical approval from ORECNI Northern Ireland, UK (09/NIR01/14) and Comité de Bioética de la Facultad de Medicina, Santa Cruz, Bolivia.

122 2.2 Whole Exome Sequencing and Ingenuity Variant Analysis

WES was performed at the Wellcome Trust Centre for Human Genetics, University of Oxford.
Briefly, genomic DNA was extracted from blood leukocytes using the QIAamp DNA Blood mini
kit (QIAGEN, Manchester, UK), quantified by the high sensitivity Qubit system (Thermo Fisher
Scientific, Loughborough, UK) and integrity of the DNA confirmed by electrophoresis on a 1%
agarose gel.

The SureSelect Human All Exon v2 kit was used for Whole Exome capture according to the manufacturer's instructions (Agilent Technologies UK, Wokingham, Berkshire, UK). The SureSelect n.2100 Bioanalyser (Agilent Technologies) allowed an assessment of the quality of the exome enriched library obtained.

Parallel sequencing was then performed by Illumina GAIIx using 150bp-paired-end reads.
Generated reads were aligned to the Human 37 reference genome with a short read mapper
(Stampy) generating data in BAM format³⁷.

Coverage of the target region was verified to be in excess of 70% (greater than 10 reads). Platypus, an in-house variant caller able to detect Single Nucleotide Variants (SNVs) and short (<50bp) insertion/deletions (INDEL), was used to detect variant sites and alleles. Once the false positive rate was reduced, the resulting variants were stored as Variant Call Format (VCF) files. Ingenuity Variant analysis (Qiagen) was used to filter and select a smaller number of candidate genes. Aligned WES reads were viewed with the IGV platform. Clustal X2.1 was used to align CRIM1 sequences and results were visualized with EsPript 3.0 (http://espript.ibcp.fr)³⁸.

142 2.3 Sanger Sequencing

Sanger sequencing was performed on genomic DNA extracted from the blood leukocytes usingQIAamp DNA Blood mini kit.

Presence of H412P in *CRIM1* was verified using the following primers: CRIM1_F: CTTCTTTTGCATGCACCCCC and CRIM1_R: TCACATGTGCAACCTTTCCTC while *CRIM1* VWFs were sequenced using genomic primers designed using Primer3³⁹ (Supplementary Table2). The PCR products were verified on a 1% agarose gel and Sanger sequenced at the Department of Zoology, University of Oxford.

150 2.4 Tissues and Impression cytology samples

Tissues samples (pterygium and normal cornea) were collected during surgeries while impression cytology samples were obtained using 4 x 4 mm strips of sterile LCR biopore membrane filter (pore size, 0.45 um; Millipore, Watford, UK) post pterygium surgery as previously described⁴⁰ and stored in RNAlater (Qiagen). Prior to IHC staining tissues were fixed in 95% ethanol for 20 minutes at room temperature. The RNA yield obtained from the impression cytology samples was never lower than 8ng/ul and never higher than 15ng/ul. The ratio of absorbance at 260 and 280

- nm (A260/280) of the RNA samples was between 1.8 and 2.2. Samples for which the RNA yield
 was too low or the quality fell outside the limits were excluded.
- 159

160 2.5 Quantitative real-time PCR

- 161 RNA extracted using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK) was quantified with
 162 the Nanodrop 1000 (Thermo Fisher Scientific) and 1 µg of total RNA was reverse transcribed
 163 into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley,
 164 UK). cDNA was used for qRT-PCR assays, performed using a Lightcycler 480 II (Roche, West
 165 Sussex UK).
- - 166 Real Time Ready Assays for *CRIM1* (assay id. 112278), *VEGFA* (assay id. 140396), *SRCAP*
 - 167 (assay id. 126413), TGF- βI (assay id. 104720), Glyceraldehyde 3-phosphate dehydrogenase
 - (GAPDH) (assay id. 141139) and hypoxanthine phosphoribosyltransferase (HPRT) (assay id.
 102079) were purchased from Roche (Burgess Hill, West Sussex, UK).
 - 170 SYBR green (Fermentas, Cambridge, UK) technology qRT-PCR was performed using BCL2
 - 171 primers (For AGCATGGGAGCCACGACCCT, Rev GGCCAAGGCCACACAGCCAA) and
 - 172 HPRT primers (For AGCTTGCGACCTTGACCAT, Rev GACCACTCAACAGGGGACAT), a
 - 173 kind gift from H. Nesbitt⁴¹.
 - 174 Data were normalised using *HPRT* and *GAPDH* as housekeeping controls for Δ Ct and $\Delta\Delta$ Ct 175 calculations⁴². *HPRT* and *GAPDH* were chosen as they have been shown to be the most stable 176 corneal housekeeping genes⁴³. For each condition all complementary cDNA samples were run in 177 duplicate in two independent experiments.

178 **2.6 HCE-S culture**

Human Corneal Epithelial cells (HCE-S), a spontaneously generated corneal cell line⁴⁴ (a kind
gift from Prof. Julie Daniels), were cultured (37°C, 5% CO₂) in Dulbecco's modified Eagle's

medium (DMEM) containing 4 g/L glucose (Thermo Fisher Scientific, UK), and supplemented
with 10% fetal bovine serum (Thermo Fisher Scientific, UK).

183 **2.7 TUNEL assay**

184 The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was 185 performed on HCE-S cells which had been reverse transfected with Lipofectamine 2000 using a 186 mock transfection with pGL4.17 [luc2/Neo] plasmid (Promega Madison, WI USA), CRIM1 wild 187 type or CRIM1 H412P plasmids. After 72 hours cells were fixed with 4% paraformaldehyde 188 (PFA) and stained using the In Situ Cell Death Detection kit (Fluorescein; Roche, Burgess Hill, 189 Surrey, UK) following the manufacturer's instructions. Images were obtained using a fluorescent 190 AxioScope A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany); 10x 191 objective. Twelve images for each condition (n=3) were quantified using ImageJ software (US 192 National Institutes of Health) and then normalised to total DAPI cells in each field.

193 **2.8 Immunohistochemistry (IHC)**

194 Pterygium and conjunctival tissues were formalin fixed and paraffin embedded. Sections of 7µm 195 were permeabilised with 0.5% Triton X-100 before staining, treated with Proteinase K solution 196 (Fisher Bioreagents, BP1700-50, 10ug/ml in PBS) and blocked with 5% goat serum (Sigma). A 197 rabbit polyclonal CRIM1 antibody (Abcam- ab189203) was incubated at 1:100, with a rabbit IgG 198 (Abcam) used as an isotype control. Secondary antibody fluorescein isothiocyanate (FITC)-199 conjugated goat anti-rabbit IgG (Santa Cruz, USA) was used at a 1:100 dilution. Each section was 200 mounted with fluorescent mounting medium (DAKO) and imaged using a 20× N Archoplan lens 201 on an AxioScope.A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany).

202 2.9 Site Directed Mutagenesis

Human CRIM1 cloned into a pcDNA3.1 plasmid was a kind gift from Dr. L Wilkinson, Institute
 for Molecular Bioscience, University of Queensland, Brisbane, Australia⁴⁵. Site directed
 mutagenesis was performed to obtain the H412P *CRIM1* clone, using the Quick Change II kit

206 (Agilent Technologies), following the manufacturer's instructions. The entire *CRIM1* sequence
 207 was checked for integrity by Sanger Sequencing (Department of Zoology, University of Oxford),
 208 primers used are listed in Supplementary Table1.

209 2.10 MTT assay

Reverse transfection was performed in 12 well plates using HCE-S cells with either 210 211 pCas9D10A_GFP (Addgene/Zhang lab), CRIM1 wild-type or H412P plasmids with 212 Lipofectamine 2000, according to the manufacturer's instructions (Falcon #353043, BD Corning 213 Life Sciences, MA, USA). The following day cells were transferred in 96 well plates and 3-(4,5-214 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium. 215 Following 2 hours of incubation, the cells were resuspended in DMSO (Dimethyl sulfoxide, 216 Sigma), absorbance was measured at 570 nm using the FLUOstar Omega (BMG Labtech, 217 Aylesbury, UK) and quantified as relative percentages compared to control conditions. 218 Absorbance measurements for the MTT were taken at 24, 48, 72 and 96 hours post transfection.

219 2.11 Western Blotting

HCE-S cells were cultured as described above. Low cell seeding densities were used to avoid cells becoming too confluent as this causes a decrease in ERK phosphorylation independent of the effect of CRIM1^{46,47}.

Proteins were extracted using Complete Lysis-M (Roche Diagnostics) and proteinase inhibitor
(Sigma-Aldrich P8340), quantification of total protein was performed using the Bradford assay
(BioRad), 25µg of the extracted proteins was then resolved on a 4-12% NuPAGE® Bis-Tris
Precast Gels (Thermo Fisher Scientific UK) using NuPAGE® MOPS SDS Running Buffer and
transferred onto Amersham TM Hybond ECL membrane (GE Healthcare Life Sciences).
Phospho ERK (#9101) and ERK (#9102) primary antibodies (Cell Signalling) were used at

dilutions of 1:100 and 1:500 respectively with an overnight incubation at 4°C. A secondary swine

anti-rabbit antibody (DakoCytomation, Ely, UK) was used at a 1:2000 dilution for 1 hour at room

temperature. Protein binding was detected by standard chemiluminescence: SuperSignal[™] West
Pico Chemiluminescent Substrate (Thermo Fisher Scientific UK) and imaged using the G:BOX
transilluminator (Syngene). Quantification was performed using GeneTools image analysis
software: average peak values of phosphoERK were normalised against the average ERK values.
All the results obtained were then normalised to the transfection control.

236

238

2.12 UV treatment and MEK1/2 inhibitor

HCE-S cells were seeded in a 24-well plate at 1×10^5 cells per well in growth medium and left to

adhere overnight at 37°C and 5% CO₂. The following day they were treated using a UVA cross-

- 239 linker (IROC Innocross AG, Ramsen, Switzerland) delivering a dose of 5.4 J/cm² as previously
- described 48 or UVB at a final dose of 0.5 J/cm² through Arcadia D3 6% lamp (Arcadia, UK) with
- an aluminium reflector at a distance of 15 cm from the cells for 34 minutes.
- The same doses of UVA and UVB irradiation were used in experiments in which the inhibitor of ERK phosphorylation (MEK1/2 inhibitor, U0126) was added to culture media an hour prior to the UV treatment as previously described²⁰, at a concentration of 10µM.
- After irradiation, HCE-S cells were incubated in culture medium at 37°C with 5% CO₂ and harvested at 1, 6, 12, 24 and 48 hours.

247 2.13 siRNA transfection

Four different siRNAs targeting the *CRIM1* sequence (Set of 4 Upgrade: ON-TARGETplus CRIM1 siRNA, LU-008492-00-0002, 2nmol, Dharmacon) were reverse transfected in HCE-S cells using Lipofectamine RNAiMAX (Fisher Thermo Scientific), following the manufacturer's instructions. The four siRNAs were transfected singularly or as a pool at a final concentration of 10nM and normalised to the results from a non-specific siRNA control (NSC4) ⁴⁹. A range of different concentrations (0.2-0.5-1-10nM) of the siRNA pool reverse transfected in HCE-S cells was subsequently tested.

256 2.14 Statistical Analysis

All error bars represent the standard error of the mean (SEM) calculated between sample replicates of the same biological group. qRT-PCR, MTT and TUNEL assays significance was estimated using a Student's t-test calculation or the Mann-Whitney U test in GraphPad Prism 5 software with data illustrated using Box plots (qRT-PCR in Fig.3). p value ≤ 0.05 was deemed to be significant (*p value ≤ 0.05 , **p value ≤ 0.01 and ***p value ≤ 0.001).

262

3. RESULTS

264 3.1 WES analysis in a Northern Irish family affected by pterygium and pinguecula

A multigenerational Northern Irish family presenting with pterygium and pinguecula, but not other eye abnormalities, was examined (Fig.1A). Pterygium affected both males and females and was diagnosed at an average age of 48 years. No history of unusual sun exposure was recorded for any family member, suggesting a familial predisposition for development of pterygium/pinguecula (Table 1).

Six members of this family underwent WES: three were affected by pterygium (II.2, II.4 and II.14, 72, 70 and 65 years old respectively), two by pinguecula (III.5 and III.6, 48 and 46 years old respectively), shown in Fig.2B, and one was unaffected (II.9, 58 years old). The other two younger unaffected members of the family (III.2 and III.3, age 49 and 34) did not participate in the WES but were subsequently analysed by Sanger Sequencing.

WES resulted in the identification of 451,153 variants in 18,858 different genes which were filtered in a stepwise manner by Variant analysis software (Ingenuity®) (Fig.1C). Selecting only those variants with a call quality \geq 50 in any case and \geq 20 in the control and a read depth \geq 10; 30,000 variants were obtained. When variants with a minor allele frequency (MAF) greater than 0.5% (pterygium prevalence in Europe is 2%) in 1000 Genomes Project, ESP EA exomes and Complete Genomics genome were excluded, the number of variants was reduced to 25,000.
Analysis using either of the two algorithms Polyphen and SIFT, predicted as deleterious 11,000
variants, while 40 variants were finally selected as displaying an autosomal dominant segregation
pattern within the family (Table 2).

284 Genes carrying mutations previously associated with pterygium such as *Ku70*, *GSTM1*, *ACE* 285 $hOGG1^{16,17,33,34}$ were not observed in the selected list of variants.

Each single variant obtained by this analysis was then manually reviewed for determination of protein structure/function in Polyphen and SIFT, conservation of the amino acid among species running BLAST or eye expression interrogating Tiger Expression database. Literature was reviewed for any possible known correlation between gene mutations and pterygium or other diseases, either of the eye or otherwise. Moreover, adequate sequencing coverage and cosegregation of the variant within the family were verified through Integrative Genomic Viewer (IGV) (Table 2).

Based on the gene expression profile and on the known role in the eye, we selected *CRIM1* as our most plausible candidate gene. Recent studies have elucidated the importance of CRIM1 expression in corneal and conjunctival development⁵⁰⁻⁵² and the same variant, H412P, was identified from another WES study on patients affected by Coloboma eye developmental disorder⁵³. Moreover, CRIM1 revealed a role in cell proliferation⁵⁴⁻⁵⁶, adhesion and migration^{57-⁵⁹, angiogenesis through VEGFA⁶⁰⁻⁶² and UV-related diseases^{63,64}, the main processes involved in pterygium development⁴.}

300

301 **3.2 CRIM1** structure and sequence analysis in individuals from Northern Ireland and Bolivia.

Presence of the variant in each affected family member, and absence from the unaffected sibling
(II.9), was confirmed by direct Sanger sequencing (Fig.2A, example) of PCR products spanning
the variant in VWFC-2.

Sequence alignment of *CRIM1* orthologues revealed residue H412 being conserved throughout
human (*Homo sapiens*), cattle (*Bos Taurus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), frog
(*Xenopous laevis*), zebrafish (*Danio rerio*) (Fig.2B).

308 CRIM1 is a 1002 amino acid transmembrane protein (once the 34 amino acid signal peptide has
309 been cleaved) and consists of a large N-terminal extracellular portion composed of 11 domains
310 (six Von Willebrand factor C, four antistasin-like and one Insulin-like Growth Factor-binding
311 Protein (IGFBP)), a 21 amino acids transmembrane domain and a small 76 amino acid C-terminal
312 cytoplasmic domain⁶⁵ (Fig.2C).

Although H412, located in the second VWFC domain, is not conserved in the other five VWFC of *CRIM1* (Fig.2D), it is conserved in the VWFC-2 domain of human chordin and in the VWFC-2 and 3 domains of human neuralin⁶⁶, two related bone morphogenic protein (BMP) antagonists,

316 like CRIM1, all of which have a proven role during embryogenesis⁴⁵ (Fig.2E).

Sequence analysis of VWFC-2 in two additional younger family members (III.2 and III.3, age 49 and 34) revealed them to possess the same H412P variant; despite these individuals not having developed any signs of the disease. This is not incompatible with the mutation predisposing to the pathology, since a pattern of reduced penetrance has been widely described in familial pterygium^{1,4,26}.

322 All the CRIM1 VWFC domains are important for interaction with BMPs 4 and $7^{45,67}$ as well as

323 with VEGFA, TGF- β and PDGF: if any of those domains are deleted, the interaction with the

324 TGF- β superfamily members is disrupted⁶². VEGFA, TGF- β s and PDGF have an important role

325 in pterygium formation^{3,68}: an altered interaction of those factors with CRIM1 VWFC domains

326 could therefore have a role in pterygium development. Sanger sequencing analysis was

327 subsequently performed for all the six VWFC domains of *CRIM1* (primers listed in

328 Supplementary Table2) in genomic DNA obtained from two ethnically different populations: 12

329 affected individuals from Northern Ireland and 9 from Bolivia (Table3).

330 While the H412P variant was found in none of the pterygium affected individuals, one patient 331 from Bolivia, B1 (Table3), presented with a cytosine to thymine transition in the first position of 332 codon 745, predicted to result in an Arginine to Cysteine (R745C) amino acid change (dbSNP: 333 rs145721446) between VWFC-4 and VWFC-5 (exon 13). Although the MAF of this variant is 334 0.000008237 (Supplementary Fig.1A) and the R745C variant is predicted to be possibly 335 damaging by PolyPhen, this arginine residue is not conserved in other species and is substituted 336 by histidine in cattle, mouse, and rat, and by serine in both frog and zebrafish (Supplementary 337 Fig.1B). The significance of this sequence variant is unclear and not explored here.

338

339 **3.3 CRIM1 expression analysis**

Global expression databases indicated *CRIM1* expression in the whole eye, without differentiating
between specific tissues. The location of CRIM1 expression in pterygium tissue and normal
conjunctiva from sporadic Northern Irish individuals was therefore assessed by
immunohistochemistry (IHC) and qRT-PCR (Fig.3).

344 CRIM1 was detected, by IHC, lengthwise across the whole pterygium tissue: from the anterior
345 head (Fig.3A) to the posterior tail (Fig.3C). Moreover, CRIM1 was observed both in the external
346 hypertrophic conjunctival epithelium and in the internal fibroblasts, in particular in the vascular
347 endothelial cells surrounding the blood vessels (Fig.3C arrows).

Within the pterygium stroma, CRIM1 was found around unusual structures such as hair follicles and inside sebaceous glands (Fig.3B). Finally, CRIM1 was detected in normal conjunctiva (Fig.3E) and in post-surgical impression cytology samples of unaffected individuals (Fig.3F).

351 *CRIM1* expression level was further investigated using qRT-PCR on RNA obtained from post-

352 surgical impression cytology samples (Fig.3F) of unrelated Northern Irish (4 pterygium and 4

353 controls) individuals, together with a sample from a Northern Irish affected family member (II.2),

found with the *CRIM1* H412P variant (Fig.3G).

355 *CRIM1* expression is significantly higher in pterygium tissues when compared to unaffected 356 conjunctival controls ($2^{-\Delta Ct}$ mean values are 4.22 ± 0.76 and 1.28 ± 0.122 , while median values 357 are 3.4 and 1.3 respectively, p = 0.028). Intriguingly, *CRIM1* expression in the Northern Irish 358 family member II.2 pterygium is lower than that of the all unaffected controls from NI ($2^{-\Delta Ct}$ value 359 of 0.32).

Thus, we have shown that CRIM1 expression, both at the RNA and protein level, is higher in pterygium than in unaffected control tissue.

362

363 3.4 Effects of UV on CRIM1 expression in corneal epithelial (HCE-S) cells

UV radiation is considered the main epidemiologic factor responsible for pterygium and 364 pinguecula development⁹⁻¹¹. Although some papers state that pterygium originates from 365 conjunctiva and Tenon's fibroblasts⁶⁹, the common consensus is that the first trigger for pterygium 366 development occurs at the limbal area^{1,2,70-72} and pterygium growth has often been described as a 367 localised limbal stem cell deficiency⁷⁰. Since the aim of this paper is studying the early events 368 369 triggering pterygium development, we considered that the best model would be a cell line coming 370 from the zone where the initial events characterizing the disease have been described: the limbus. 371 For this reason, for *in vitro* experiments we chose the HCE-S cell line, which spontaneously originated from corneal cells of the limbal area⁴⁴. 372

The effects of UV light exposure upon HCE-S cells *in vitro* were therefore investigated. The average daily dose of UV light has been estimated to be 60-70 J/cm² in central Europe⁷⁴. Considering one hour exposure daily in general population, we irradiated an HCE-S monolayer to a dose of 5.4 J/cm² UVA^{20,75}, and a dose of 0.5 J/cm² UVB.

Following UVA exposure, *CRIM1* expression started increasing from 3 hours, becoming significantly different from untreated control by 6 hours and continuing up to 24 hours $(2^{-\Delta\Delta Ct} \pm$ SEM values at 3, 6 and 24 hours are respectively: 0.90 ± 0.15 , 8.59 ± 0.22 p ≤ 0.05 and $10.39 \pm$ 380 $0.40 \text{ p} \le 0.01$) (Fig.4A). UVB resulted in a significant increase in *CRIM1* expression at 24 hours381after the treatment when compared to the untreated control $(2^{-\Delta\Delta Ct} \pm \text{SEM} \text{ values at } 3, 6 \text{ and } 24$ 382hours are respectively: 1.31 ± 0.03 , 0.90 ± 0.01 and 1.95 ± 0.008 , the last with a p ≤ 0.05). Since383irradiation with UVA has similar, but greater effects on *CRIM1* expression than UVB, subsequent384experiments were conducted using UVA alone.

ERK phosphorylation, previously shown to be increased in pterygium cells following UVA exposure ^{19,20}, was assessed in UVA-treated HCE-S cells as described above. This resulted in a 5.6-fold increase in ERK phosphorylation compared to the untreated control at 6 hours after UVA treatment, rising to 32.1-fold higher at 24 hours (Fig.4B). Treatment of HCE-S cells with the MEK1/2 inhibitor, U0126, prior to UVA irradiation resulted in a complete inhibition of UVAinduced ERK phosphorylation.

Since UVA significantly elevated both *CRIM1* expression and ERK phosphorylation at 24 hours after treatment, the effects of inhibition of ERK phosphorylation on *CRIM1* expression following UV irradiation were studied. Surprisingly, inhibition of ERK phosphorylation in HCE-S cells significantly (p<0.001) potentiated the UV-induced increase in *CRIM1* expression at 24 hours (UVA + inhibitor: 3.75 ± 0.12 , UVA only: 1.82 ± 0.15 , inhibitor only 0.77 ± 0.02 , untreated control: 0.63 ± 0.07 ; values expressed as $2^{-\Delta Ct}$) (Fig.4C).

397 Several studies have shown that increased ERK activity is associated with a decreased 398 expression of the anti-apoptotic transcription factor, B*CL2* and an increased apoptosis⁷⁶. 399 Increased expression of *BCL2* and decreased expression of apoptosis markers has been observed 400 in pterygia^{1,77}. With this in mind, the expression level of *BCL2* was investigated following UVA 401 treatment activating the ERK pathway in HCE-S cells.

402 Either UVA treatment or inhibition of ERK phosphorylation in HCE-S cells alone significantly

403 decreased *BCL2* expression $(2^{-\Delta\Delta Ct} \pm \text{SEM} \text{ values for ERK-I: } 0.53 \pm 0.05 \text{ and UVA } 0.48 \pm 0.06;$

404 both $p \le 0.05$) (Fig.4D). In comparison to those, when HCE-S cells were treated with MEK1/2

405 inhibitor, U0126, and UVA irradiation, *BCL2* expression was significantly increased $(2^{-\Delta\Delta Ct} \pm 406)$ 406 SEM of 1.45 ± 0.13; p < 0.001) and restored levels seen in untreated cells (p = 0.06).

407 UV irradiation has therefore a fundamental role in triggering a series of events, including the
408 increase of *CRIM1* expression and the phosphorylation of ERK, corresponding to a decrease in
409 *BCL2* levels in HCE-S cells.

410

411 3.5 CRIM1 intracellular pathway triggered by UVA

412 CRIM1, ERK and BCL2, as shown above, are interrelated in playing a pivotal role in the 413 intracellular pathway triggered by UV exposure in HCE-S.

414 To further investigate the relationship between those factors, we sought to assess the effects of 415 knocking down expression of *CRIM1* following siRNA transfection. Four siRNAs targeting 416 *CRIM1*, both separately and in a pool, efficiently knocked down endogenous *CRIM1* expression 417 in HCE-S cells to less than 30% of untreated level, 48 hours after transfection ($p \le 0.001$) 418 (Supplementary Fig.2).

Endogenous levels of *CRIM1* expression in HCE-S, normalised to cells transfected with NSC siRNA, increased after UVA treatment by 1.7 ± 0.05 fold, $p \le 0.05$. Because levels of *CRIM1* expression appear critical and finely regulated following UV irradiation, the concentration of siCRIM1 was titrated (0.2nM, 0.5nM, 1nm and 10nM) to establish the concentration at which *CRIM1* expression was the same as non-UVA treated, NSC4-tranfected HCE-S cells (Supplementary Fig.3). The siCRIM1 concentration at which this was achieved was 0.5nM (Fig.5A).

Since pterygium is considered a proliferative rather than degenerative condition^{4,70} and UV light
 alters cell proliferation at the limbal area as the first event promoting pterygium formation⁷⁸, an
 MTT proliferation assay was then performed on HCE-S cells after UVA treatment (Fig.5B). UVA
 significantly increased HCE-S proliferation at 72 hours (Δabsorbance at 72-24 hours, control

430 0.53 ± 0.02 OD vs UVA 0.6 ± 0.03 OD; $p \le 0.05$). When cells were transfected with 0.5nM 431 siCRIM1 prior to UVA treatment, HCE-S proliferation was further increased relative to UVA 432 treatment alone (Δ absorbance at 72-24 hours: 0.7 ± 0.02 OD, with $p \le 0.05$ compared to UVA 433 and $p \le 0.01$ compared to the mock NSC4 control).

As previously shown (Fig.4B), ERK phosphorylation was significantly increased 24 hours
after UV exposure. Transfection of cells with 0.5nM siCRIM1 prior to UV exposure almost
completely abolished the increase in ERK phosphorylation (Fig.5C).

Finally, ERK and *CRIM1* regulation were related to *BCL2* expression levels (Fig.5D), which decreased significantly 24 hours after UVA treatment of HCE-S cells when compared with the untreated control ($2^{-\Delta\Delta Ct}$ values for UVA treated HCE-S: 0.61 ± 0.06 , p ≤ 0.01). However, prior transfection with 0.5nM siCRIM1 prevented the decrease in *BCL2* expression, and was not significantly different from mock NSC4 transfected cells ($2^{-\Delta\Delta Ct}$ values of siCRIM1+UVA: 0.96 ± 0.08).

The latest results obtained using siCRIM1 were able not only to confirm the previous data showing a UVA-mediated increase in *CRIM1* expression, ERK phosphorylation and a decrease in *BCL2* expression, but also to demonstrate that by modulating only *CRIM1* expression, the whole signalling pathway leading to cell proliferation in response to UVA exposure can be regulated.

448

449 **3.6** Effects of wild type and H412P mutant CRIM1 over-expression in HCE-S cells

Based on the outlined pathway involving CRIM1 regulation upon UV exposed HCE-S cells, the
consequences of the H412P *CRIM1* variant in a pterygium pathogenic context were investigated.
The H412P variant was introduced into the human *CRIM1* sequence by site directed mutagenesis
and the complete *CRIM1* sequence checked by Sanger sequencing (Supplementary Table1),

454 confirming the presence of the c.1235 A>C substitution and absence of any other variation in the
 455 sequence.

456 HCE-S cells were transfected with an empty plasmid, wild type or the *CRIM1*:p.H412P 457 expression plasmid. A significant *CRIM1* overexpression with respect to endogenous *CRIM1* was 458 revealed by qRT-PCR both at 48 hours (*CRIM1* wt 6 ± 0.82 , p<0.01 and *CRIM1* H412P 5.8 \pm 459 0.97, p<0.05) and at 72 hours (*CRIM1* wt 6.4 \pm 1.075, p<0.01 and *CRIM1* H412P 6.3 \pm 1.4 460 p<0.01) after transfection (Fig.6A).

461 Since a decrease in proliferation was reported in vascular endothelial cells after transient CRIM1 overexpression⁵⁴, an *in vitro* MTT proliferation assay was then performed in HCE-S cells 462 463 transfected with CRIM1 wild type or H412P constructs (Fig.6B). Compared to mock transfected 464 HCE-S cells, wild type CRIM1 overexpression had a relevant anti-proliferative effect, which was 465 most significant at 72 hours post-transfection (Δ absorbance 72-24 hours 0.60 ± 0.02 OD; p<0.01). This effect was not observed in the CRIM1 H412P transfected cells (Aabsorbance 72-24 hours 466 467 0.52 ± 0.02 OD), which did not differ significantly from the mock transfected control 468 (Δ absorbance 72-24 hours 0.57 \pm 0.01 OD).

469 ERK phosphorylation is increased, simultaneously with CRIM1 expression, in vascular 470 endothelial cells after VEGFA treatment⁷⁹. Here, ERK phosphorylation was strongly increased in 471 the wild type *CRIM1* transfected HCE-S compared to mock transfected control and to H412P 472 *CRIM1* transfected cells at 72 hours post-transfection (Fig.6C).

473 Other gene expression levels, possibly affected by *CRIM1* H412P variant, were investigated 474 by qRT-PCR: *VEGFA* for its role in pterygium angiogenesis^{3,72} and its direct interaction with 475 *CRIM1*⁷³, Transforming Growth Factor beta I (*TGF-\beta I*) for its involvement in several other eye 476 diseases⁸⁰ and *BCL2* for its importance in apoptosis, previously documented in pterygium^{77,81}. 477 HCE-S cells were transfected with wild type and H412P *CRIM1* plasmids and gene expression

478 was assessed at 48 and 72 hours after transfection (Fig.6D). Levels of VEGFA and TGF- βI

479 expression were not significantly different between the wild type and the H412P CRIM1 480 transfected cells (*VEGFA*: wild type 48 hours 0.8351 ± 0.0740 , H412P 48 hours 0.8966 ± 0.0630 , wild type 72 hours 1.1447 ± 0.0940 , H412P 72 h 1.0443 ± 0.1100 , *TGF-* βI : wild type 48h 0.7410 481 482 \pm 0.0730, H412P 48h 0.8630 \pm 0.0480, wild type 72h 0.9428 \pm 0.0650, H412P 72h 1.1810 \pm 0.1080, all values are expressed in $2^{-\Delta Ct}$). In contrast, a significant decrease in *BCL2* expression 483 484 level was observed in the wild type CRIM1 transfected cells with respect to H412P mutant and 485 mock transfected cells (p value <0.05) both at 48 and 72 hours (wild type 48 hours: $0.5453 \pm$ 486 0.0720, H412P 48 hours: 1.1647 ± 0.1800 and wild type 72 hours: 0.5977 ± 0.0240 , H412P 72 hours: 1.2376 \pm 0.1350, all values are expressed in 2^{- Δ Ct}), suggesting that overexpression of 487 CRIM1 may play a role in up-regulation of apoptosis, which is normally induced in cornea and 488 conjunctiva by UV radiation^{82,83}. 489

490 The effect of increased *CRIM1* expression upon apoptosis in HCE-S cells was investigated in 491 CRIM1 transfected cells by TUNEL assay (Fig.6E). At 72 hours after transfection the wild type 492 *CRIM1* transfected HCE-S cells showed a significantly higher rate of apoptosis compared to either 493 the H412P CRIM1 or mock transfected HCE-S. The percentage of apoptotic (TUNEL positive), 494 wild type CRIM1 transfected cells (25.6 ± 1.8) was significantly higher than either H412P CRIM1 495 plasmid or mock transfected HCE-S cells $(3.9 \pm 0.4 \text{ and } 2.7 \pm 0.5 \text{ respectively; } p<0.001)$ (Fig.6E). 496 We have shown here that *CRIM1* plasmid overexpression exerts the same effects of UV induced 497 CRIM1 increased intracellular levels, in terms of decreased cell proliferation, increase in ERK 498 phosphorylation and reduction in BCL2 expression, followed by a higher rate of apoptosis. 499 The presence of H412P variant in CRIM1 however abolished any of those effects, supporting a

role for this variant in the increased susceptibility of members of the Northern Irish family topterygium and pinguecula.

4. **DISCUSSION**

504 UV light presents a chronic stimulus to the eye surface, altering the normal processes of growth 505 control in cornea and conjunctiva, and is associated with several pathologies affecting the anterior eye⁸⁴ such as photokeratitis⁸⁵, climatic droplet keratopathy¹⁰, cortical cataract⁸⁶, squamous cell 506 carcinoma⁸⁷ and pterygium^{9,21}. Pterygium, resulting in 1% of all the ocular surgeries in developed 507 countries⁸⁸ and presenting a 12% surgery recurrence rate⁵, represents a substantial cost to National 508 509 Health Services and the community, estimated in Australia as US \$100M per year⁸⁹. In the 510 identified Northern Irish family (Fig.1), affected by pterygium or pinguecula despite not being 511 exposed to high levels of sunlight, genetic predisposition may play a fundamental role in the 512 etiologic process (Table 1). A WES approach to candidate gene identification with in silico and literature analysis led to selection of H412P variant in CRIM1 gene for several reasons. Firstly, 513 514 the substitution of a highly conserved, positively charged histidine (H) residue with an apolar 515 proline (P) is predicted to interfere with CRIM1 structure and function.

516 Moreover, the interaction between F1 domains of fibronectin, structurally similar to VWF 517 domains of CRIM1 where H412P is located, and VEGF is enhanced at acidic pH $(5.5-7)^{66}$. As 518 Histidine works at this pH range (pKa₃=6), a mutation in this residue may interfere with the 519 electrostatic binding of other ligands.

CRIM1's VWF domains (Fig.2) were also shown essential for interaction with VEGFA⁶², the 520 angiogenic factor overexpressed in the vascularised pterygium tissue following UV exposure^{3,72}. 521 CRIM1 is thus proposed as a VEGFA antagonist^{60,73}, possibly resisting pterygium formation by 522 preventing angiogenesis as it has been shown to prevent proliferation^{54,55}. Proliferation in 523 cardiomyocytes is reduced by CRIM1 upregulation following miR199a silencing^{55,56}, the same 524 miRNA which was found overexpressed in pterygium⁹⁰. In addition to proliferation and 525 angiogenesis, tissue remodelling through cell adhesion and migration also occurs during 526 pterygium formation⁴. This process has been shown to be regulated by CRIM1 in neurons⁵⁷, lung 527

528 cancer⁵⁹ and lens epithelial cells⁵⁸, the latter showing a premature fibre differentiation when 529 *CRIM1* is lost⁹¹.

As previously described, pterygium etiopathogenesis is generally ascribed to UV eye exposure and *CRIM1* was found to be involved in UV related diseases such as bovine eye cancer⁶³ and melanoma⁶⁴. Finally, recent studies have elucidated the importance of *CRIM1* expression during the anterior eye development^{50,51,58}.

CRIM1 was thus selected as our best candidate responsible for pterygium pathogenesis within 534 535 the NI family. However, a reduced penetrance, as previously described in pterygium^{1,26,31}, makes interpretation of genetic data challenging³⁶. While two unaffected members of the NI family (III.2 536 537 and III.3) harbour the H412P variant in CRIM1, no affected member was found that did not have the H412P variant. Moreover, given the younger age of the unaffected members (34 and 48 years 538 539 old), penetrance in this case could be age-related, as described in MEN1 syndrome, in which tumour occurrence increases with the age, becoming fully penetrant only after sixty vears⁹². It is 540 541 therefore possible that III.2 and III.3 family members may never develop pterygium or will do so 542 later in their lifetime; in any case, they should be monitored closely for development of pterygium 543 in the future.

Neither the *CRIM1* H412P variant nor any other alteration within the six VWFC domains of *CRIM1* were found in other affected unrelated individuals from Northern Ireland.

546 Only one other patient from Bolivia was identified with a missense variant located between 547 VWFC-4 and VWFC-5: R745C (Supplementary Fig.1). While this arginine residue is not 548 conserved between the species, the introduction of a cysteine residue may facilitate disulphide 549 bridge formation with highly conserved cysteine residues in the previous or subsequent VWFC 550 domain, vital to the VWF domain's structure^{45,93}.

551 While no variants in *CRIM1* were found in the NI patients, CRIM1 expression was elevated

throughout the pterygium tissue compared to unaffected conjunctiva (Fig.3). In particular, *CRIM1*

553 expression was observed around pterygium blood vessels which, together with its previously

described expression in endothelial cells during capillary formation^{60,61} and its interaction with VEGFA⁷³, suggests a role for CRIM1 during the critical angiogenic processes of pterygium formation^{1,21}. CRIM1 was also detected in unusual structures such as hair follicles and sebaceous glands, previously identified as characteristic features of some pterygia⁷⁰. These structures are representative of an uncontrolled transdifferentiation program⁹⁴ occurring in pterygium in addition to an epithelial-mesenchymal transition (EMT) or fibrosis process⁹⁵, and likely reflects similarities between pterygia, limbal dermoids and hair epithelia⁹⁶.

561 Comparing *CRIM1* expression levels between pterygium-affected and -unaffected individuals, 562 those were significantly increased in the affected samples (Fig.3G), while the lowest *CRIM1* 563 expression was recorded for the affected family member (II.2). A high *CRIM1* expression in 564 pterygium-affected individuals could therefore represent a defensive cellular response mechanism 565 against UV damaging effects, which is impaired by the H412P variant within the NI family.

566

567 Involvement of CRIM1 in UV-related diseases either in the eye^{63} or the $skin^{64}$ is not surprising 568 considering that UV irradiation affects the balance between proliferation and apoptosis in several 569 cell types, including skin keratinocyes^{97,98}, corneal epithelium⁸² and pterygium basal epithelial 570 cells⁷⁷. However, while the eye is exposed to both UVA and UVB radiation, the intensity of UVA 571 radiation reaching the Earth's surface has 10-100 times the intensity of UVB⁹⁹ and it is known to 572 induce oxidative stress in exposed cells and tissues, including pterygium¹⁰⁰. Accordingly, UVA 573 induced a more rapid and larger response than UVB in HCE-S cells (Fig.4A).

574 Exposure of HCE-S cells to UVA radiation leads to an increase in *CRIM1* (Fig.4A) expression. 575 The elevated ERK phosphorylation upon UVA irradiation we observed (Fig.4B) was previously 576 described in UVA or UVB irradiated pterygium and conjunctival cells^{19,20}. While inhibition of 577 ERK phosphorylation alone had no effect on the expression of *CRIM1*, it potentiated the *CRIM1* 578 up-regulation when HCE-S cells were treated with UV (Fig.4C). This underscores the importance 579 of UV irradiation as the primary trigger for the intracellular pathway but underlines also the capacity of ERK phosphorylation to regulate *CRIM1* expression levels. Moreover, inhibition of
 ERK phosphorylation in HCE-S cells treated with UV abolished the UV-induced decrease in
 BCL2 expression (Fig.4D): UV decreases *BCL2* levels through ERK phosphorylation, which
 results therefore in upstream *BCL2* regulation within this pathway.

584 The role of CRIM1 in the cellular response to UV exposure was confirmed in HCE-S cells in 585 which CRIM1 over-expression induced by UV treatment was inhibited by siRNA transfection 586 (Fig.5A). An increased cell proliferation in siCRIM1-treated cells upon UV exposure (Fig.5B), 587 suggests that CRIM1 has a role in protecting against pterygium formation by minimising cell 588 proliferation in response to UV radiation. When siCRIM1 was added to UV treatment, HCE-S 589 cells decreased ERK phosphorylation to that seen in untreated cells (Fig.5C) and restored BCL2 590 basal expression (Fig.5D), revealing that also CRIM1 is able to control ERK phosphorylation as 591 well as *BCL2* expression and locates it upstream of those two factors within the pathway. Those 592 experiments showed how CRIM1 exerts a central role within the UV triggered intracellular 593 pathway and silencing its expression is enough to counter all those alterations we examined.

594 Previous studies have shown how CRIM1 alterations influences cellular function: multiple exon deletions of *CRIM1* lead to syndromic disease⁵² while a minor alternately spliced *Crim1* isoform 595 596 determines perinatal lethality with multiple organ dysfunction⁵¹; however, the effects of *CRIM1* 597 missense mutations still remain unexplored. Therefore, based on the intracellular mechanism 598 described, the effects of the H412P variant found within the Northern Irish kindred were studied. 599 CRIM1 over-expression in HCE-S cells leads to reduced proliferation (Fig.6B) and a parallel 600 increase in apoptosis (Fig.6D), as expected following the previous results where CRIM1 was 601 shown having a protective role in pterygium formation, counteracting the UV-induced 602 proliferation (Fig.4B). This is in accordance with the downregulation of vascular endothelial cell proliferation after transient CRIM1 overexpression⁵⁴ and with the increase in cardiomyocyte 603 604 proliferation following CRIM1 silencing through miR-199a^{55,56}. Since the same miR-199a was found upregulated in pterygium⁹⁰, this could analogously increase cell proliferation in pterygium 605

606 formation through *CRIM1* silencing, counteracting the protective effects of *CRIM1* 607 overexpression.

608 *CRIM1* overexpression was also able to increase ERK phosphorylation (Fig.6B) following UVA 609 irradiation (Fig.4B). This result confirms previous data (Fig.5C) showing that the effects of 610 *CRIM1* overexpression lie upstream of ERK phosphorylation and demonstrates that it does not 611 need UV to exert its function.

All the effects of *CRIM1* overexpression in HCE-S cells were almost completely abolished by the
H412P variant in *CRIM1* (Fig.6).

The results obtained delineated a finely regulated pathway where either UV (Figure 4B) or elevated CRIM1 levels (Figure 6C) increase ERK phosphorylation, demonstrating that CRIM1 lies upstream of, and is responsible for ERK phosphorylation.

- CRIM1 expression was increased upon UV exposure and this increase was potentiated when the
 U0126 inhibitor was added to the UV-irradiated cells. This suggests a feedback mechanism in
 which ERK phosphorylation, increased by elevated *CRIM1* levels, serves to limit the rise in *CRIM1* expression triggered by UV (Fig.7). UV is therefore necessary for the activation of the
 whole pathway because the U0126 inhibitor alone, without UV treatment, does not increase
 CRIM1 expression (Figure 4C). For the first time an increase in *CRIM1* expression has been
 directly correlated with a consequent activation of the ERK pathway in corneal cells.
- Based on the *in vitro* experimental evidence and on the feedback mechanism outlined, H412P can be considered either a loss-of-function or a dominant-negative mutation. Similarly, a study on a shortened mouse *Crim1* isoform was unable to conclude whether the mechanism was hypomorphic (reduced activity) or dominant negative⁵¹, although the latter have been described in growth factor receptors which bind the same CRIM1 interactors such as PDGF¹⁰¹ or VEGF¹⁰² and in VWF domains similar to those found in CRIM1^{103,104}.
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5. CONCLUSIONS

In summary, we have shown that *CRIM1* plays an important role in the response of corneal cells to UV irradiation by activation of a finely regulated intracellular pathway where increased *CRIM1* expression counteracts cell proliferation and increases apoptosis. We demonstrate that *CRIM1* H412P variant impairs this response, suggesting its involvement in the increased susceptibility to pterygium observed in a Northern Irish family. Further investigation of the role of CRIM1 in UV triggered pathways and pterygium holds promise as a target for the prevention and treatment of development or recurrence of pterygium or other UV related diseases.

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648



652 Figure 1. Northern Irish family affected by pterygium or pinguecula and WES analysis. (A). Pedigree 653 of a Northern Irish family affected with pterygium. Open symbols denote unaffected individuals; 654 filled black symbols denote pterygium affected individuals and filled black symbols with open circles inside denote pinguecula affected individuals. Squares represent male and circles represent female 655 656 individuals. Slashed symbols denote deceased family members and question marks are for individuals 657 who have not participated in the study. Although all family members were invited to participate in 658 this study, only those who gave informed consent were enrolled. (B). Pinguecula visible at the limbal 659 area, between the cornea and conjunctiva (arrowhead). The image was obtained from family member 660 III:5. (C). WES variants screening. Ingenuity variant analysis screened the 451,153 variants obtained 661 with WES using four subsequent filters: Confidence to ensure quality, Common Variants (MAF < 662 0.05), Predicted deleterious using Polyphen and SIFT and Genetic, assuming an autosomal dominant inheritance pattern. A final In silico analysis based on the residue conservation through BLAST and 663 664 Clustal Omega, expression analysis using TiGER database and literature research allowed selection 665 of a single candidate variant in CRIM1 gene.



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Figure 2. CRIM1 (Cysteine RIch transMembrane BMP regulator 1) sequence in the NI family, 667 conservation and structure analysis. (A). Electropherogram of Sanger sequencing performed in the 668 669 family members confirmed the c.1235 A>C heterozygous transversion mutation in affected 670 individuals and which was homozygous for wild-type A allele in the unaffected control. (B). Clustal X2.1 multiple sequence alignment of orthologous CRIM1 protein sequences from Human 671 672 (NP_057525.1), Cattle (NP_001192227.1), Mouse (NP_056615.1), Rat (NP_001162574.1), Frog 673 (NP_001163917.1), Zebrafish (NP_997986.1) indicates that the H412P missense mutation occurs in 674 a highly conserved residue. EsPript 3.0. Legend: Red box, white character: strict identity; Red character: similarity in a group; Blue frame: Similarity (C). Schematic ideogram of Human CRIM1, 675 676 characterised by multiple domains: one transmembrane (blue), six von Willebrand factor (VWF) (yellow), four antistasin-like (green) and one IGFBP (red). The position of the H412P and the R745C 677 678 mutation are indicated. (D). Clustal X2.1 alignment of the six VWFC domains in CRIM1 shows that, 679 other than in VWFC-2, H412 is not conserved in the other five VWFC domains. (E). Clustal X2.1

- alignment of CRIM1 with other BMP antagonists indicates that the residue H412 is conserved in the
- 681 VWFC-2 domain of human chordin and in the VWFC-2 and 3 domains of human neuralin.
- 682 across groups (if more than 70% of the residues within the frame share similar physico-chemical

683 properties).



686 Figure 3. CRIM1 expression analysis in pterygium and conjunctiva from Northern Ireland. A-F 687 images represent immunohistochemical (IHC) staining of CRIM1 expression (green) and nuclei 688 (DAPI, blue) in pterygium and conjunctival tissues obtained from sporadic Northern Irish patients. 689 Scale bar, 100µm (A). CRIM1 expression in a pterygium head, visible in both the external epithelial 690 layer (E) as well as the internal stroma (S). (B). CRIM1 expression shown in peculiar structures 691 identified in pterygium stroma: a hair follicle (empty arrowhead) and a sebaceous gland (plain 692 arrowhead). (C). CRIM1 expression in pterygium tail, arrowheads indicate CRIM1 expression in 693 cells surrounding the blood vessels. (D). Negative IgG control in pterygium tail tissue. (E). CRIM1 694 expression in conjunctival epithelial tissue of an unaffected individual. (F). IHC of a post-surgical impression cytology sample obtained from unaffected superficial epithelial conjunctival cells: 695 696 CRIM1 is expressed in all the cells captured on the membrane filter. (G) CRIM1 expression is

- 697 increased in pterygium patients from Northern Ireland. qRT-PCR analysis of *CRIM1* carried out in 698 cDNA from post-surgical impression cytology samples: NI conjunctival individual controls, NI 699 pterygium individuals and one affected NI family member (II.2 in Fig. 1). All values are expressed 690 in $2^{-\Delta Ct} \pm$ SEM. n=2 with two technical replicates each sample.
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Figure 4. UV treatment of HCE-S cells increases CRIM1 expression, ERK phosphorylation and 703 704 decreases BCL2 expression. (A). qRT-PCR revealed significantly increased CRIM1 expression levels 705 in HCE-S cells at 6 ($p \le 0.05$) and 24 hours ($p \le 0.01$) after UVA treatment and at 24 hours ($p \le 0.05$) 706 after UVB treatment compared to the untreated control. (B). Western blot analysis of HCE-S cell 707 lysates revealed an increase in ERK phosphorylation at 6 and 24 hours after UVA irradiation with 708 respect to ERK phosphorylation levels of untreated control. Pretreatment of cells with U0126 709 inhibitor abolished ERK phosphorylation in UV treated cells. (C). gRT-PCR was used to evaluate 710 CRIM1 expression following UV treatment in the presence of MEK1/2 inhibitor, U0126. Inhibition 711 of ERK pathway activation enhances the increase in CRIM1 expression seen in UV irradiated HCE-712 S cells whilst inhibition of ERK phosphorylation alone has no significant effect. (D). BCL2 713 expression measured by qRT-PCR. HCE-S cells were UVA irradiated with and without U0126 and

- 714 harvested 24 hours after treatment. A significant decrease in *BCL2* expression was observed when
- the cells were treated with either U0126 or UVA alone but not in combination when compared to the
- 716 HCE-S untreated cells. qRT-PCR data represent fold change of the $2^{-\Delta\Delta Ct}$ mean \pm SEM compared to
- 717 untreated HCE-S. n=3 with three technical replicates each.
- 718





Figure 5. siCRIM1 0.5nM is able to restore normal HCE-S conditions after UVA treatment. (A). 720 721 qRT-PCR shows the amount of siCRIM1 (0.5nM) able to restore endogenous CRIM1 levels in 722 HCE-S after UVA exposure. For all the experiments HCE-S cells were treated with: NSC4, UVA + NSC4 and UVA+ siCRIM1 (0.5nM) and harvested 24 hours later. Data represent $2^{-\Delta Ct} \pm SEM$. n=3 723 with three technical replicates for each sample. (B). An MTT assay demonstrates an increased 724 725 proliferation upon UVA exposure, which is further increased if CRIM1 expression is restored to 726 pre-treatment endogenous levels (siRNA 0.5nM), confirming the antiproliferative effect of CRIM1. 727 n=6 with eight technical replicates for each condition. (C). Western Blot analysis shows ERK 728 phosphorylation (pERK) at 24 hours after UVA treatment in HCE-S cells. The increased ERK 729 phosphorylation due to UVA exposure was brought back to normal levels following transfection 730 with 0.5nM CRIM1 siRNA. (D). BCL2 expression, measured by qRT-PCR, decreases upon UVA

- radiation eliciting apoptosis but is restored in HCE-S cells treated with siCRIM1. Data represent 2⁻
- $\Delta\Delta Ct \pm SEM$ with respect to untransfected HCE-S. n=3 with three technical replicates each sample.

Figure 6. CRIM1 wild type overexpression in HCE-S cells results in a decrease in cell proliferation 735 736 and an increase in ERK phosphorylation and apoptosis. (A). qRT-PCR showing CRIM1 737 overexpression in mRNA obtained from HCE-S cells transfected with Human CRIM1 wild type and 738 mutant (H412P) in pcDNA3.1 expression plasmid. Both CRIM1 wild type (wt) and H412P mutant 739 were significantly overexpressed at 48 and 72 hours after transfection with respect to the mock control. Data represent fold change of the $2^{-\Delta Ct}$ mean \pm SEM. n=3 with 3 technical replicates for each 740 741 condition. (B). An MTT assay of HCE-S cell proliferation was performed at 72 hours after 742 transfection with empty plasmid (mock), CRIM1 wt and H412P mutant constructs. CRIM1 wt, when overexpressed, has an anti-proliferative effect when compared to the mock transfected control 743 744 (p<0.001). Overexpression of the mutated H412P CRIM1 does not have the same anti-proliferative

745	effect. n=6 with 8 technical replicates for each condition. (C). ERK phosphorylation (pERK) was
746	detected by Western Blot analysis at 72 hours post transfection with mock, CRIM1 wt and H412P
747	plasmids in HCE-S cells. CRIM1 wt overexpression resulted in a high level of ERK phosphorylation
748	in comparison to <i>CRIM1</i> H412P and mock control. (D). qRT-PCR analysis of <i>VEGFA</i> , <i>TGF-βI</i> and
749	BCL2 expression in HCE-S cells transfected with mock, CRIM1 wt and H412P plasmids and
750	harvested after 48 and 72 hours. No significant variation of VEGFA and TGF- βI expression was
751	observed between mock, CRIM1 wt and CRIM1 H412P while BCL2 expression significantly
752	decreased in H412 wt CRIM1 transfected cells compared with mock transfected HCE-S both at 48
753	and 72 hours post transfection. Data represent fold change of the $2^{-\Delta Ct}$ mean \pm SEM respect to mock
754	transfected HCE-S. n=3 with three technical replicates each. (E). TUNEL assay in HCE-S cells
755	transfected with wt and H412P mutant CRIM1 plasmids. TUNEL-positive cells are stained green and
756	nuclei are stained blue with DAPI. Overexpression of CRIM1 wt results in increased apoptosis
757	compared to either the CRIM1 H412P or mock transfected cells. Scale bar, 100 µm. TUNEL assay
758	quantification was performed in 12 fields per condition using ImageJ. n=3
750	

Figure 7. CRIM1 regulates pERK in a looped pathway. Here, based on our experimental evidence, 764 765 we propose, within a schematic, an intracellular pathway triggered by UV that may act as a protective mechanism against pterygium development. When cells are exposed to UV light, CRIM1 766 767 expression is increased, which in turn increases ERK phosphorylation. This, in turn, blocks further 768 CRIM1 expression in the feedback loop shown. Inhibition of ERK phosphorylation prevents this 769 negative feedback, resulting in further increases of CRIM1 expression. 770 ERK phosphorylation induces decreased expression of the anti-apoptotic BCL2, initiating cell 771 apoptosis. This proposed pathway was shown to be impaired in the case of the H412P mutation in 772 CRIM1, found in the Northern Irish family affected by pterygium.

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- 774

775

Supplementary Figure 1. R745C variant found in a pterygium affected patient from Bolivia. (A).
Electropherogram showing the novel c.2299C>T transition variant from the Bolivian patient (B1),
which corresponds to the R745C variant. (B). Multiple *CRIM1* sequence alignment shows that the

779 R745 residue is not conserved across species; it is conserved however the absence of cysteine between

780 VWFC-4 (residues 677-735) and VWFC-5 (residues 751-809).

CRIM1

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Supplementary Figure 2. siRNAs targeting *CRIM1* efficiently knock down its expression in HCE-S cells. *CRIM1* expression obtained using qRT-PCR of HCE-S cell cDNA 48 hours post transfection. All four siRNAs tested were able to knock down *CRIM1* expression, including the pool of four siRNAs used at the same final concentration of 10 nM. qRT-PCR data represent fold change of the $2^{-\Delta\Delta Ct}$ mean ± SEM with respect to untreated HCE-S. n=2 with three technical replicates each.

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Supplementary Figure 3. A dose response curve shows that 0.5nM siRNA pool restores HCE-S endogenous *CRIM1* levels. *CRIM1* expression obtained using qRT-PCR of HCE-S cell cDNA 48 hours post transfection with UVA and the siRNA pool at different concentrations. qRT-PCR data represent fold change of the $2^{-\Delta\Delta Ct}$ mean ± SEM with respect to untreated HCE-S. n=2 with three technical replicates each.

TABLES

Family member	Year of birth	Sex	Eye condition	Age of diagnosis	Lived abroad	Time in sunny climates	Wear sunglasses
II.2	1944	F	pterygium	40 years	No	1/year, 3 weeks	Sometimes
II.4	1946	F	pterygium	59 years	Canada 13 years	1/year, 2 weeks	No
II.9	1958	М	unaffected	-	No	1/year, 3 weeks	Yes
II.14	1951	М	pterygium	62 years	No	1/year, 1 week	Yes
III.2	1967	М	unaffected	-	No	1/year, 1 week	Yes
III.3	1982	F	unaffected	-	No	1/year, 1 week	Sometimes
III.5	1968	F	pinguecula	38 years	Canada 13 years	1/year, 2 weeks	Sometimes
III.6	1970	F	pinguecula	40 years	Canada 13 years	1/year, 2 weeks	Sometimes

Table 1. Questionnaire results. The Northern Irish family members participating in the study are
798 listed with age of pterygium diagnosis, averaged 48 years old. The family were shown not to have
799 had excessive exposure to the sun, spending time in sunny climates from 1 to 3 weeks per years with
800 most stating that they wear sunglasses.

Gene Symbol	Chr	Position	Gene Region	Protein Variant	Conservation to:	Gene expressed in:	Associated Diseases
CTRC	1	15764869	Promoter		Human	pancreas, liver, spleen	chronic pancreatitis
KIF21B	1	200978030	Exonic	p.T105M	Elephant	eye (cornea), brain, blood	multiple sclerosis, inflammatory bowel disease
GALNT14	2	31348076	Intronic; Exonic; 5'UTR	p.A58V	Human	kidney, PNS, blood, eye	breast cancer
CRIM1	2	36706700	Exonic	p.H412P	Lamprey	placenta, kidney, PNS, eye	syndactyly, neuronitis, bovine ocular carcinoma
SPTBN1	2	54886366	Exonic	p.T2107A; p.T2094A	Dog	tongue, soft tissue, eye	fissured tongue, and buphthalmos
VRK2	2	58313555	Exonic; ncRNA	p.G113E; p.G90E	Elephant	lymph node, testis, colon	vaccinia, and hypoxia
PLEK	2	68615546	Exonic	p.N229H	X_tropicalis	bone marrow, lymph node, eye	aarskog-scott syndrome, centronuclear myopathy
HNMT	2	138772049	3'UTR	•	Dog	bladder, kidney, liver, eye	asthma, eosinophilia-myalgia syndrome
ITGB6	2	160994200	Exonic	p.H469N	Mouse	pancreas, tongue, stomach, eye	bullous keratopathy, mouth disease
WDR12	2	203760891	Exonic	p.L169S	Zebrafish	blood, intestine, thymus, eye	gigantism, and neuronal ceroid lipofuscinosis
CLDN16	3	190106072	Exonic	p.A56fs*16	Rhesus	kidney, ovary, uterus	nephrocalcinosis, and hypomagnesemia primary
CLDN16	3	190106074	Exonic	p.A56P	Rhesus	kidney, ovary, uterus	nephrocalcinosis, and hypomagnesemia primary
PRIM2	6	57398226	Exonic	p.G310V	Human	thymus, ovary, mammary, eve	······································
STK31	7	23811795	Exonic: ncRNA	n N598_S600delinsKKI	Elephant	testis	
TYW1	7	66660242	Exonic	n H632R	Human	stomach heart colon eve	fanconi's anemia and cytochrome p450
GLDC	9	6556206	Exonic	n I717V	Zebrafish	small intesting kidney liver eve	glycine encenhalonathy
IFNA5	9	21304891	Exonic	p.1717 v	Elephant	sman mestne, kluley, iver, eye	hemorrhagic fever
DMRTA1	9	22451120	Exonic	p.01228	Rhesus		metabolic acidosis prostatitis
MIR/1289	9	91360776	microRNA	p.112421	Dog		incubone actuosis, prosuntis
HBG2	11	5276282	Promoter		Rhesus	spleen thymus liver	sulfhemoglohinemia, cavernous hemangioma
OR/C16	11	55339462	Promoter		Human	spicen, mynus, nver	neuronitis
OR10AG1	11	55735808	Exonic	n H/13D	Rhesus		neuronitis
C12orf56	12	64712620	Exonic: Intronic	p.1143D	Human		neuronnus
ATP8B4	15	50223420	Exonic: ncRNA	p.R5130	Lamprey	blood, bone marrow	intrahepatic cholestasis
DMXL2	15	51773329	Exonic	p.D1356N; p.D1992N	Chicken	blood, testis, heart, eye	gynecomastia, and myocardial infarction
TPSD1	16	1306802	Exonic	p.I87V	Human		asthma, allergic and inflammatory disorders
TPSD1	16	1306817	Exonic	p.A92T	Human		asthma, allergic and inflammatory disorders
NOMO1	16	18544469	Exonic	p.R418H	Chicken	intestine, cervix, colon, eye	pseudoxanthoma elasticum (PXE)
SRCAP	16	30731568	Exonic	p.R968H	Chicken	Larynx, thymus, spleen, eye (cornea)	floating-harbor syndrome, Eosinophilic angiocentric fibrosis
ZNF319	16	58031913	Exonic	p.A86V	Elephant	bladder, blood, tongue	
PDPR	16	70154480	Exonic	p.T29A	Human	heart, bone, Eye	sarcosinemia
				p 454 455insH:			
ZFHX3	16	72832474	Exonic	p.1368 1369insH	X tropicalis		prostate cancer, acute myocardial infarction
WWOX	16	78466409	Exonic	p.L272F	Rhesus	mammary, tongue, PNS, eye	toxic pneumonitis, and aspiration pneumonitis
GSDMA	17	38122680	Exonic	p.V128L	Human		atopy, gastric cancer
DNAI2	17	72306188	Intronic; Exonic	p.V460V	Elephant	testis, uterus, lung, brain	ciliary dyskinesia
MAN2B1	19	12774537	Exonic	p.P248L	Dog	bone, PNS, intestine, eye	alpha-mannosidosis, alpha-mannosidosis, adult form
CYP4F2	19	15989730	Exonic	p.T472A	Mouse	intestine, blood, liver, muscle	warfarin sensitivity, cytochrome p450
PRKD2	19	47204207	Exonic	p.V167M; p.V324M	Lamprey	Spleen, tongue, ovary, eve	polycystic kidney disease, gastric cancer
NI DD7	10	55451405	Evonio	n P2610	V tropicalic	1 , 6, , ,	aestational transphalactic peoplean hudetidiform male
TTC2	21	38560004	Exonic	p.r201Q	X tropicalis	Tongue uterus baset ava	down syndrome critical ragion, down syndrome
1103	21	30309884	EXONIC	p.018055	A_uopicans	rongue, uterus, neart, eye	down syndrome cruccal region, down syndrome

Come Semilar	Turneletter Innert	SIFT Function	SIFT	PolyPhen-2 Function	JLEND ID	NHLBI ESP	Co-segregation within the family (IGV)					
Gene Symbol	I ranslation Impact	Prediction	Score	Prediction	absnp iD	European Frequency (%)	II.2	II.4	111.5	III.6	II.14	II.9
CTRC					144717165		Het	Het	Hom WT	Het	Het	Hom WT
KIF21B	missense	Damaging	0,01	Benign	150551633	0,01	Het	Het	Het	Het	Het	Hom WT
GALNT14	missense	Tolerated	0,26	Benign	41280621		Het	Het	Het	Het	Het	Hom WT
CRIM1	missense	Damaging	0,01	Probably Damaging	113372122	0,29	Het	Het	Het	Het	Het	Hom WT
SPTBN1	missense	Tolerated	0,72	Benign	147989241	0,16	Het	Het	Het	Hom WT	Het	Hom WT
VRK2	missense	Damaging	0,02	Benign	147530902	0,36	Het	Het	Het	Hom WT	Het	Hom WT
PLEK	missense	Tolerated	0,52	Benign	34338164	1,14	Het	Het	Het	Hom WT	Het	Hom WT
HNMT							Het	Het	Het	Hom WT	Het	Hom WT
ITGB6	missense	Tolerated	0,65	Benign	142197545	0,29	Het	Het	Hom WT	Het	Het	Hom WT
WDR12	missense	Damaging	0	Possibly Damaging		0	Het	Het	Hom WT	Het	Het	Hom WT
CLDN16	frameshift				56086318	0	Het	Het	Het	Het	Het	Hom WT
CLDN16	missense	Damaging	0,04	Benign	3214506	0	Het	Het	Het	Het	Het	Hom WT
PRIM2	missense	0 0		Ç	77436138		Het	Het	Het	Het	Het	Hom WT
STK31	in-frame					0	Het	Het	Het	Het	Het	Hom WT
TYW1	in-frame	Tolerated	0,5	Benign		0	Het	Het	Het	Het	Het	Hom WT
GLDC	missense	Tolerated	1	Benign	117460214	0,03	Het	Het	Hom WT	Het	Het	Hom WT
IFNA5	missense	Damaging	0	Probably Damaging	140371188	0.59	Het	Het	Hom WT	Hom WT	Het	Hom WT
DMRTA1	missense	Tolerated	0,54	Benign	145718826	0,38	Het	Het	Hom WT	Hom WT	Het	Hom WT
MIR4289							Het	Het	Het	Hom WT	Het	Hom WT
HBG2					113622787		Het	Het	Het	Hom WT	Het	Hom WT
OR4C16							Het	Het	Hom m	Het	Het	Hom WT
OR10AG1	in-frame	Activating	1	Benign		0	Het	Het	Hom m	Het	Het	Hom WT
C12orf56	missense	Tolerated	0,13		367932023	0,03	Het	Het	Hom WT	Het	Het	Hom WT
ATP8B4	missense	Damaging	0	Probably Damaging		0	Het	Het	Hom WT	Het	Het	Hom WT
DMXL2	missense	Tolerated	0,17	Benign	144241909	0,02	Het	Het	Hom WT	Het	Het	Hom WT
TPSDI	missense	Tolerated	0,31	Benign	2401930	0	Het	Het	Het	Het	Het	0
TPSD1	missense	Tolerated	0,75	Benign	3993983	0	Het	Het	Het	Het	Het	0
NOMOI	missense	Democrated	0,25	Benign Dessible Demosine	140359200	0,12	Het	Het	Hom WI	Hom w I	Het	Hom WI
ZNE210	missense	Damaging	0	Possibly Damaging	3688/6335	0,01	Het	Het	Hom WT	Het	Het	Hom w I
ZNI'319	missense	Tolerated	0,08	Denign	200460749	0	Het	Het		Het	Het	U
7FHX3	in-frame	Tolerated	1	Denigii	200409748	0	Het	Het	Hom WT	Het	Het	Hom WT
WWOX	missense	Tolerated	0.7	Banian	186745328	0.46	Hot	Hot	Hom WT	Hot	Hot	Hom WT
GSDMA	missense	Activating	1	Benign	180743328	0,40	Het	Het	Het	Het	Het	Hom WT
DNAI2	synonymous	rictivating	•	Demgn	148947094	0.05	Het	Het	Hom WT	Het	Het	Hom WT
MAN2B1	missense	Damaging	0,01	Possibly Damaging	117843968	0,42	Het	Het	Het	Het	Het	Hom WT
CYP4F2	missense	Tolerated	0,39	Benign	4020346	0	Het	Het	Hom WT	Hom WT	Het	Hom WT
PRKD2	missense	Tolerated	0,15	Benign	45455991	0,98	Het	Het	Hom WT	Het	Het	Hom WT
NLRP7	missense	Damaging	0	Probably Damaging		0	Het	Het	Het	Hom WT	Het	Hom WT
TTC3	missense	Tolerated	0,55	Possibly Damaging		0	Het	Het	Het	Hom WT	Het	Hom WT

Table 2. Candidate genes analysis. The table shows the 40 genes obtained from Ingenuity and the analysis done for each of the associated variants. The selection was mainly based on the expression of the gene in the eye (TIGER database), the SIFT and Polyphen predictions and known diseases association (literature review). Subsequently the aminoacid conservation through vertebrate species (BLAST analysis) and co-segregation of the mutation in pterygium (II.2, II.4, II.14) and pinguecula (III.5, III.6) affected members and not in the II.9 unaffected family member (IGV analysis) was considered. This screening led to the selection of CRIM1 as the best candidate considering those parameters.

- 813 A.

Patient NI	Year of birth	Sex	Eye condition
1	1942	М	pterygium
2	1950	М	minor pterygium
3	1931	М	pterygium
4	1954	М	pterygium
5	1946	М	pterygium
6	1975	М	pterygium
7	1941	М	pterygium
8	1958	F	pterygium
9	1984	F	pterygium
10	1934	М	pterygium
11	1974	F	pterygium
12*	1967	F	pterygium family

818 B.

Patient Bolivia	Year of birth	Sex	Eye condition
B1	1965	F	pterygium
B2		М	pterygium
В3	1949	М	pterygium
B4	1957	М	pterygium
В5	1999	М	pterygium
B6	1952	М	pterygium
B7	1947	F	pterygium
B8	1941	М	pterygium
B9	1990	М	pterygium

Table 3. Individual pterygium participants. (A). A list of pterygium affected individuals collected in 821 the UK and the relevant information obtained through the questionnaire. *Patient 12 denotes the 822 pterygium affected II.1 family member previously studied²⁷. (B). List of pterygium affected 823 individuals collected in Bolivia and the year of birth and sex of participants.

CRIM1 sequence	Primer sequence (5' to 3')
T7_F	TAATACGACTCACTATAGGG
Seq1_R	GCAGAATGTGCAGTCGTCTT
Seq1_F	TGATCGAGGGTTATGCTCCT
Seq2_F	TACTACGTGCCCGAAGGAGA
Seq2_R	GGCACTTTCACAGGGTTTGT
Seq3_F	TGCCGGGAATGCTACTGT
Seq3_R	ACAGAAGGGCAGGACTCAGA
Seq4_F	CTGAGTCCTGGAAGCCTGAC
Seq4_R	CCTGGAGGTGACCCATATCT
Seq5_F	AACCATCGAGGAGAGGTTGA
Seq5_R	TCGTCTTCCGTCTTTTGAAAC

Supplementary Table 1. Primers used to validate that the whole of the CRIM1 sequence was inserted
into the pcDNA3.1 plasmid and to check the presence of the H412P mutation introduced by site
directed mutagenesis.

CRIM1 VWFs	Primer sequence (5' to 3')
Exon6_F (VWF1)	TTGAAAAACATCAAAGGACACAA
Exon6_R (VWF1)	CCATGTATGCTCCTGTTAATCTG
Exon7_F (VWF2)	GATGACTAGAACCCAGGGAAAA
Exon7_R (VWF2)	AGCAGACATTATGCCCAAGG
Exon11_F (VWF3)	GCCTGTTTCTCCTGTGCAGT
Exon11_R (VWF3)	TGCAAGGCAGAAGTCATTTG
Exon12_F (VWF4)	CCAGGCTTTCAAGAGTTGGA
Exon12_R (VWF4)	GGGTCCCACAGAATGACAAC
Exon13_F (VWF5)	CTGGCCAACAGCATCTTCTT
Exon13_R (VWF5)	GACATGTCAAGCAGGGAAAAA
Exon14_F (VWF6)	AAGATCGTGTGCGTTGTCAC
Exon14_R (VWF6)	GTCGAGCTCTGCTTCGATTT

841 Supplementary Table 2. Primers used to verify the presence of other mutations in all the VWF

domains of CRIM1.

844 8. REFERENCES

- Chui, J., Di Girolamo, N., Wakefield, D. & Coroneo, M. T. The pathogenesis of pterygium:
 current concepts and their therapeutic implications. *The ocular surface* 6, 24-43 (2008).
- B47 2 Das, P. *et al.* Limbal epithelial stem-microenvironmental alteration leads to pterygium
 development. *Molecular and cellular biochemistry* 402, 123-139, doi:10.1007/s11010-0142320-z (2015).
- Bianchi, E. *et al.* Immunohistochemical profile of VEGF, TGF-beta and PGE(2) in human
 pterygium and normal conjunctiva: experimental study and review of the literature. *Int J Immunopathol Pharmacol* 25, 607-615 (2012).
- 4 Detorakis, E. T. & Spandidos, D. A. Pathogenetic mechanisms and treatment options for
 ophthalmic pterygium: trends and perspectives (Review). *International journal of molecular medicine* 23, 439 (2009).
- Solution Structure
 Solution Structure</l
- Kaji, Y. *et al.* Immunohistochemical localization of advanced glycation end products in
 pinguecula. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 244, 104-108,
 doi:10.1007/s00417-005-0047-y (2006).
- Jakobiec, F. A., Rashid, A., Bozorg, M. S. & Dana, R. Unusual large uniocular elastoid and
 collagenous pinguecula. *Graefe's archive for clinical and experimental ophthalmology* = *Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 252, 11731175, doi:10.1007/s00417-014-2649-8 (2014).
- 867 8 Lemercier, G., Cornand, G. & Burckhart, M. F. [Pinguecula and pterygium: histologic and
 868 electron microscopic study (author's transl)]. *Virchows Archiv. A, Pathological anatomy and*869 *histology* 379, 321-333 (1978).
- Moran, D. J. & Hollows, F. C. Pterygium and ultraviolet radiation: a positive correlation. *British Journal of Ophthalmology* 68, 343-346 (1984).
- Taylor, H. R. *et al.* Corneal changes associated with chronic UV irradiation. *Archives of Ophthalmology* **107**, 1481-1484 (1989).
- McCarty, C. A., Fu, C. L. & Taylor, H. R. Epidemiology of pterygium in Victoria,
 Australia. *British Journal of Ophthalmology* 84, 289-292 (2000).
- Zhong, H. *et al.* Ethnic Variations in Pterygium in a Rural Population in Southwestern
 China: The Yunnan Minority Eye Studies. *Ophthalmic Epidemiol* 23, 116-121,
 doi:10.3109/09286586.2015.1099685 (2016).
- 879 13 Gazzard, G. *et al.* Pterygium in Indonesia: prevalence, severity and risk factors. *British*880 *journal of ophthalmology* 86, 1341-1346 (2002).
- Lombardo, M., Pucci, G., Barberi, R. & Lombardo, G. Interaction of ultraviolet light with
 the cornea: clinical implications for corneal crosslinking. *Journal of cataract and refractive surgery* 41, 446-459, doi:10.1016/j.jcrs.2014.12.013 (2015).
- 15 Coroneo, M., Müller-Stolzenburg, N. & Ho, A. Peripheral light focusing by the anterior eye
 and the ophthalmohelioses. *Ophthalmic Surgery, Lasers and Imaging Retina* 22, 705-711
 (1991).
- Kau, H. C., Tsai, C. C., Hsu, W. M., Liu, J. H. & Wei, Y. H. Genetic polymorphism of hOGG1 and risk of pterygium in Chinese. *Eye (Lond)* 18, 635-639, doi:10.1038/sj.eye.6700738 (2004).
- Tsai, Y. Y. *et al.* Null type of glutathione S-transferase M1 polymorphism is associated with
 early onset pterygium. *Mol Vis* 10, 458-461 (2004).
- Bi Girolamo, N., Wakefield, D. & Coroneo, M. T. UVB-mediated induction of cytokines
 and growth factors in pterygium epithelial cells involves cell surface receptors and

- intracellular signaling. *Invest Ophthalmol Vis Sci* 47, 2430-2437, doi:10.1167/iovs.05-1130
 (2006).
- Bi Girolamo, N., Coroneo, M. T. & Wakefield, D. UVB-elicited induction of MMP-1
 expression in human ocular surface epithelial cells is mediated through the ERK1/2 MAPKdependent pathway. *Investigative ophthalmology & visual science* 44, 4705-4714 (2003).
- Chao, S. C. *et al.* Ultraviolet-A irradiation upregulated urokinase-type plasminogen activator
 in pterygium fibroblasts through ERK and JNK pathways. *Invest Ophthalmol Vis Sci* 54,
 901 999-1007, doi:10.1167/iovs.12-10469 (2013).
- 21 Coroneo, M. T. Pterygium as an early indicator of ultraviolet insolation: a hypothesis. *The* 303 *British journal of ophthalmology* 77, 734-739 (1993).
- Gallagher, M. J., Giannoudis, A., Herrington, C. S. & Hiscott, P. Human papillomavirus in pterygium. *The British journal of ophthalmology* 85, 782-784 (2001).
- 906 23 Piras, F. *et al.* Detection of human papillomavirus DNA in pterygia from different
 907 geographical regions. *The British journal of ophthalmology* 87, 864-866 (2003).
- BOOTH, F. Heredity in one hundred patients admitted for excision of pterygia. *Australian and New Zealand journal of ophthalmology* 13, 59-61 (1985).
- 910 25 Ang, M. *et al.* Prevalence of and racial differences in pterygium: a multiethnic population
 911 study in Asians. *Ophthalmology* **119**, 1509-1515 (2012).
- 26 Zhang, J. D. An investigation of aetiology and heredity of pterygium. Report of 11 cases in a family. *Acta Ophthalmol (Copenh)* 65, 413-416 (1987).
- P14 27 Romano, V., Steger, B., Kovacova, A., Kaye, S. B. & Willoughby, C. E. Further evidence
 P15 for heredity of pterygium. *Ophthalmic genetics*, 1-3, doi:10.3109/13816810.2015.1111911
 P16 (2016).
- 917 28 Hecht, F. & Shoptaugh, M. G. Winglets of the eye: dominant transmission of early adult
 918 pterygium of the conjunctiva. *J Med Genet* 27, 392-394 (1990).
- 919 29 Contrucci Faraldi, N. & Gracis, G. Pterygium on twins. *Ophthalmologica* 172, 361-366
 920 (1976).
- 30 Hill, J. C. & Maske, R. Pathogenesis of pterygium. *Eye (Lond)* 3 (Pt 2), 218-226, doi:10.1038/eye.1989.31 (1989).
- 31 Islam, S. I. & Wagoner, M. D. Pterygium in young members of one family. *Cornea* 20, 708710 (2001).
- 92532Tsai, Y.-Y. *et al.* Null type of glutathione S-transferase M1 polymorphism is associated with926early onset pterygium. *Molecular vision* 10, 458-461 (2004).
- 33 Tsai, Y. Y. *et al.* Pterygium and genetic polymorphism of DNA double strand break repair
 gene Ku70. *Mol Vis* 13, 1436-1440 (2007).
- 92934Demurtas, P. *et al.* Association between the ACE insertion/deletion polymorphism and930pterygium in Sardinian patients: a population based case-control study. *BMJ open* **4**,931e005627, doi:10.1136/bmjopen-2014-005627 (2014).
- General State
 Stat
- 93436Taylor, J. C. *et al.* Factors influencing success of clinical genome sequencing across a broad935spectrum of disorders. 47, 717-726, doi:10.1038/ng.3304 (2015).
- 936 37 Nesbit, M. A. *et al.* Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3.
 937 *Nature genetics* 45, 93-97 (2013).
- Robert, X. & Gouet, P. Deciphering key features in protein structures with the new
 ENDscript server. *Nucleic acids research* 42, W320-W324 (2014).
- 940 39 Untergasser, A. *et al.* Primer3—new capabilities and interfaces. *Nucleic acids research* 40, e115-e115 (2012).
- 94240Moore, J. E. *et al.* Effect of tear hyperosmolarity and signs of clinical ocular surface943pathology upon conjunctival goblet cell function in the human ocular surface. Investigative9440.44
- 944 ophthalmology & visual science 52, 6174-6180, doi:10.1167/iovs.10-7022 (2011).

- 945 41 Nesbitt, H. *et al.* Nitric Oxide Up-Regulates RUNX2 in LNCaP Prostate Tumours:
 946 Implications for Tumour Growth In Vitro and In Vivo. *Journal of cellular physiology* 231,
 947 473-482 (2016).
- 94842Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT949method. *Nature protocols* 3, 1101-1108 (2008).
- 43 Kulkarni, B., Mohammed, I., Hopkinson, A. & Dua, H. S. Validation of endogenous control
 951 genes for gene expression studies on human ocular surface epithelium. *PloS one* 6, e22301,
 952 doi:10.1371/journal.pone.0022301 (2011).
- 44 Notara, M. & Daniels, J. T. Characterisation and functional features of a spontaneously
 immortalised human corneal epithelial cell line with progenitor-like characteristics. *Brain Res Bull* 81, 279-286, doi:10.1016/j.brainresbull.2009.08.009 (2010).
- Wilkinson, L. *et al.* CRIM1 regulates the rate of processing and delivery of bone
 morphogenetic proteins to the cell surface. *J Biol Chem* 278, 34181-34188,
 doi:10.1074/jbc.M301247200 (2003).
- Vinals, F. & Pouyssegur, J. Confluence of vascular endothelial cells induces cell cycle exit
 by inhibiting p42/p44 mitogen-activated protein kinase activity. *Molecular and cellular biology* 19, 2763-2772 (1999).
- 47 Kaya, A. I. *et al.* Cell contact-dependent functional selectivity of beta2-adrenergic receptor
 bigands in stimulating cAMP accumulation and extracellular signal-regulated kinase
 phosphorylation. *The Journal of biological chemistry* 287, 6362-6374,
 doi:10.1074/jbc.M111.301820 (2012).
- Moore, J. E. *et al.* Protection of corneal epithelial stem cells prevents ultraviolet A damage
 during corneal collagen cross-linking treatment for keratoconus. *The British journal of ophthalmology* 98, 270-274, doi:10.1136/bjophthalmol-2013-303816 (2014).
- Allen, E. H. *et al.* Allele-Specific siRNA Silencing for the Common Keratin 12 Founder
 Mutation in Meesmann Epithelial Corneal DystrophyAllele-Specific siRNA Silencing. *Investigative ophthalmology & visual science* 54, 494-502 (2013).
- 97250Lovicu, F., Kolle, G., Yamada, T., Little, M. & McAvoy, J. Expression of Crim1 during973murine ocular development. *Mechanisms of development* **94**, 261-265 (2000).
- 974 51 Pennisi, D. J. *et al.* Crim1KST264/KST264 mice display a disruption of the Crim1 gene
 975 resulting in perinatal lethality with defects in multiple organ systems. *Developmental*976 *Dynamics* 236, 502-511 (2007).
- 97752Beleggia, F. *et al.* CRIM1 haploinsufficiency causes defects in eye development in human978and mouse. *Hum Mol Genet* 24, 2267-2273, doi:10.1093/hmg/ddu744 (2015).
- Hocking, J. C. *et al.* Morphogenetic defects underlie Superior Coloboma, a newly identified
 closure disorder of the dorsal eye. 14, e1007246, doi:10.1371/journal.pgen.1007246 (2018).
- Nakashima, Y. *et al.* Inhibition of the proliferation and acceleration of migration of vascular
 endothelial cells by increased cysteine-rich motor neuron 1. *Biochem Biophys Res Commun*462, 215-220, doi:10.1016/j.bbrc.2015.04.118 (2015).
- 55 Eulalio, A. *et al.* Functional screening identifies miRNAs inducing cardiac regeneration.
 Nature 492, 376-381, doi:10.1038/nature11739 (2012).
- 98656Ferguson, S. W. *et al.* The microRNA regulatory landscape of MSC-derived exosomes: a987systems view. Scientific reports 8, 1419 (2018).
- 988 57 Ponferrada, V. G. *et al.* CRIM1 complexes with ss-catenin and cadherins, stabilizes cell-cell
 989 junctions and is critical for neural morphogenesis. *PloS one* 7, e32635,
 990 doi:10.1371/journal.pone.0032635 (2012).
- 58 Zhang, Y. *et al.* Crim1 regulates integrin signaling in murine lens development.
 992 Development, doi:10.1242/dev.125591 (2015).
- Solution 2993 Solution 2993 Solution 2993 Solution 2994 Solution 2994 Solution 2994 Solution 2995 Solutio

- 99660Fan, J. *et al.* Crim1 maintains retinal vascular stability during development by regulating997endothelial cell Vegfa autocrine signaling. Development 141, 448-459 (2014).
- 99861Glienke, J., Sturz, A., Menrad, A. & Thierauch, K. H. CRIM1 is involved in endothelial cell999capillary formation in vitro and is expressed in blood vessels in vivo. Mech Dev 119, 165-1000175 (2002).
- Wilkinson, L. *et al.* Crim1KST264/KST264 mice implicate Crim1 in the regulation of
 vascular endothelial growth factor-A activity during glomerular vascular development. *J Am Soc Nephrol* 18, 1697-1708, doi:10.1681/asn.2006091012 (2007).
- Pausch, H. *et al.* Identification of QTL for UV-protective eye area pigmentation in cattle by
 progeny phenotyping and genome-wide association analysis. *PLoS One* 7, e36346,
 doi:10.1371/journal.pone.0036346 (2012).
- 100764Gerber, T. et al. Mapping heterogeneity in patient-derived melanoma cultures by single-cell1008RNA-seq. Oncotarget (2016).
- Kolle, G., Georgas, K., Holmes, G. P., Little, M. H. & Yamada, T. CRIM1, a novel gene
 encoding a cysteine-rich repeat protein, is developmentally regulated and implicated in
 vertebrate CNS development and organogenesis. *Mechanisms of development* 90, 181-193
 (2000).
- 101366O'Leary, J. M. *et al.* Solution structure and dynamics of a prototypical chordin-like cysteine-1014rich repeat (von Willebrand Factor type C module) from collagen IIA. *The Journal of*1015*biological chemistry* 279, 53857-53866, doi:10.1074/jbc.M409225200 (2004).
- 101667Kinna, G. *et al.* Knockdown of zebrafish crim1 results in a bent tail phenotype with defects1017in somite and vascular development. *Mechanisms of development* **123**, 277-287 (2006).
- 101868Kria, L., Ohira, A. & Amemiya, T. Immunohistochemical localization of basic fibroblast1019growth factor, platelet derived growth factor, transforming growth factor-beta and tumor1020necrosis factor-alpha in the pterygium. Acta Histochem **98**, 195-201 (1996).
- Wu, M. *et al.* Overexpression of low-density lipoprotein receptors stimulated by vascular
 endothelial growth factor in fibroblasts from pterygium. *Biomed Pharmacother* 93, 609-615,
 doi:10.1016/j.biopha.2017.06.090 (2017).
- 102470Chui, J. et al. Ophthalmic pterygium: a stem cell disorder with premalignant features. Am J1025Pathol 178, 817-827, doi:10.1016/j.ajpath.2010.10.037 (2011).
- 1026 71 Dushku, N. & Reid, T. W. Immunohistochemical evidence that human pterygia originate
 1027 from an invasion of vimentin-expressing altered limbal epithelial basal cells. *Curr Eye Res*1028 13, 473-481 (1994).
- 1029 72 Gebhardt, M. *et al.* Differential expression of vascular endothelial growth factor implies the
 1030 limbal origin of pterygia. *Ophthalmology* 112, 1023-1030,
 1031 doi:10.1016/j.ophtha.2005.01.023 (2005).
- 1032 73 Wilkinson, L. *et al.* Crim1KST264/KST264 mice implicate Crim1 in the regulation of vascular endothelial growth factor-A activity during glomerular vascular development.
 1034 *Journal of the American Society of Nephrology* 18, 1697-1708 (2007).
- 1035 74 Marionnet, C., Tricaud, C. & Bernerd, F. Exposure to non-extreme solar UV daylight:
 1036 spectral characterization, effects on skin and photoprotection. *International journal of* 1037 molecular sciences 16, 68-90 (2014).
- 1038 75 Courtney, D. G. *et al.* siRNA silencing of the mutant keratin 12 allele in corneal limbal
 1039 epithelial cells grown from patients with Meesmann's epithelial corneal dystrophy. *Invest*1040 *Ophthalmol Vis Sci* 55, 3352-3360, doi:10.1167/iovs.13-12957 (2014).
- 104176Cagnol, S. & Chambard, J. C. ERK and cell death: mechanisms of ERK-induced cell death--
apoptosis, autophagy and senescence. *The FEBS journal* **277**, 2-21, doi:10.1111/j.1742-
4658.2009.07366.x (2010).
- 104477Liang, K. *et al.* Expression of cell proliferation and apoptosis biomarkers in pterygia and1045normal conjunctiva. (2011).

- 1046 78 Kwok, L. S. & Coroneo, M. T. A model for pterygium formation. *Cornea* 13, 219-224 (1994).
 1048 79 Nakashima, Y. & Takahashi, S. Induction of cysteine-rich motor neuron 1 mRNA
- 1048/9Nakashima, Y. & Takanashi, S. Induction of cystelle-rich motor neuron 1 mRNA1049expression in vascular endothelial cells. *Biochemical and biophysical research*1050communications 451, 235-238, doi:10.1016/j.bbrc.2014.07.108 (2014).
- 1051 80 Kannabiran, C. & Klintworth, G. K. TGFBI gene mutations in corneal dystrophies. *Human* 1052 *mutation* 27, 615-625 (2006).
- 105381Tan, D. T., Tang, W. Y., Liu, Y. P., Goh, H. S. & Smith, D. R. Apoptosis and apoptosis1054related gene expression in normal conjunctiva and pterygium. *The British journal of*1055ophthalmology 84, 212-216 (2000).
- 105682Mencucci, R. et al. Effects of riboflavin/UVA corneal cross-linking on keratocytes and1057collagen fibres in human cornea. Clinical & experimental ophthalmology 38, 49-56 (2010).
- 1058 83 Buron, N. *et al.* Differential mechanisms of conjunctival cell death induction by ultraviolet
 1059 irradiation and benzalkonium chloride. *Investigative ophthalmology & visual science* 47,
 1060 4221-4230 (2006).
- 106184Yam, J. C. & Kwok, A. K. Ultraviolet light and ocular diseases. International1062ophthalmology 34, 383-400 (2014).
- 1063 85 Cullen, A. P. Photokeratitis and other phototoxic effects on the cornea and conjunctiva.
 1064 *International journal of toxicology* 21, 455-464 (2002).
- 1065 86 DILLON, J., Zheng, L., MERRIAM, J. C. & GAILLARD, E. R. The optical properties of
 1066 the anterior segment of the eye: implications for cortical cataract. *Experimental eye research*1067 68, 785-795 (1999).
- 106887Newton, R., Reeves, G., Beral, V., Ferlay, J. & Parkin, D. Effect of ambient solar ultraviolet1069radiation on incidence of squamous-cell carcinoma of the eye. *The Lancet* 347, 1450-14511070(1996).
- 1071 88 Lucas, R. M., McMichael, A. J., Armstrong, B. K. & Smith, W. T. Estimating the global
 1072 disease burden due to ultraviolet radiation exposure. *International journal of epidemiology*1073 37, 654-667 (2008).
- 1074 89 Hirst, L. W. Distribution, risk factors, and epidemiology of pterygium. *Pterygium, Kugler* 1075 *Publications, The Hague, The Netherlands*, 15-27 (2000).
- 107690Raghunath, A. & Perumal, E. Micro-RNAs and their roles in eye disorders. Ophthalmic1077research 53, 169-186, doi:10.1159/000371853 (2015).
- 107891Tam, O. H. *et al.* Crim1 is required for maintenance of the ocular lens epithelium.1079*Experimental eye research* 170, 58-66, doi:10.1016/j.exer.2018.02.012 (2018).
- Machens, A. *et al.* Age-related penetrance of endocrine tumours in multiple endocrine
 neoplasia type 1 (MEN1): a multicentre study of 258 gene carriers. *Clinical endocrinology* **67**, 613-622 (2007).
- 1083 93 Vitt, U. A., Hsu, S. Y. & Hsueh, A. J. Evolution and classification of cystine knot1084 containing hormones and related extracellular signaling molecules. *Molecular*1085 endocrinology 15, 681-694 (2001).
- Pearton, D. J., Ferraris, C. & Dhouailly, D. Transdifferentiation of corneal epithelium:
 evidence for a linkage between the segregation of epidermal stem cells and the induction of
 hair follicles during embryogenesis. *The International journal of developmental biology* 48,
 197-201, doi:10.1387/ijdb.031744dp (2004).
- 1090 95 Engelsvold, D. H. *et al.* miRNA and mRNA expression profiling identifies members of the
 1091 miR-200 family as potential regulators of epithelial-mesenchymal transition in pterygium.
 1092 *Exp Eye Res* 115, 189-198, doi:10.1016/j.exer.2013.07.003 (2013).
- Watson, S. *et al.* Limbal dermoid epithelium shares phenotypic characteristics common to
 both hair epidermal and limbal epithelial stem cells. *Current eye research* 38, 835-842,
 doi:10.3109/02713683.2013.780625 (2013).

- He, Y.-Y., Council, S. E., Feng, L. & Chignell, C. F. UVA-induced cell cycle progression is
 mediated by a disintegrin and metalloprotease/epidermal growth factor receptor/AKT/Cyclin
 D1 pathways in keratinocytes. *Cancer research* 68, 3752-3758 (2008).
- 109998Assefa, Z., Van Laethem, A., Garmyn, M. & Agostinis, P. Ultraviolet radiation-induced1100apoptosis in keratinocytes: on the role of cytosolic factors. *Biochimica et Biophysica Acta*1101(BBA)-Reviews on Cancer 1755, 90-106 (2005).
- 1102 99 Moan, J. 7 Visible Light and UV Radiation. *Radiation*, 69 (2001).
- 1103 100 Tsai, Y. Y. et al. Oxidative DNA damage in pterygium. Molecular vision 11, 71-75 (2005).
- 104 101 Mercola, M. *et al.* Dominant-negative mutants of a platelet-derived growth factor gene.
 1105 *Genes & development* 4, 2333-2341 (1990).
- 1106 102 Millauer, B., Shawver, L. K., Plate, K. H., Risaui, W. & Ullrich, A. Glioblastoma growth 1107 inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature* **367**, 576-579 (1994).
- 108 103 Casari, C. *et al.* The dominant-negative von Willebrand factor gene deletion p.
 1109 P1127_C1948delinsR: molecular mechanism and modulation. *Blood* 116, 5371-5376
 1110 (2010).
- 1111 104 Castaman, G., Eikenboom, J. C., Missiaglia, E. & Rodeghiero, F. Autosomal dominant type
 1112 1 von Willebrand disease due to G3639T mutation (C1130F) in exon 26 of von Willebrand
 1113 factor gene: description of five Italian families and evidence for a founder effect. *British*1114 *journal of haematology* 108, 876-879 (2000).