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The impact of Fam84b in retinal homeostasis

Vliv proteinu Fam84b na homeostázu retiny

Diploma thesis

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ABSTRAKT

Fam84b je jen málo prostudovaný protein, jehož funkce v eukaryotické buňce je stále nejasná. V této práci je představen myší model s vyřazeným genem FAM84B (FAM84B KO) s detailní charakteristikou jeho fenotypu v oční sítnici. Morfologie sítnice byla u FAM84B KO myší studována pomocí optické koherentní tomografie a histologie. Tyto dvě metody ukázaly dynamické změny vycházející z fotoreceptorové a z pigmentové epiteliální vrstvy. Fenotyp se zhoršoval s věkem a patologické oblasti se postupně rozšiřovaly, ale také pronikaly hlouběji do vnitřních retinálních vrstev. Porovnání lokalizace standardních retinálních buněčných markerů ukázalo, že u FAM84B KO myší dochází k postupnému rozrušení vrstev sítnice spolu s deformací retinální makrostruktury. Sítnice mutovaných myší také vykazují nižší reakci na stimulaci světlem, což bylo prokázáno pomocí elektroretinografie. Fam84b je homologní s HRASLS enzymatickou rodinou, která tlumí signalizaci asociovanou s Ras. K ověření, zda má Fam84b podobnou funkci, byly porovnány hodnoty fosforylovaných a aktivovaných downstream Ras efektorů mezi lyzáty sítnice FAM84B KO a kontrolních nemutovaných zvířat. U KO myší byla naměřena redukce pERK1 (pY204), která poukazuje na možnou funkci v rámci downstream Ras signalizace. Předběžné ko-imunoprecipitační pokusy odhalily specifickou interakci mezi Fam84b a GRIPAP1, neuronálním scaffold proteinem účastnícím se JNK signalizace, která by mohla být hlavním propojením mezi tajemným Fam84b a destruktivní retinální degenerací vzniklou při jeho absenci.

Klíčová slova

Fam84b, retina, degenerace, Ras, MAPK, Ras signalizace, knockout, myš

ABSTRACT

Fam84b is a largely unstudied protein, where its function in eukaryotic cells is unclear. This thesis work presents a FAM84B knockout mouse model and characterises the resulting retinal phenotype in detail. FAM84B KO mice were morphologically assessed by optical coherence tomography and histological processing, revealing dynamic changes stemming from the photoreceptor and pigmented epithelial layers. This potent phenotype progresses with age, spreading inwards towards the inner retinal layers, as well as laterally to adjacent retinal regions. Comparative localisation of standard retinal cell markers demonstrates that FAM84B KO retinal layering becomes increasingly disorganised, together with deformation of the retinal macrostructure. Due to this, KO mice experience reducing responses to light, as demonstrated by electroretinography, where overall retinal efficiency falls. Fam84b shows homology to the HRASLS enzyme family, which are capable of attenuating Ras-associated signalling. To investigate whether Fam84b possesses a similar function, the level of phosphorylated and activated downstream Ras effectors were compared between wild type and FAM84B KO mouse retinal lysates. A reduction of pERK1 (pY204) in KO lysates suggests that Fam84b holds some function related to this pathway downstream of Ras. Preliminary co-immunoprecipitation experiments revealed a specific interaction between Fam84b and GRIPAP1, a neuronal scaffold for the JNK pathway, which may provide a crucial link between the elusive Fam84b and the destructive retinal degeneration present in its absence.

Key words

Fam84b, retina, degeneration, Ras, MAPK, Ras signalling, knockout, mouse

PROHLÁŠENÍ

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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1 ABBREVIATIONS

1.1 List of abbreviations

AMD	Age-related macular degeneration
BCMP101	Breast cancer membrane associated protein-101
CCP	Czech Centre for Phenogenomics
ERG	Electroretinogram
ERK	Extracellular signal-related kinase
ESCC	Oesophageal squamous cell carcinoma
Fam84b	Family with a sequence similarity member 84b (protein)
FAM84B	Family with a sequence similarity member 84b (gene)
GAP	GTPase-activating protein
GEF	Guanosine-nucleotide exchange factor
GDP	Guanosine diphosphate
GRIPAP1	GRIP1-associated protein 1
GTP	Guanosine triphosphate
HGP	Human Genome Project
hom	Homozygous
IMPC	International Mouse Phenotyping Consortium
IMPreSS	International Mouse Phenotyping Resource of Standardised Screens
JNK	c-Jun N-terminal kinase
KO	knock-out
LRAT	Lecithin: retinol acyltransferase
MAPK	Mitogen-activated protein kinase
OCT	Optical Coherence Tomography
PKC- α	Protein kinase C-alpha
PLA/AT	Phospholipase A2 and acyltransferase activity
RP	Retinitis pigmentosa
RTK	Receptor tyrosine kinase

SD-OCT	Spectral domain Optical Coherence Tomography
SNP	Single nucleotide polymorphism
TD-OCT	Time domain Optical Coherence Tomography
VEGF	Vesicular endothelial growth factor

1.2 Retinal layers

RNFL	Retinal nerve fibre layer
GCL	Ganglion cell layer
IPL	Inner plexiform layer
INL	Inner nuclear layer
OPL	Outer plexiform layer
ONL	Outer Nuclear layer
RPE	Retinal pigmented epithelium

2 BACKGROUND

Pathological disease, in all its shapes and forms, has haunted man for millennia. Over the last century, our methods to avoid, prevent, manage or treat particular diseases have evolved rapidly as our understanding of the intricate biological systems around us has expanded. This continuing surge in knowledge is due, in part, to the accessibility of animal models. The very concept of animal models provides astounding opportunities to study a biological system *in vivo*, and when we manipulate the system, we learn more about how the system works.

It is estimated that around 95% of animal studies utilise live mice as a model (Vandamme, 2014), of which are used for a variety of experimental objectives. One example of this would be in the production of newly designed drugs, which implements a stage of animal testing to validate efficient delivery to, and efficacy in, the target area or to screen for associated side effects. It's true that despite the similarities between the human and mouse genomes - which pack a comparable amount of protein coding genes - ~20,000 and ~25,000 for human and mouse respectively (Piovesan et al., 2019); (Guenet, 2005), there are cases in which mice respond to specific clinical treatments where as humans do not. It is because of this; many drugs fall short of reaching a place in the market. The anti-angiogenic drug endostatin is a part of many successful treatments to manage malignancies in mice, however shows limited worth in human therapy regimes (Perlman, 2016). This reminds us that even though animal models have shown their importance for understanding the mechanisms of complex disease processes; we must carefully select valid models to best represent our study outcomes.

Another example of utilising mouse models in scientific research, relevant to this thesis work, would be the manipulation of the mouse genome to uncover the functionality of genes. This may come in the form of, but not limited to, whole or partial gene knock-ins and knock-outs (KO). These techniques can be either precisely targeted to occur at specific loci in the genome, e.g. in the CRISPR/Cas9 system, or executed more randomly, e.g. via radiation or ENU induced mutation events. The aim here is to initiate changes in the host's genome in order to disrupt the normal expression of a gene, which leads to an altered or lack of, functionality in its corresponding protein. The resulting phenotype, if any, can then be assessed for abnormalities using a series of standardised tests adjusted to the study question. This is exactly the base theory behind the collaborative effort of the International Mouse Phenotyping Consortium (IMPC).

2.1 IMPC phenotyping

The completion of the Human Genome Project in 2003, determined the identification and mapping of all human genes at a single base pair resolution. This major achievement of the largest scientific venture to date was a massive step forward for our potential understanding of genetic functionality, regulatory mechanisms and categorisation of individual and pathogenic differences in our innate hard-wiring. Additionally, this paved the way to the development of many valuable online tools such as the NCBI database, the Ensembl project and the UCSC genome browser. Nevertheless, this new wealth of information left us oblivious to the activity, expression profiles and disease susceptibility of an enormous number of genes. According to the Mouse Genome Database (<http://www.informatics.jax.org>) back in 2011, only around 30% of mouse genes possessed specific knockout mutation models (Eppig et al., 2011), undoubtedly only reflecting the particular interests of investigators. This importantly emphasised the need for a new approach to fill the gaps with the revelation of 'mouse clinics and pilot large-scale phenotyping efforts' (Brown and Moore, 2012). In that same year, the IMPC was established.

The main goal of the IMPC (www.mousephenotype.org) is to provide a comprehensive, functional catalogue of the mouse genome by the cooperation of research institutions around the world. Each participating centre is responsible for the production of null mutations in assigned protein coding genes to create KO lines, and to perform unbiased phenotyping 'screens' to assess major biological functions. This includes a KO mouse's performance in motor tasks, gross morphology and essential biochemical processes. All resulting data is then concatenated and made freely available to the scientific community. Since ~99% of mouse genes possess a detectable homologue in the human genome (Mouse Genome Sequencing Consortium, 2002), this approach of utilising mice to systematically phenotype ~20,000 KO inbred mouse strains (one for each gene) can be considered a worthy tactic to enhance our understanding of each gene in our own genome. Furthermore, this concept may also be applied to the non-coding genome in the future.

The C57Bl/6 inbred mouse strain is the most widely used in biomedical research, and a sub-strain derived from this, C57Bl/6NCrJ, is employed to undergo this series of standardised pilot testing. An inbred mouse strain is ideal for this purpose as, theoretically, each mouse in the colony possesses the same homozygous allele for every sequence in the genome, due to many generations or rounds of back-crossing (Bryant, 2011). This means that findings in the phenotyping assessments are likely due to the KO of that particular gene, and suitable, non-mutated controls can be taken from the same colony for comparison. However, care must be taken when comparing two different produced KO lines, as continued breeding for multiple generations can lead to unique single-nucleotide polymorphisms (SNPs) in that line, branching off and creating a new mouse strain drifting genetically from that of its lineage.

Traits of C57Bl/6N lines include their high breeding activity, capacity for learning (Bryant, 2011), and high susceptibility to weight gain and diabetes (Podrini et al., 2013). However, a comparative study of C57Bl/6 sub-strains revealed that C57Bl/6N mice are homozygous for the single nucleotide *Crb1*^{rd8} mutation, whereas C57Bl/6J and C57Bl/6J are not. This leads to age-related retinal degeneration and outer retinal dysplasia manifestation (Mattapallil et al., 2012), a topic discussed in further detail later. The *rd8* mutation in *Crb1*, which codes for a protein important for the correct formation of adherens junctions at areas of cell-cell contact, has been successfully corrected via transcription activator like effector nucleases, or TALENs, technology (Low et al., 2014). This reduction in dysplastic vulnerability in C57Bl/6N may allow researchers to maintain their mouse colonies background with a little more faith in the future. This example highlights that there is no perfect mouse line for all research, and while this sub-strain may be advantageous for embryonic, behavioural and metabolic studies, those utilising it for their research must be aware of potential hindrances when evaluating data.

Each new KO mouse strain generated is initially assessed for fertility and viability. Fertility, refers to the proficiency of homozygous (hom) males and females in mating, and male-(in)fertility or female-(in)fertility or both is deciphered for that particular KO strain. The postnatal viability, sub-viability and lethality of hom mice are also recorded. These preliminary assessments provide clues as to whether the gene knocked out codes for a protein actively involved in the fertilisation process, or is vital for embryonic development.

Figure 1 shows a schematic of the IMPC adult phenotyping pipeline, which summarises the examinations performed at each fixed week of age. Mandatory tests and non-mandatory tests, dependent upon the centre's capacity, are specified. A minimum of 7 females and 7 males of each new KO C57Bl/6NCrl mouse strain, including the equivalent number of non-mutated control animals, enters and proceeds through standardised and unbiased testing at 9 weeks of age. It is important to mention before summarising the pipeline in detail, that these primary tests take a glance at potential abnormalities in essential mouse functions. To further articulate glitches in the particular system and comprehend the detected phenotypical phenomena, secondary testing and investigations are performed at the interest of individual participating centres, or by those accessing the IMPC KO mouse database. The pipeline outline that follows is based upon the wealth of information on collected parameters, general procedures and purpose of each screen situated on the IMPC online resource (<https://www.mousephenotype.org/impress>), and the website of the Czech Centre for Phenogenomics (CCP; <https://www.phenogenomics.cz>), the contributing centre where this thesis work was carried out.

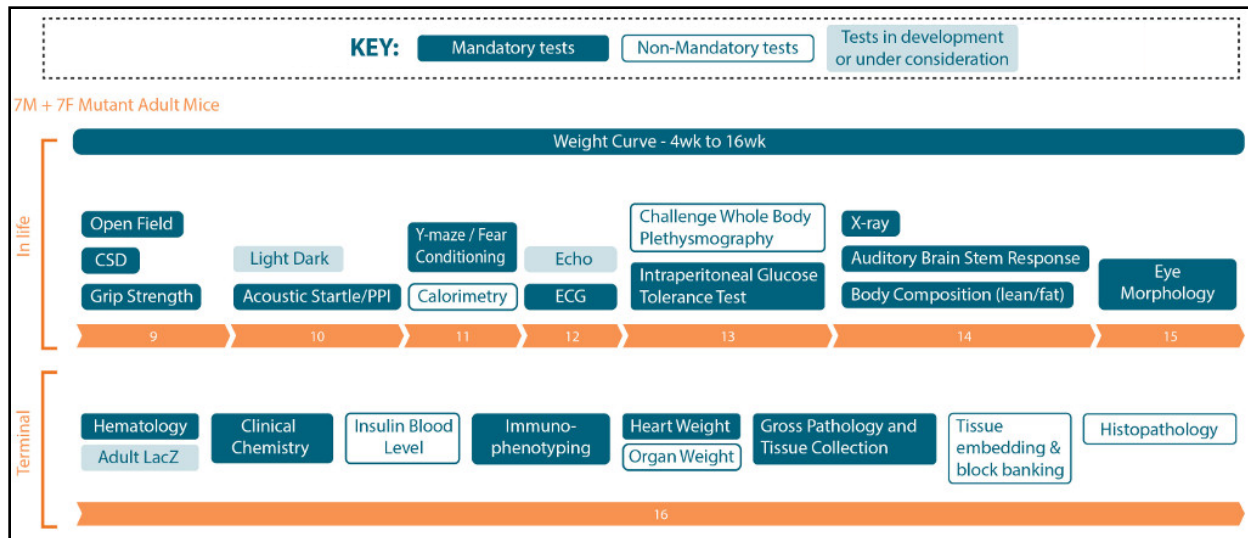


Figure 1: The IMPReSS Adult Mouse Phenotyping Pipeline. Mice that enter the pipeline undergo a series of standardised tests which are organised to occur consistently at the same week of life, e.g. Grip Strength test always at 9 weeks of age. The number in orange below each test name corresponds to the age of the animal (in weeks) when it undergoes that test. Screens in weeks 9-15 occur on live animals, whereas terminal screens follow in week 16. The elected tests assess major biological functions including their performance in motor tasks, gross morphology and essential biochemical processes. M, male; F, female; CSD, Combined SHIRPA and dysmorphology PPI, prepulse inhibition; Echo, echocardiogram; ECG, electrocardiogram. (Adapted from IMPReSS | International Mouse Phenotyping Resource of Standardised Screens, n.d.)

The journey starts with the assessment of typical neurological behaviours and evidence of physical abnormalities. The Open Field test evaluates a mouse's natural tendency to explore a lit arena, which may reveal increased/decreased levels of anxiety. The SHIRPA and dysmorphology test (or CSD for short) combines checks for irregular locomotion, such as difficulties walking or tremors, with unexpected behaviours, such as vocalisation or aggression. The final mandatory test at week 9 is the measurement of forelimb and hind limb grip strength, which may signal either reduced muscular power or neuromotor control. At 10 weeks, the mice undertake the Acoustic Startle Response paired with prepulse inhibition, a test that can be utilised as a primary screen for hearing impairment, to reflect the animal's ability to filter essential and non-essential sensory information, and predict psychotic imbalances in conditions that include autism spectrum disorders and schizophrenia (Swerdlow et al., 2008). The Fear Conditioning test is also carried out to assess aversive learning and memory by pairing conditioned and unconditioned stimuli. The optional Light-Dark box test also builds on information about the animal's anxiety and willingness to explore based on light avoidance behaviour.

Week 11 brings Indirect Calorimetry to explore the detailed profile of mouse energy metabolism. Here, O₂ consumption and CO₂ expenditure are measured to unravel changes in lipid and carbohydrate usage via the calculation of the respiratory exchange ratio, or RER. Quantification of day/ night activity and food and water intake is also incorporated for a more accurate determination of the mouse's management of energy.

At 12 weeks of age, screening for irregularities in cardiac activity occurs by analysing the conscious murine electrocardiogram (ECG), as well as an assessment of cardiovascular structure and functionality by utilising ultrasound technology in the echocardiogram (Echo). Here, parameters include ventricle dynamics in systole and diastole, blood flow efficiency, in addition to chamber and blood vessel wall thickness. The following week includes challenges related to respiratory function and glucose tolerance, which monitor the animal's natural breathing frequency and airflow capacity, and glucose metabolism respectively. These tests may unveil any asthma-related or diabetic phenotypes.

An assessment of skeletal anatomy, bone mineral content density, as well as full-body composition reviews the mice's gross morphology via microCT and radiographic absorptiometry at 14 weeks of age. This period is shared with the analysis of the Auditory Brainstem Response, which comprises of acoustically stimulating anaesthetised mice and evaluating their hearing sensitivity. Subcutaneously placed electrodes record the electrical response to stimuli from their inception in the cochlea, through to the inferior colliculus. Parameters such as response amplitude and latency, as well as the architecture of individual peaks can be then used to summarise hearing capability, an approach also used in human diagnoses.

At the age of 15 weeks, mice undergo a rigorous evaluation of their ocular morphology whilst under anaesthesia. Data produced from this particular week inspired - and enclosed the most relevant information for - this thesis work. Firstly, the pupillary light reflex is verified before forced pupil dilation, which allows rotating Scheimpflug imaging of the eye from the anterior corneal surface to the posterior lens surface. The Scheimpflug principle permits increased focal depth and image sharpness by arranging three plains (focal, lens and image) in a non-parallel way to intercept each other at the point of focus. The obtained images can be translated into thickness profiles, quantifiable opacities and densitograms of each structure, revealing cataracts (Wegener and Laser-Junga, 2009), keratoconus (corneal thinning), dystrophies (accumulation of abnormal material) and astigmatism (irregular shaping) in the cornea and lens (Faria-Correia and Ambrósio Júnior, 2016). At CCP, collection and analysis of this data is achieved by utilising the Pentacam® (Oculus) technology platform. Although this device was designed to provide gold-standard ophthalmic testing in humans, it stands in as an incredibly useful tool for inspecting the anterior segments of mouse eyes, at least until a rodent-orientated adaptation becomes available.

Following the anterior segment comes the posterior section evaluation, which specifically refers to cross-sectional imaging of the retina and optic disc morphology via Optical Coherence Tomography (OCT). A schematic of the OCT principle is shown in Figure 2. This non-invasive procedure facilitates the detection of changes and pathologies related to retinal thickness, layer architecture and blood vessel count and patterning without the need for sample preparation. Deviations in these parameters are the foundation of a number of dynamic retinal diseases, discussed in more detail in the next chapter. OCT is a technique that has improved considerably since its introduction by Huang et al. (1991), who extended low-coherence interferometry to a level of representative tomography with the time-domain (TD)-OCT. Here, a low coherent light source is split and directed towards a reference mirror and the sample eye, and interference occurs on reflection and recombination of backscattered light beams based on their time-delay. Altering the position of the reference mirror allowed differing depths of the tissue to be detected; generating a reflectivity or interference profile which can be reconstructed into cross-sectional images (b-scan) of the retina (Gabriele et al., 2011). Initially, TD-OCT was restricted to ~400 axial scans (a-scan) per second. This slow scanning speed was due to the need to physically move the reference mirror, amplifying motion-related artefacts such as breathing. The establishment of spectral-domain (SD)-OCT overcame this, offering significantly improved imaging speed and resolution simultaneously with the use of

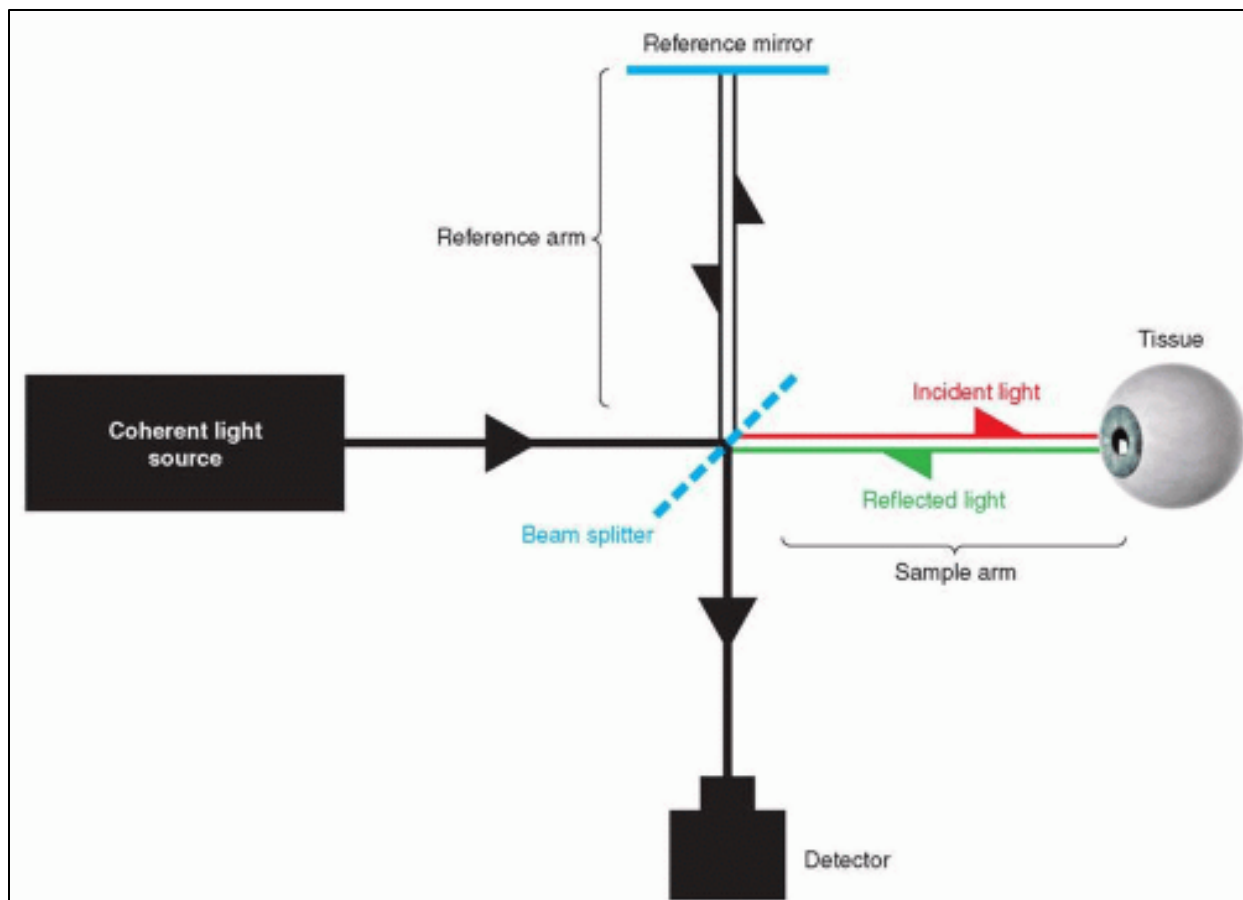


Figure 2: Optical Coherence Tomography principle. The design is based upon a Michelson interferometer. A coherent light source is split and guided towards the sample and a reference mirror at a known length. Backscattered light is reflected which creates an interference profile, allowing transformation into detailed images of the retina. In SD-OCT, the light source would be of broadband width, and a spectrophotometer sits in the detector position. (Ocular Anatomy by Optical Coherence Tomography, accessed 12/11/2019).

broadband light sources and reflected wave length separation via a spectrometer (Aumann et al., 2019). Here, the reference mirror remains stationary to speed up image acquisition times.

The Spectralis® (Heidelberg Engineering, Germany) device combines SD-OCT and confocal scanning laser ophthalmology (cSLO), where visualisation of an elected part of the fundus and corresponding retinal cross-section at the same time is made possible. Moreover, the axial resolution achieved lies between 3-10µm, higher than that of other medical imaging techniques, such as OCT's acoustic cousin; ultrasound, and magnetic resonance imaging (MRI). Although not initially required for an IMPC primary screen; this modularly built platform can be extended to fluorescence imaging modes such as fluorescein angiography - for detailed assessments of retinal blood vessels, and autofluorescence - for the detection of age-related build-up of damaging autofluorescent material. If any abnormalities are perceived, these secondary screens can be employed to investigate further.

Another important secondary screen within the sphere of visual evaluation is the role of the electroretinogram (ERG). ERG is an additional non-invasive technique that has had medical and veterinary significance for over a century. It provides objective, electrical information about different retinal cell types during their evoked response to brief light stimulations at varying intensities and frequencies (Balicka, Trbolová and Vrbovská, 2016). Typically, small electrodes are placed on the corneas of anaesthetised mice after overnight dark-adaptation; ensuring the separation of scotopic (rod driven) and photopic (cone driven) responses by initially desensitising cone and cone-related retinal cell types (Kremers and Tanimoto, 2018). Responses that are absent, slower or reduced in particular components of the produced waveform can be correlated with global or cell-type specific abnormalities and known retinal diseases. Consequently, ERG adds an extra dimension to the anterior and posterior morphology by complementing it with retinal functional efficiency.

The major components of ERG responses are shown in Figure 3, with a comparison between typical scotopic and photopic responses in mice. The initial negatively deflected a-wave corresponds to the response generated by the photoreceptors, while the positively deflected b-wave includes the responses of bipolar and Muller cells. Oscillatory potentials are immediately obvious in scotopic curves; their origin is still debated but the combined intraretinal interactions of bipolar, amacrine and ganglion cells have been suggested as the source (Gauvin et al., 2016). Finally, the slow positively deflected c-wave represents the activity of the retinal pigmented epithelium (RPE).

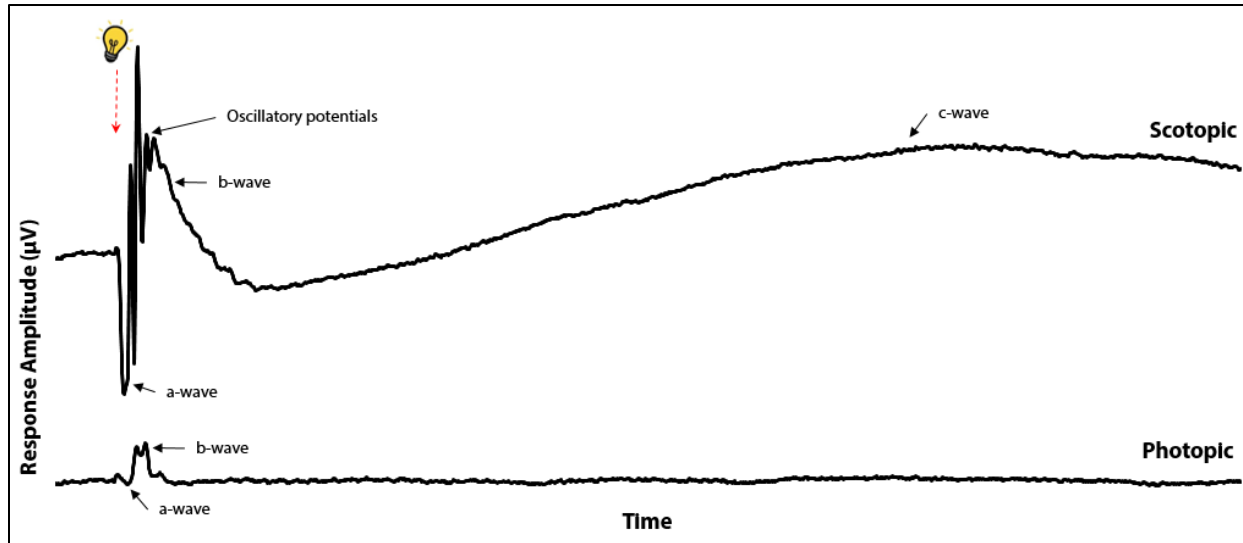


Figure 3: Typical electroretinogram responses in C57Bl/6NCrl mice. Top, an example of a produced curve by an 18 week old wild type C57Bl/6NCrl mouse that has been dark-adapted overnight. A negatively deflected a-wave corresponding to the hyperpolarisation of the photoreceptors is the first recognisable event. This is followed by a positively deflected b-wave with oscillating potentials occurring concurrently. These events are due to the collective interactions between the inner retinal neurones. A slow positive c-wave corresponds to the activity of the retinal pigmented epithelium. Bottom, an example response of the same mouse that has been allowed to adapt to light. The a- and b-waves are typically much smaller due to the reduced sensitivity of cones compared to rods. Oscillatory potentials and the c-wave are difficult to distinguish or absent. Both displayed curves are the average of 30 responses to a light flash of 10 cds/m². The lightbulb and red dashed arrow show the time in which the light stimulation occurred.

Following sacrifice and blood sampling at week 16, mice enter the terminal section of the pipeline. Whole blood counts, quantifying e.g. red and white blood cells, can establish the presence of any haematological imbalances in KO mice. Immuno-phenotyping further breaks down the description of immune cell sub-populations via flow cytometry, and may disclose any genetic predispositions to immune deficiencies, inflammatory diseases or allergies. Following this, serum or urine samples are examined for their level of particular organic and inorganic analytes. Measurements of essential enzymes such as, but not limited to, Alkaline Phosphatase and Lactate Dehydrogenase are also included in this comprehensive clinical chemistry scope, and may be linked back to any acknowledged irregularities in previous screens.

Finally, after completion of fluid sampling, a complete necropsy is carried out, where a standardised checklist of internal and external organs is seized, weighed and their dimensions noted. Fixation and archiving of these tissues allows the opportunity for additional processing, and detailed histopathological assessments to further investigate abnormal phenotypes detected in earlier live examinations.

As we have seen, the IMPC standardised testing pipeline is carried out by its participating centres, and aims to provide a complete KO mouse strain catalogue for all protein-coding genes that will be generously available to the scientific community. This information may inspire projects or be simply used as a reference for genetic and protein research around the world, but importantly, will help to fill the gaps in the existing knowledge of human and mouse genes with unidentified functions. The next section will focus on the current understanding of the retina and its associated diseases.

2.2 The retina and associated diseases

The retina is a 10-layer, sensory structure with a fundamental design consistent across all vertebrates. Its ability to perceive and process light stimuli, and transmit the complex photographic output to the brain is owed to the dedicated neuronal circuits that work in parallel or against each other. The retinal neurome was estimated to be composed of around 55 functionally distinct cell types (Masland, 2001), but with developments in imaging, electrophysiological and molecular biology techniques, this number continues to increase as we delve deeper into the morphology, activity and expression patterns of individual cell types. There are 5 classes of neurones in the vertebrate retina; photoreceptors, with the photosensitive pigments; horizontal, bipolar and amacrine cells, the processing interneurons; and the retinal ganglion cells, where the electrical information is passed to the brain via the optic nerve. A schematic of mammalian retinal structure is shown in Figure 4. Each of the classes contain a number of sub-types, which have fine-tuned structures and responsive to all kinds of contextual stimuli. Of course, with most systems in the body, proneness to malfunctions and disease is an entity that can occur, and the retina is no different.

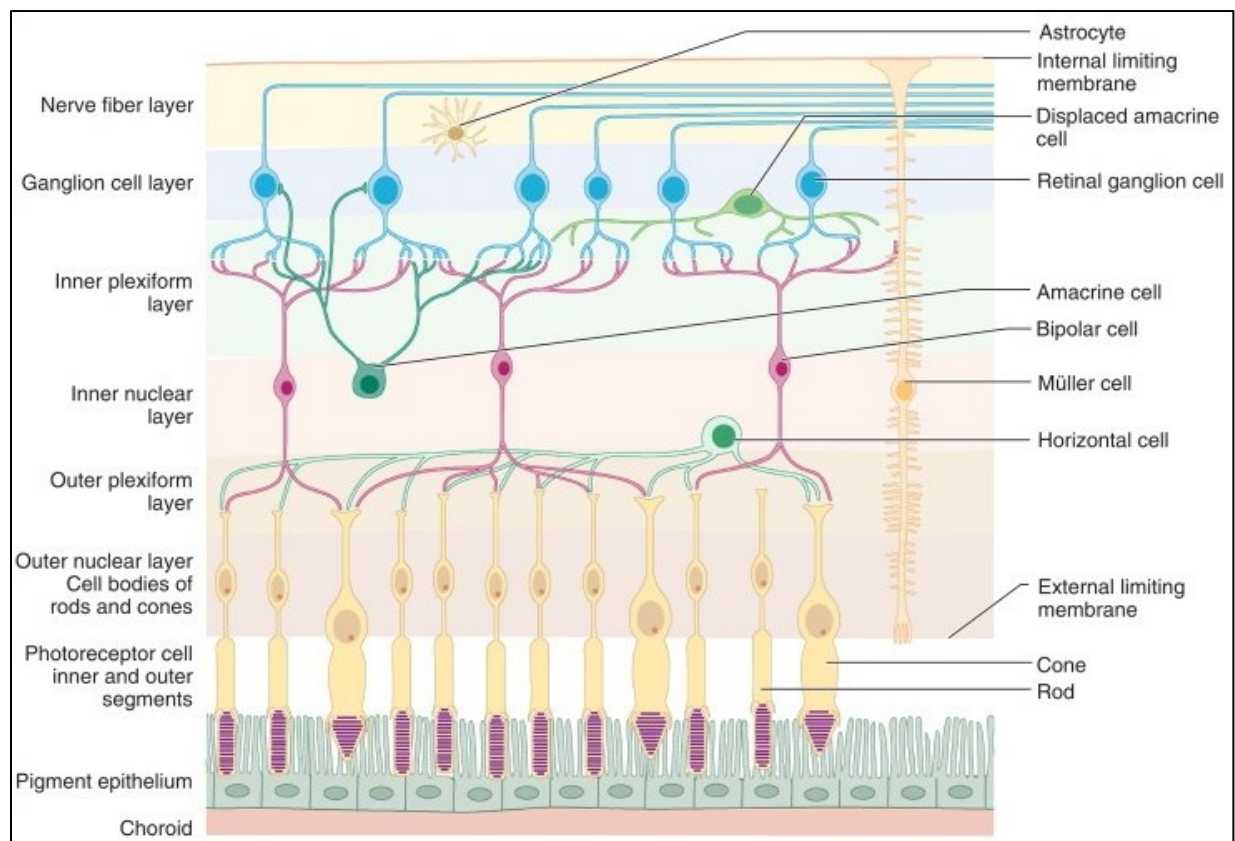


Figure 4: Schematic of mammalian retinal structure. The retina is organised into distinct layers that can be distinguished using imaging techniques such as OCT, and histology. The names of each of these layers are annotated on the left-hand side; from the nerve fibre layer adjacent to the vitreous, to the outer choroid layer. The functional cell types of the retina are labelled, of which can localise to a single layer or span multiple layers. Image from Lee et al., (2015).

Age-related macular degeneration (AMD) is one the major causes of vision loss in the developed world, affecting more than 10% of people over 65 years of age (Al-Zamil and Yassin, 2017). AMD is a progressive disease that specifically leads to degeneration of photoreceptor and retinal pigmented epithelium (RPE) layers around the macula, with the presence of its trademark drusen - lipid rich material build-up between the RPE and Bruch's membrane. The initial presentation of this retinal morphology is known as dry-AMD, and a representative example is shown in Figure 5. Advanced stages of the disease transform from local RPE and photoreceptor atrophy and depigmentation, to an exudative form (aka. wet-AMD). This entails choroidal neovascularisation, where the vasculature begins to grow through Bruch's membrane, inevitably damaging the overlying RPE layer. RPE detachment can also occur due to the increasing pressure caused by expanding drusen (Seth, Sigler and Adelman, 2011). The pathophysiology of AMD is poorly understood, but is associated with a number of risk factors, including age, genetic background and diet and lifestyle choices. Additionally, treatment plans such as laser photocoagulation and vascular endothelial growth factor (VEGF) inhibitors have shown promising benefits, but by no means cure or revert the already impaired retinas (Al-Zamil and Yassin, 2017).

The importance of the photoreceptors is paramount, harbouring the photosensitive pigments that excite, initiate the phototransduction cascade, and transmit the signal onto the subsequent retinal neurones. However, the RPE monolayer provides essential support to the whole retina. It participates in the enzymatic recycling of retinoids (Sears and Palczewski, 2015); acts as the blood-retinal barrier; absorbs stray photons; phagocytoses photoreceptor outer segments; and secretes proteins required for normal retinal function. The RPE is therefore indispensable in its ability to strictly control fluid, ion and glucose levels, and to protect against photoreceptor toxicity (Simó et al., 2010). Death of these layers is incredibly worrying for an individual's visual capability, especially considering that unlike teleosts, the mammalian RPE cannot regenerate (Hanovice et al., 2019). Animal models for AMD are available but challenging to recreate fully. They rely on mimicking certain aspects of AMD, such as the choroidal neovascularisation in later stages of the disease via laser exposure (Lambert et al., 2013). This difficulty is because mice lack a cone-dense macula region, they generally do not live long enough for full case studies, and AMD genetics remains complex.

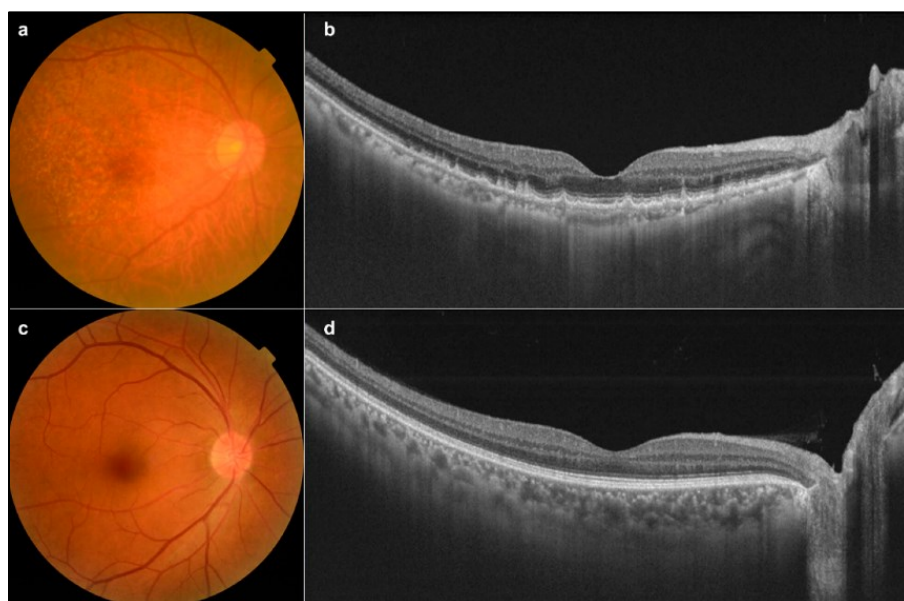


Figure 5: Representative example of dry-AMD via OCT imaging. (a,b) Fundus image and corresponding OCT b-scan of an elderly female diagnosed with dry age-related macular degeneration (AMD). Numerous soft drusen can be seen - the accumulation of lipid deposits under the retinal pigmented epithelium layer. In later stages of AMD, drusen increase in size and retinal detachment becomes apparent. (c,d) Normal fundus photography and corresponding OCT b-scan of an elderly control male. (Heo et al., 2020).

Conversely, the genetics of retinitis pigmentosa (RP) has been well described, with mutations in more than 70 genes shown to be associated with this inherited retinopathy. The RHO gene in particular is accountable for up to 30% of autosomal dominant RP cases (Dias et al., 2018). For example, mutated pre-mRNA processing factor PRPF31, a component of the U4/U6•U5 tri-snRNP, leads to unsuccessful splicing of rhodopsin intron 3 (Yuan et al., 2005). An aberrant rhodopsin protein could then be inefficiently placed at the outer segment, incorrectly folded, or imperfectly post-translationally modified depending on the penetrance of its mutation. Affecting around 2.5 million people worldwide, pigment deposits typically manifest in rod outer segments of the peripheral retina, resulting in night blindness and tunnel vision, before invading the outer nuclear layer and eventually more central areas (Dias et al., 2018), closer to the optic disc and macula. Rod and cone apoptosis soon follows, with the collapse of the inner retinal neurones in later stages. At this clinical phase of RP progression, pigment proliferation can coincide with anatomical remodelling of the retina. In areas of long-term photoreceptor loss, pigmented epithelial cells start to migrate towards the inner layers of the retina, resulting in a bone-spicule like appearance, RPE

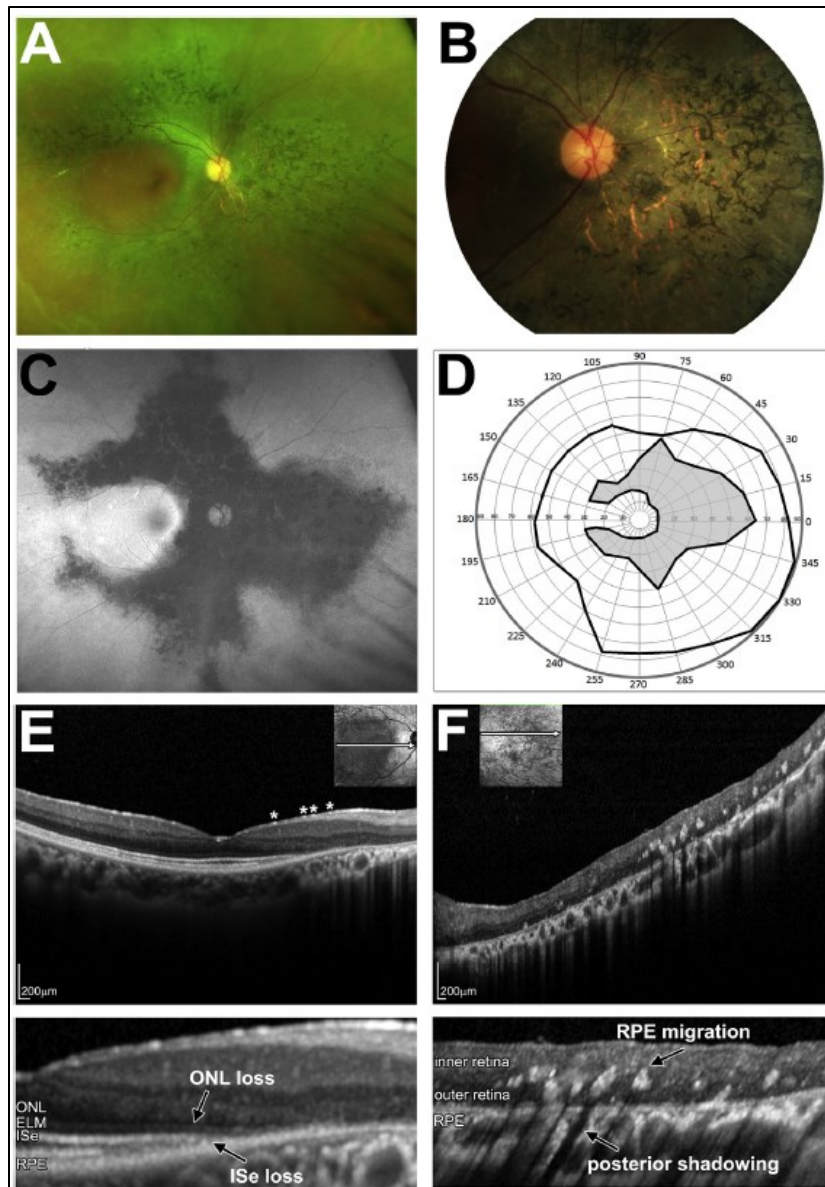


Figure 6: Clinical presentation of retinitis pigmentosa in a 50 year old patient. (A) Widefield image of the posterior pole displaying irregularities in pigment localisation, predominantly in the nasal and superior of the mid-peripheral retina. (B) Fundus photography revealing a prominence of bone spicules. (C) Extensive hypo-autofluorescence is detected in areas of RPE degeneration, and hyper-autofluorescence of the central retina. (D) Model of the dysfunctional retinal areas in the example eye mapped from visual field testing. (E) Spectralis (Heidelberg Engineering) OCT cross section of the macula displaying atrophy of the outer retina, and a typical loss of the photoreceptor layers. (F) Spectralis OCT cross-section demonstrating pigment migration towards the inner retina - the cause of the bone spicule appearance seen in (A) and (B). The migrating pigments cause shadowing due to their hyper-reflectivity. ONL, Outer nuclear layer; ELM, external limiting membrane; RPE, retinal pigmented epithelium; ISe, photoreceptor inner segment. Image from a review by Kalloniatis et al., (2016).

dysfunction, and inner retina breakdown (Kalloniatis et al., 2016). An example visualisation of the complexity of an RP phenotype is shown by fundus photography and OCT in Figure 6.

Rd1 and rd10 hom mice both express a random mutation to disable and reduce phosphodiesterase 6 (PDE6) activity, respectively. This leads to a build-up of cGMP in the photoreceptors, a prolonged hyperpolarised state, and eventual cell death. Both of these mouse models display early onset retinal layer degeneration and detachment from the RPE/ choroid by P27, and are subsequently good models to study RP. Further, rd10 mice more closely follow the time scale of degeneration in humans due to their reduced, rather than lack of, PDE6 activity (Pennesi et al., 2012), allowing a greater time window to study retinal layer death. Here, the advantage of tracking these retinal phenotypes by OCT rather than conventional histology is – despite the reduced resolution – that the same animals can be examined repeatedly, enabling progressive observations of degeneration. Additionally, more accurate thickness quantification can take place due to the abolishment of histological processing artefacts.

Overall, therapies for retinal diseases have only succeeded to manage or delay inevitable blindness, which is a depressing thought. Nevertheless, our molecular toolbox has improved dramatically over recent years. Since the retina is relatively immune-privileged, foreign molecules are fairly well tolerated – making inherited retinal diseases excellent candidates for molecular therapies. Vazquez-Dominguez, Garanto and Collin (2019) provided an extensive review of the current progress of such therapies in the treatment of retinal diseases. It encases the benefits and risks of many methodologies currently in the pipeline. Gene augmentation therapy, which aims at the replacement of a faulty gene via DNA or mRNA delivery, so far seems to be leading the way as approval for the first one-time retinal gene therapy Luxturna™ was granted in 2018. Luxturna™ utilises AAV2 delivery of a working copy of the RPE65 gene through sub-retinal injection (Novartis, 2018). Going forward this may be an effective strategy for treating recessive inherited eye conditions as more defective genes are discovered and characterised.

Genome editing tools such as zinc-finger nucleases, TALENs, and the increasingly powerful CRISPR/Cas system are also gaining traction in this field, yet these types of therapies require the cells of the target area to be alive, and hence, patients suffering from the later stages of retinal diseases may benefit more from an optogenetic approach (Vazquez-Dominguez, Garanto and Collin, 2019). Optogenetics is essentially manipulating control of neural tissue through light stimulation, and studies in mice are already showing promising results. Garita-Hernandez et al., (2019) recently showed that optogenetic transformation of either neonatal or adult mouse retinal cells can successfully recoup their perception of visual stimuli. Here, instead of correcting a gene, the forced expression of a microbial opsin (halorhodopsin) is capable of responding to light and opening chloride ion channels of transplanted photoreceptors, causing the necessary hyperpolarisation and neurotransmitter release to stimulate the retinal interneural networks. This system would essentially bypass the damaged, or lack of, photoreceptors in the patient. Yet, it will take time before it reaches the human medicinal market, as some microbial opsin issues still need to be ironed out; such as their narrow window of wavelength stimulation, their decreased sensitivity compared to mammalian rhodopsin, as well as the question of how much visual acuity could realistically be achieved in the patient. Other therapeutic attempts for retinal diseases include post-translational gene silencing or pathway specific therapies (Vazquez-Dominguez, Garanto and Collin, 2019), which require a greater knowledge of retinal homeostasis than we possibly currently hold.

IMPC screening prompted the development of a FAM84B gene KO mouse strain by CCP, our preliminary investigations revealed specific morphological changes within the retinal layers. Here, a degenerative phenotype mirroring aspects of AMD and retinitis pigmentosa was apparent; the next section will discuss current knowledge of the FAM84B gene and its respective protein transcript.

2.3 Family with a sequence similarity member 84b (Fam84b)

The FAM84B gene, located at cytogenetic location 8q24.21, is an elusive gene where its function in healthy cells is unclear, and so far, remains incompletely characterised. The protein transcript entails a primary structure of the 310aa long encoded by two exons, including a proline rich region at position 47-76 (The UniProt Consortium, 2018). Its first literature citing was under its initial alias Breast cancer membrane associated protein-101 (BCMP101), where Adam et al., (2003) identified it as a potential breast cancer risk gene due to a 3-fold increase in mRNA expression in human breast cancer tissues. Their immunocytochemistry experiments revealed a widespread intracellular localisation of the protein, as well as highly concentrated expression at the plasma membrane of MDA-MB-468 and T-47D mammary cancer cell lines. Interestingly, this was particularly in areas of cell-to-cell contact. Although informative, no insights of Fam84b's function arose from this apart from a novel interaction with the tumour-suppressing α -catenin. This is a fascinating notion considering α -catenin (depending on its dimerisation status) is directly involved in the regulation of the actin cytoskeleton at adherens junctions (Drees et al., 2005), and alterations here are a gateway to increased metastatic potential.

Since then, a handful of articles have provided correlational evidence for a potential role for Fam84b in tumourigenesis. Huang et al., (2006) reported an overexpression of Fam84b in a cohort of patients with oesophageal squamous cell carcinoma (ESCC), when compared to matched healthy epithelia. More recently, Hsu et al., (2015) showed that high Fam84b expression congregates in pre-treated oesophageal cancerous lesions; however, knockdown can delay ESCC xenograft tumour growth. This study also negatively correlated Fam84b expression with a patient's response to neoadjuvant chemoradiation therapy, leading to a proposal to implement Fam84b as a biomarker to aid therapeutic strategies. Furthermore, Fam84b has also been explicitly linked to initiating metastasis and castrate resistance in prostate cancer. Wong et al., (2017) emphasised that Fam84b is not only expressed in DU145 xenograft tumours, but also in even higher levels at the tumour edges of induced lung and bone metastasis. Curiously, this corresponded to the areas that were adjacent to healthy lung tissue, and all of the castrated NOD/SCID mice used in this work exhibited an elevated level of Fam84b expression compared to those not. This association between the evolution of metastatic castration resistant prostate cancer, decreased disease-free survival and Fam84b amplification is also backed up by Lin et al., (2017).

Despite the increasing evidence that Fam84b may be a protagonist in the development of the mentioned malignancies, a function for this enigmatic protein is still yet to be tied down. 8q24 is a well-studied region of the genome, and Fam84b's relationship with its genomic neighbours may be an important element in the journey to unravelling its routine function and suspected oncogenic ability.

2.4 The 8q24 locus

The 8q24 chromosomal locus is described as a 'gene desert' due to its low CpG count and protein-coding gene scarcity across its 2Mb span. It is commonly reported as a risk locus for SNPs and amplification in a number of cancers including prostate (Freedman et al., 2006) (Wong et al., 2017) (Du et al., 2015) (Jiang et al., 2019), breast (Turnbull et al., 2010) (Bertucci et al., 2012), bladder (Kimeney et al., 2008), colorectal (Pomerantz et al., 2009) and ovarian (Braem et al., 2011). Here, a number of GWAS studies have revealed SNP risk alleles at 8q24 for the above cancers (reviewed in Huppi et al. 2012 and Bishop et al., 2016).

8q24 is believed to be an epigenetically dynamic region of the genome, which can create inter-chromosomal interactions with other genome regions involved in cancer susceptibility, such as those with genes participating in Wnt/ β -catenin signalling activity or those where the genes for proto-oncogenic transcription factors E2F1 and TCF3 reside. This suggests some regulatory mechanism for the 8q24 region through physical contact (Du et al., 2015).

The FAM84B gene sits at the centromeric end of the 8q24 locus (shown in Figure 7), with POU5F1B located just distal to it. POU5F1B was initially viewed simply as a pseudogene of OCT4, one of the four Yamanaka pluripotency factors and a key transcription factor involved in stem cell reprogramming. Nevertheless, it has since been shown to demonstrate weak transcriptional activity (Huppi et al., 2012), and work cooperatively with Myc to promote gastric tumour formation and growth, most likely via its 95% homology with OCT4a (Hayashi et al., 2015). LncRNAs such as PRNCR1 (prostate cancer non-coding RNA) and PVT1 (plasmacytoma variant translocation 1), and a cluster of miRNAs are also transcribed from a region just downstream of MYC (Huppi et al., 2012), where attempts to decode the latter are now being attempted (Anauate et al., 2019).

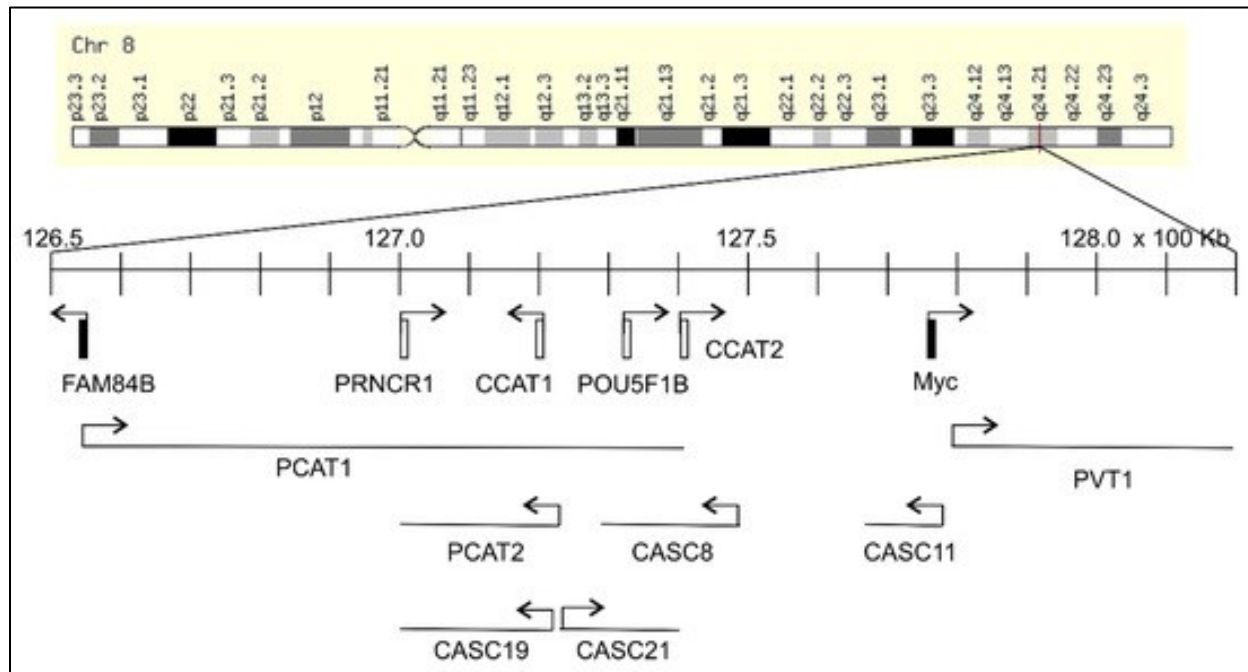


Figure 7: The 8q24 chromosomal locus. A gene desert in which FAM84B and MYC are the only protein-coding genes present. The positions and transcription direction of these, the OCT4 pseudogene POU5F1B, and a number of lncRNAs and miRNAs are shown, including the prostate cancer associated transcripts PCAT1 and PCAT2, and the SNP hotspot PVT1. Image is a summary of an analysis of the Genome Reference Assembly Human Genome build 38 by Gu et al., (2020).

Research focus on this region has centralised around the well-known family of proto-oncogenic transcription factors MYC, situated towards the telomeric end of 8q24. MYC expression is a point of convergence in various signalling pathways and contributes to a number of cellular processes such as cell growth and differentiation, apoptosis and protein translation; and many anti-cancer therapies involve targeting the reduction of Myc-Max dimerisation (Dang, 2012). As it is acknowledged as the protein-coding gene with the highest amount of somatic copy number alterations associated with cancer (Beroukhi et al., 2010), it is therefore possible that chromosomal alterations that target MYC could also lead to the co-amplification of neighbouring regions, leading to a higher expression of Fam84b in the cancerous tissue. At least 85% of prostate cancers follow this pattern (Gu et al., 2020), yet in cohorts of oesophageal and prostate cancer patients, increases in Fam84b expression do not always correlate with either MYC gene copy number or protein expression (Huang et al., 2006; Wong et al., 2017). Although this does not deny that large sections of 8q24 are co-amplified with MYC in cancer, the specific increase in Fam84b expression alone, without MYC amplification, reinforces the idea that it possesses a function relevant to the initiation or enhancement of certain malignancies.

2.5 Fam84b structure and homologies

When we examine Fam84b's primary structure more closely, a few interesting themes arise. Sequence alignments have revealed a homology to the Lecithin retinol acyltransferase (LRAT) enzyme family at amino acids 119-212 (Shinohara et al., 2011; Jiang et al., 2019). This unveiled another and more recent Fam84b alias; LRATD2 (LRAT domain-containing 2). The first of the LRAT family to be characterised, LRAT itself, plays an important role in the visual cycle where it performs one of the three steps in the RPE required to reset the chromophore to continue vision. Here, stimulation of opsin by photons leads to the photoisomerisation of 11-*cis*-retinal to all-*trans*-retinal, rendering it unable to respond to more photons on initiation of the phototransduction cascade. After its disassociation from opsin, it is reduced to all-*trans*-retinol (aka. vitamin A), and shuttled to the RPE where it accepts an acyl group from the *sn*-1 position of phosphatidylcholine via LRAT (Sears and Palczewski, 2015). Subsequent steps of isomerisation by RPE-65 and oxidation by 11-*cis*-retinol dehydrogenase complete the recycling of retinoids for the reappearance of a photosensitive pigment. LRAT null mice present photoreceptor degeneration of up to 35% by 8 weeks of age when compared to wild-type mice (Sears and Palczewski, 2015) and the sequence similarity between Fam84b and LRAT provides a plausible enzymatic potential for Fam84b in the retina.

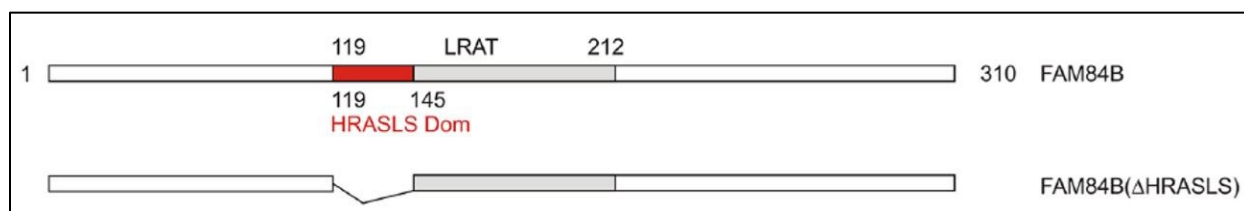


Figure 8: Fam84b Protein Structure. Top: Fam84b aligns with HRASLS at residues 119-145 (red) and LRAT at 145-212 (grey). Bottom: Jiang et al., (2019) achieved deletion of the HRASLS domain in DU145 cells.

In humans, five tumour-suppressor proteins, HRASLS1-5, also make up a part of the LRAT family, of which HRASLS2 and HRASLS4 are lacking from the rodent genome. All members of this sub-family possess phospholipase A2 and acyltransferase activity (PLA/AT), and hold functions in wide-range of fields from lipolysis regulation in white adipose tissue to the activation of transglutamases in the epidermis, as well as their dysregulation in various cancers via PLA/AT (Mardian, Bradley and Duncan, 2015). Fam84b aligns with HRASLS's N-termini at amino acids 119-145 (shown in Figure 8), and interestingly, the homologous region between the two actually encompasses the HRASLS catalytic domain, within which the highly conserved Cys-His-His catalytic triad is situated. Pang et al., (2012) performed a detailed analysis of the crystal structure and enzymatic activity of HRASLS1, showing that Cys¹¹³ on helix α 3 acts as the essential reactive nucleophile, the His²³ on the nearby β -strand forms ion pairing with Cys¹¹³, and His³⁵ aids in the maintenance of the triad's correct orientation. Substitution of any of these residues severely impacts the efficiency of nucleophilic attacks on the *sn*-1 or *sn*-2 acyl groups of phospholipids. The determination of HRASLS2 and HRASLS3's crystal structure also supported the collaborative action of Cys¹¹³-His²³-His³⁵ in these enzymes (Ren et al., 2010; Golczak et al., 2012). In addition, full-length HRASLS proteins seem to be required for their activity. Despite the observation that it is the N-terminal domains of HRASLS enzymes that are capable of inducing apoptosis and cell senescence (Wei et al., 2015), it cannot do it alone. Deletion of the hydrophobic C-terminus weakens their associations with membranes, resulting in a lack of signal anchorage and consequently, no detectable activity (Pang et al., 2012; Wei et al., 2015).

Shinohara et al., (2011) postulated a phylogenetic tree of the LRAT family members (shown in Figure 9), which incorporated Fam84a and Fam84b based on sequence homology. Fam84a and Fam84b share a 43% sequence identity; however, the former has received next to no research attention.

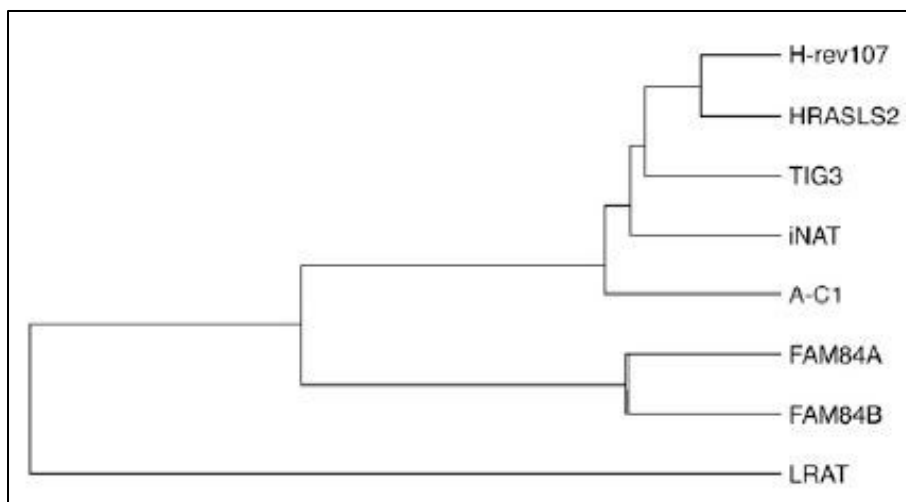


Figure 9: The LRAT family tree. An analysis by Shinohara et al., (2011) based upon their primary sequences. H-rev107 aka. HRASLS3 is the most studied of the HRAS-like suppressor (HRASLS) family. TIG3 aka. HRASLS4; iNAT aka. HRASLS5; A-C1 aka. HRASLS1; LRAT (Lecithin:Retinol Acyltransferase).

The HRASLS family acquired their name from their abilities to reduce Ras-related signalling (Mardian, Bradley and Duncan, 2015). In particular, HRASLS3 has been shown to suppress EGF-induced Ras activation in HtTA cells by up to 86% in a dose-dependent manner (Wang et al., 2014). The exact molecular mechanism for this has yet to be unravelled, however Wang et al., (2014) provided vital evidence; demonstrating that H-Ras is in fact an acylation target of HRASLS3, which can be obscured by PLA/AT inhibitors. Here, HRASLS3-mediated acylation reduced the level of active H-Ras-GTP and its downstream effectors, whereby suppression of the O-acyltransferase activity rescued H-Ras-driven signalling. Their results additionally showed that HRASLS3 acylation interferes with H-Ras C-terminal palmitoylation; an essential post-translational modification for its maturation and affinity to membranes (Ahearn et al., 2011). On the other hand, HRASLS3 has also been shown to form a stable complex with K-Ras-GDP (Han, Jeong and Jang, 2017), yet K-Ras does not undergo C-terminal palmitoylation (Ahearn et al., 2011), revealing potential inconsistencies in the said theory.

As Fam84b demonstrates homology to the catalytic portion of the HRASLS family, we can speculate that it may carry out a similar function. This includes a probable link between Fam84b's overexpression in cancers mentioned previously, and alterations in Ras signalling; a pathway often deregulated in malignant cells. Unfortunately, it appears Fam84b displays a lack of PLA/AT activity, most likely due to a substitution of cysteine to serine in the supposed catalytic triad (Shinohara et al., 2011). The sequence alignment in Figure 10 illustrates this and, if accurate, implies that Fam84b establishes a Ser-His-His catalytic triad instead. This unconventional triad is present in nature; utilised by proteases of cytomegaloviruses (Ekici, Paetzel and Dalbey, 2008). Albeit, there is no mention in the literature of its occurrence in mammalian proteins as of yet. With all considered, Fam84b must have some functional relevance as deletion of the HRASLS-like domain renders DU145 cells no longer capable of triggering growth and invasion *in vitro* (Jiang et al., 2019), and its potential effect on the dynamics of Ras signalling pathways is a sensible place to start looking.

		HRASLS domain (119-145)	
FAM84B	yqksfapgsaalstytpenllnkckpgdlvefvsqagyphwavyvgnfqvvhhrleivn	152	
HRASLS1	f-----nd-----cfslnypgnpcpgdlievf-rpgyqhwalylgdgyviniapvdgip	50	
HRASLS5	wssipkpenegklikqaaegkprprpgdlieif-rigyehwaiyveddcvvhlapseef	165	
HRASLS4	-----masphqepkpgdlieif-rlgyehwalylgdgyvihlapseyp	43	
HRASLS2	-----malarprprlgdlieis-rfgyahwaiyvgdgyvvhlapaseia	43	
HRASLS3	-----mrapipepkpgdlieif-rpfyrhwaiyvgdgyvvhlappseva	43	
	. ***:*. : * ***:*. : *:.. .		
		Active	Active
FAM84B	-----sfltdasqgrgrvndl-yrykplsssavvrnalavhgak	192	
HRASLS1	a-sftsaksvfsskalvkmqlldvvgndtyrinnkydetypplpveeiikrsefvgqe	109	
HRASLS5	evg--sitsifsnravvkysrledvlgcswkvnkldgtylplpvdkiqrctkmvnki	223	
HRASLS4	gagsssvfsvlsnsaevkrerledvvggccyrvnnsldheyqprpveviissakemvgqk	103	
HRASLS2	gagaasvlsaltnkaivkkellsvvggdnryrvnnkhddrytplsniivkraeelvgqe	103	
HRASLS3	gagaasvmsaltdkaivkkellydvagsdkyqvnkhddkysplpcskiiqraeelvgqe	103	
	. * . : : * . * * . : : :		
FAM84B	e-relswrnsesfaawcrygkrefkiggelrigkqpyrlqiqlsaqrshtlefqsledli	251	
HRASLS1	vaynllvndcehfvtllrygegvseqanraistv-----e-fvtaavgvfsflglf	159	
HRASLS5	vqysliegncehfvnlgrygvprsqqvehalmeg-----a-kaagavisavvdsik	273	
HRASLS4	mkysivsrrncehfvtqlrygksrckqvekakvev-----g-vatal-gilvvagcs	152	
HRASLS2	lpysltsdncehfvnhlrygvsrsdqvtgavttv-----g-vaagllaaslvgil	153	
HRASLS3	vlykltseencehfvnrlrygvarsdqvrdiviaa-----s-vagmglaamsligvm	153	
	.: : * * . *** .		
		Active	
		Cat	
FAM84B	mek-rrndqigraavlqelathlhpaeeegdsnvartppppgrppapsseedgeavah	310	
HRASLS1	pkg-qrakyy-----	168	
HRASLS5	-----pkpita-----	279	
HRASLS4	fairryqkkata-----	164	
HRASLS2	larskrerq-----	162	
HRASLS3	fsrnkrqkq-----	162	

Figure 10: Sequence alignment of Fam84b and HRASLS1-5. The HRASLS family maintain a conserved NCEHFV motif (underlined). The residues that make up the catalytic triad (Cys113, His23 and His35) are marked. The Cys113 nucleophile (Cat) is substituted for Ser113 in Fam84b. HRASLS, HRAS-like suppressor; S, serine; NCEHFV motif includes the amino acids: asparagine (N), cysteine (C), glutamic acid (E), histidine (H), phenylalanine (F), and valine (V). Alignment was performed by Jiang et al., (2019).

2.6 Ras GTPases

The Ras superfamily consists of proteins capable of hydrolysing guanosine triphosphate (GTP) to guanosine diphosphate (GDP) to elicit continuous alterations in eukaryotic cell activity. They are central regulatory components that either directly or indirectly affect nearly all aspects of inter- and intracellular communication (Rajalingam et al., 2007). These small GTPases can be divided into five main subfamilies of Ras, Rho, Rab, Arf/Sar and Ran based on their sequence, structure and function. All Ras-related proteins function as binary molecular switches with intrinsic GTP hydrolysis and GDP/GTP exchange capabilities. Tight control by two main classes of regulatory proteins is offered by; guanosine-nucleotide exchange factors (GEFs), which stimulate the formation of the active GTP-bound enzyme; and GTPase-activating proteins (GAPs), which help to accelerate GTPase activity leading to the inactive GDP-bound form (Wennerberg, Rossman and Der, 2005). This means they exist in “on” and “off” states, where the “on” conformation has much higher affinity for its downstream effectors, leading to the initiation of the directed cellular effect. The proto-oncogenic Ras family specifically are implicated in around 30% of human cancers, where glycine 12, glycine 13 or glutamine 61 are mutated in more than 95% of Ras-related malignancies. This renders Ras proteins incapable of GTP hydrolysis, and relentlessly sustained in the “on” signalling state (Han, Jeong and Jang, 2017).

The Rho subfamily influence cytoskeletal dynamics via their interaction with the plasma membrane, and hence have a hand in cell polarity, migration and extracellular matrix remodelling. The largest subfamily, Rab, regulates vesicle trafficking and microtubule dynamics, where each organelle and each membrane type employ at least one of its own unique Rab proteins to ensure specificity. The Arf subfamily are also involved in vesicular trafficking, their N-terminal myristoylation aids functions such as the recruitment of coat proteins (e.g. clathrin and COPI) during vesicle formation. Finally, the last major subfamily Ran, is vital for protein and RNA translocation through the nuclear pore complex, in which the direction of transport into, or out of the nucleus is determined by its GDP or GTP bound status and interaction with a GAP or GEF (Song et al., 2019).

The founding Ras family serve as signalling nodes, initiating and controlling intracellular growth factor signalling upon their activation by extracellular stimuli. Activation leads to the transcription of a diverse number of genes involved in cell proliferation, differentiation and survival, in a context-specific manner. However, this can only occur if Ras and its associated GEF are in direct proximity, which is accomplished by their recruitment to the inner side of the plasma membrane. C-terminal prenylation and palmitoylation are the essential post-translation lipid modifications that arise during Ras maturation, in which it cannot elicit its affect without. Ras prenylation encompasses farnesylation of a cysteine residue at the CaaX motif (in which “C” is cysteine, “a” is an aliphatic amino acid and “X” can be any amino acid) by farnesyltransferases (Simanshu, Nissley and McCormick, 2017). The addition of a hydrophobic 15-carbon farnesyl lipid leads to the accumulation of Ras on the cytoplasmic side of the endoplasmic reticulum, where the aaX is cleaved and the remaining farnesylcysteine undergoes methylesterification. This results in the C-terminally remodelled Ras capable of inserting into membranes. In the case of dually lipidated H-Ras, N-Ras and K-Ras4a, these events are a prerequisite to further S-palmitoylation processing at the cytoplasmic face of the Golgi apparatus to permit trafficking to the plasma membrane, where it can actively signal. Ras that is both farnesylated and palmitoylated then has >100-fold higher affinity for membranes (Ahearn et al., 2011). This is often referred to ‘affinity trapping’ the protein.

Studies in yeast have revealed DHHC (Asp-His-His-Cys) palmitoyl acyltransferases (DHHC-PATs) that can facilitate the addition of palmitoyl groups to cysteine residues upstream of CaaX on Ras *in vitro* (Swarthout et al., 2005). On the other hand, inconsistencies have arisen in the DHHC-PATs that control mammalian Ras palmitoylation *in vivo*, possibly due to PAT compartmentalisation, or the increased complexity of eukaryotic Ras regulation (Young et al., 2013). Palmitoylation, unlike farnesylation, is a reversible modification. After a set amount of time at the plasma membrane, Ras proteins are depalmitoylated and released into the cytoplasm. This is followed by re-joining with the Golgi apparatus for another cycle of palmitoylation and vesicular transport to the plasma membrane. So far, Ras farnesylation seems to be an automatic housekeeping process (Ahearn et al., 2011) due to the lack of evidence for prior regulatory steps. Additionally, much is still to be learnt about the regulation of palmitoylation cycling. Ras depalmitoylation by acyl protein thioesterases may be driven by Ras-GTP loading and activity (Baker et al., 2003), or the distribution of palmitoylated-Ras between lipid rafts and more soluble membranes (Agudo-Ibáñez et al., 2015). However, the mechanisms or proteins recognising the need to remove Ras from the plasma membrane are unclear.

Once Ras is settled in the plasma membrane, its availability to be activated comes into effect. There are many downstream effects of Ras, in which the end point is changes in gene expression to stimulate the appropriate alterations in cellular physiology. Ras effectors, such as Raf, have a strong affinity for Ras-GTP, and their binding via a Ras-binding domain triggers the activation of a mitogen-activated protein kinase (MAPK) cascade. MAPK signalling pathways are key intermediaries between the binding of extracellular stimuli to their receptor at the plasma membrane, and transcriptional activity, regulating cellular processes such as growth, differentiation, apoptosis and immune responses (Rajalingam et al., 2007). In mammals, there are four major MAPK pathways which result in the activation of; extracellular signal-related kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), p38, and ERK5. The ERK1/2 pathway was the first to be identified, and is activated mainly through growth factor and hormone stimulation at the membrane. Here, the binding of epidermal growth factor (EGF) to a tyrosine-linked EGF receptor (EGFR) leads to the dimerisation and activation of the cross-phosphorylation tyrosine kinase activity of the dimerised receptor. The EGFR's tyrosine residues become phosphorylated allowing docking proteins with an SH2 domain to bind the phosphotyrosines (e.g. GRB2). A GEF, such as SOS, docks via an SH3 domain and stimulates the removal of GDP from Ras, permitting Ras to bind a GTP molecule in its active conformation. Fibroblast growth factor receptor and platelet-derived growth factor receptor also follow this Ras-activating route this with their respective ligands.

MAPK pathways are three-tiered signal transduction cascades, using phosphorylation as their key tool to transmit instructions for cellular fate. A MAP3K, e.g. Raf (Rajalingam et al., 2007), receives input from various sources, but most notably drawn to the plasma membrane to interact with an active small GTPase such Ras-GTP. An intermediate tier of kinases (MAP2Ks) are dependent upon a MAP3K for activation, which go on to activate MAPKs at the final tier of the cascade. MAPKs are considered the pathway's effectors, which phosphorylate a large number of substrates to stimulate the appropriate directed response. Amplification and much cross-talk occurs along MAPK cascades and other signalling pathways in order to elicit a robust and coordinated response. ERK1/2, the MAPK functioning downstream of Raf and MEK1/2, phosphorylates transcription factors c-fos and c-jun in order to activate the transcription of many genes involved in cell proliferation, such as the oncogene MYC. Other Ras effectors include PI3K, which through a phosphoinositol-dependant mechanism can activate NF- κ B to contribute to cell cycle progression, BCL-X proteins influencing cell survival and apoptosis, and the mTOR pathway regulating metabolic growth. Ras can also initiate modifications in cell motility and migration, through the activation of other small GTPases Rac and Rho (Gimple and Wang, 2019).

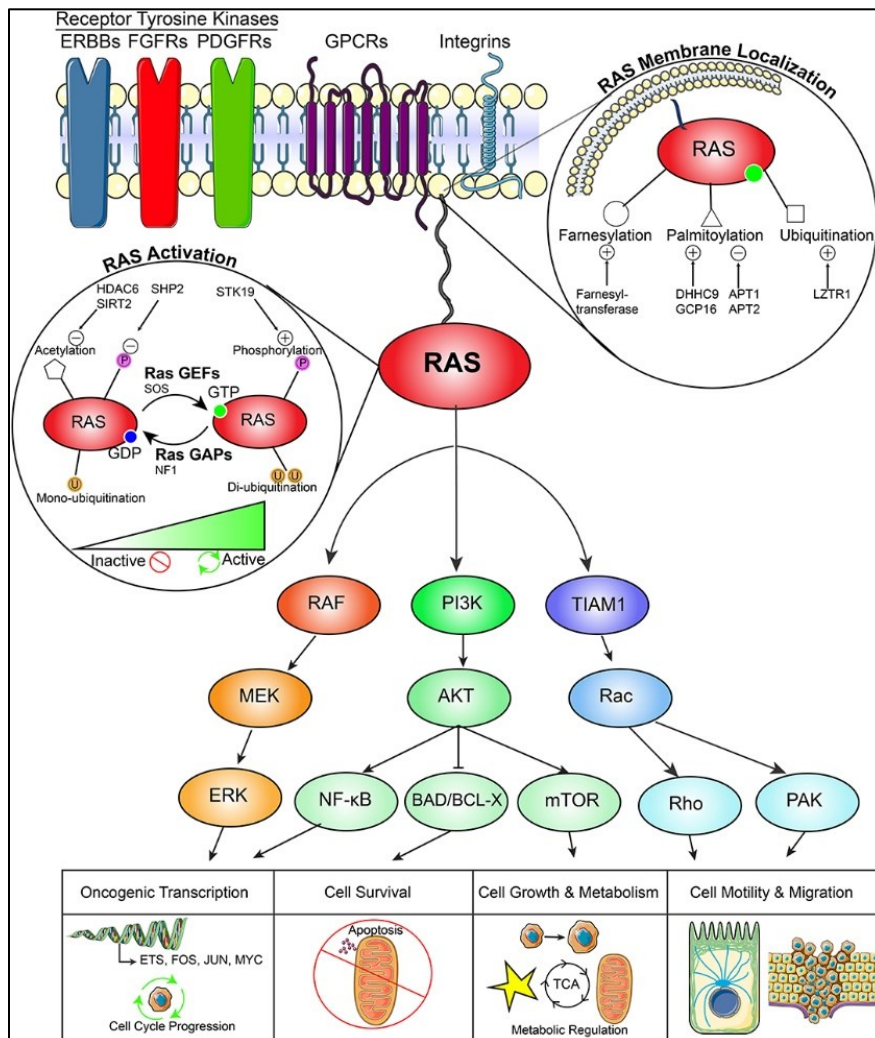


Figure 11: Overview of Ras' role in eukaryotic cells. Ras is activated via events initiated by tyrosine kinase-linked receptors, G-protein coupled receptors and integrin the plasma membrane. Ras' affinity for the plasma membrane is increased by lipid modifications to its C-terminus. Activation of Ras is dependent on its GDP or GTP bound status, regulated by GEFs and GAPs. There are many downstream effects of Ras, the diagram shows three examples of pathways that result in varied changes in cellular activity. Image from a review by Gimple and Wang et al., 2019.

Certainly, the downstream effects of Ras are extremely diverse – and too extensive to discuss them all here. This is made more complex by the huge amount of cross-talk between its effector pathways, and regulation on Ras itself. Figure 11 shows a brief overview of Ras activation, maturation, and some examples of its downstream effector pathways that influence and maintain homeostasis. Deviations in any of these regulatory mechanisms or well-controlled signalling cascades may prove problematic for the cell, or the organism as a whole. It is clear that the superfamily Ras GTPases are central for the proper functioning of mammalian cells. As Wang et al., (2017) observed HRASLS enzymatic activity is capable of interrupting Ras palmitoylation, we can consider the possibility that the homologous Fam84b may also play a part in the regulation of Ras affinity to the membrane, and consequently, its signalling activity.

Fam84b is currently an uncharacterised protein. This thesis work will present a FAM84B KO mouse model and describe the associated phenotypical findings. It will also show work that has been completed to attempt to discover where this protein fits into maintaining retinal homeostasis. Our knowledge of genes, molecular pathways and their associated pathophysiology has taken giant leaps over recent years, but there is still much learn. With practical and effective animal models at our side, our fundamental understanding of biological systems will continue to grow, which will aid us in improving quality of life and treating disease more effectively.

3 THESIS AIMS AND HYPOTHESES

3.1 Aims

- To fully characterise the retinal phenotype unveiled in FAM84B KO mice
- To determine the expression of Fam84b within retinal tissue
- Find clues about the function of Fam84b by discovering its interacting protein partners
- Analyse the effect of FAM84B KO on a RAS-MAPK signalling pathway in the retina

3.2 Hypotheses

- KO of FAM84B leads to retinal degeneration in C57Bl/6NCrl mice
- Fam84b reduces RAS-associated signalling in the normal retina

4 MATERIALS & METHODS

4.1 Animal husbandry

Animals were raised at the Czech Centre for Phenogenomics (Institute for Molecular Genetics of the ASCR, v.v.i., Czech Republic) in individually ventilated cages with a 12/12hr light/dark cycle. They were allowed access to food and water ad libitum.

4.2 Genotyping the FAM84B locus in mice

The Transgenic and Archiving Module at the Czech Centre for Phenogenomics designed and generated a C57Bl/6NCrl mouse line with the FAM84B gene knocked out using the CRISPR/Cas9 system. This line was used for IMPC-directed primary screening at the Czech Centre for Phenogenomics. Responsibility for the breeding pairs of this line was transferred to me.

4.2.1 DNA isolation

Between 3-5 weeks of age, 0.5cm of C57Bl/6NCrl mouse tails were cut into a microfuge tube. Tails were incubated overnight at 55°C with 0.5ml DNA digestion buffer (1M Tris HCL pH8.5, 1M NaCl, 0.5M EDTA, 10% SDS, dH₂O) and 0.2mg/ml Proteinase K. DNA was isolated using 0.5ml phenol/chloroform/isoamyl alcohol (25:24:1) per tail and mixing vigorously, centrifuging at 13,500rpm for 4.5mins, adding 0.5ml 96% ethanol to the upper phase and inverting until a DNA precipitate formed. Precipitates were centrifuged at 13,500rpm for 4.5mins, and supernatants removed. 70% ethanol (-20°C) was added to pellets, centrifuged at 13,000rpm for 4.5mins and the supernatants discarded. Pellets were dried at 37°C for at least 4 hours and resuspended in 200µl dH₂O.

4.2.2 Polymerase chain reaction

DNA was amplified by means of PCR. The reaction mixture template is shown in Table 1. Primers used for amplification of the target region are shown in Table 2. The PCR amplification protocol is shown in Protocol 1.

Table 1: Reaction mixture template for FAM84B genotyping protocol

Reagent	Volume (µl)
Dream Taq buffer 10x (ThermoFisher Scientific, USA)	2.5
dNTPs (10mM) (ThermoFisher Scientific, USA)	0.5
Primer forward (10µM)	1.0
Primer reverse (10µM)	1.0
Dream Taq polymerase (ThermoFisher Scientific, USA)	0.2
DNA	3.0
dH ₂ O	16.8
Total volume	25.0

Table 2: Primer sequences for FAM84B genotyping protocol

Primer name	Sequence
Fam84b FW	5'-GCACCAGTGCCAATGACTCTG-3'
Fam84b REV	5'-GGTGCAAATGCACCACCTGG-3'

Protocol 1: PCR amplification protocol for FAM84B genotyping

Step	Temperature (°C)	Time	
Denaturation	95	5min	
Denaturation	95	30sec	x38
Annealing	61	40sec	
Elongation	72	75sec	
Final Elongation	72	5min	
End	12	∞	

4.2.3 Gel electrophoresis

PCR products were separated by gel electrophoresis by loading 10µl onto a 2% agarose (SeaKem LE Agarose, East Port Life Science, Czech Republic) gel. Gels were run at 80V until products were appropriately separated. Animals were designated as; wild type if the PCR product was 514bp, homozygous if the product was 250bp, and heterozygous if both 514bp and 250bp products were present.

4.3 Optical coherence tomography

4.3.1 Experimental procedure

Mice were anaesthetised by injecting 20% Zoletil – 0.03g/kg tiletamin and 0.03g/kg zolezepam (Virbac, France) intramuscularly. Pupils were dilated by applying drops of Atropin-POS® 0.5% (Ursapharm, Czech Republic). Once pupils were dilated, Visidic 2g/mg (Dr. Gerhard Mann Chem-pharm, Germany) was applied to eyes to prevent corneal dehydration, and PMMA contact lenses (Cantor&Nissel, United Kingdom) were placed on the eyes to assist image acquisition. Images of the fundi and retinas of animals were obtained by spectral-domain optical coherence tomography using HRA+OCT Spectralis® PLUS (Heidelberg Engineering, Germany). Anaesthetised mice were placed on a platform with the eye to be measured perpendicular to the camera. The fundus and retina were brought into focus and the optic nerve head centralised, and the scan angle was set at 30°. Fundus images 5.3 x 5.3mm in size were taken. Retinal cross-sectional images 5.3 x 4.4 x 1.9mm in size were taken, with a distance of 74µm between the 61 b-scans in 'high resolution mode'. Images were obtained of animals aged 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 weeks of age to track potential changes in FAM84B KO and wild type mouse retinas.

4.3.2 Retinal thickness quantification

OCT images were exported from the Spectralis® PLUS (Heidelberg Engineering, Germany) acquisition software into AVI format. AVI files were transformed into 3D TIFF format using a MATLAB (MathWorks, USA) script written at the Czech Centre for Phenogenomics (Institute of Molecular Genetics of the ASCR v.v.i., Czech Republic). The thickness of each retinal layer of mouse eyes was determined using The Iowa Reference Algorithms (Retinal Image Analysis Lab, Iowa Institute for Biomedical Imaging, Iowa City, IA, USA). The segmentation of each retinal layer for each eye was checked manually, and altered to best represent the boundaries of each layer if required. Quantification of layer thickness was exported and analysed separately in Microsoft Excel. Thickness values were averaged from the whole retina except the periphery and the area surrounding the optic nerve head. The total retinal thickness was defined as the distance from the inner limiting membrane to the outer boundary of the retinal pigmented epithelium. The inner retinal thickness was defined as the distance from the inner limiting membrane to the outer boundary of the outer nuclear layer. The outer retinal thickness was defined as the distance from the

outer boundary of the outer nuclear layer to outer boundary of the retinal pigmented epithelium. Data obtained from animals aged 12, 24, 36, 48 and 60 weeks were used for layer thickness analysis.

4.3.3 Retinal damage quantification

FAM84B KO mice were examined for the amount of degeneration present in their retinas using the HRA+OCT Spectralis® PLUS integrated software. Each obtained retinal cross-sectional image was used to define the boundaries of degenerated retinal tissue in each z-stack of the scan, and marked on the corresponding fundus image to define an area representing degenerated retinal tissue. The HRA+OCT Spectralis® PLUS integrated software quantified the area of these regions of degenerated tissue automatically. These were added together to provide a total area of degeneration (within the scanned region) for each eye.

4.3.4 Grading severity of FAM84B KO retinal degeneration

Values generated for each eye described in section 4.3.3 were used to grade the severity of retinal degeneration in FAM84B KO animals. Cross sectional b-scan images were also used to define the criteria, by inspecting if the RNFL and GCL layers at the vitreo-retinal border showed deformities. Table 4 defines the criteria that each FAM84B KO retina was assessed by. Each animal received a grade for each eye. This grading system was subsequently used to split eyes into categories for electroretinography experiments and analyses. A full breakdown of which category animals were placed in is shown in Supplementary Table 1.

Table 4: Retinal degeneration category criteria

Criteria	Degeneration Category			
	Wild type	Mild	Moderate	Severe
FAM84B KO homozygous genotype	X	✓	✓	✓
Total degeneration surface area <1.5mm ²	-	✓	✓	X
Total degeneration surface area >1.5mm ²	-	X	X	✓
Deformation of RNFL and GCL	-	X	✓	X

4.3.5 Statistical analysis

The total, inner and outer retinal thickness of wild type and FAM84B KO mice at 12, 24, 36, 48, 60 weeks of age was compared for statistical differences using a two-tailed Student's ttest. KO retinal thickness was compared against the wild type thickness of the same age group. If the returned p-value was <0.05, then the Bonferroni correction was applied as a post-hoc test. The p-values were then expressed as; not significant, p<0.05, p<0.01, p<0.001, or p<0.0001 depending on the significance level. Age groups were also compared for differences within the same genotype using the same methodology.

4.4 Electrophysiology

4.4.1 Experimental procedure

Wild type and FAM84B KO mice were assessed by single-flash whole-field electroretinography using the Electrophysiological Test Unit RETIanimal (Ronald Consult, Germany). Animals were measured at 12, 30, 48 and 60 weeks of age to monitor changes in the two groups over time. Mice were dark-adapted overnight (at least 12hours) in Red/Black IVC cages (Techniplast, Italy), then anaesthetised by injecting 20% Zoletil – 0.03g/kg tiletamin and 0.03g/kg zolezepam (Virbac, France) intramuscularly. Pupils were dilated by applying drops of Atropin-POS® 0.5% (Ursapharm, Czech Republic). Once pupils were dilated, Visidic 2g/mg (Dr. Gerhard Mann Chem-pharm, Germany) was applied to eyes to prevent corneal dehydration. Small golden ring electrodes (3mm in diameter) were placed onto the corneas of both eyes as the active electrodes, and the pupils centred inside. The reference electrode was placed under the skin just above the snout, and a ground electrode was placed under the skin in the animal's back close to its hind legs. The anaesthetised mice were then exposed to white light flash stimulations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 cds/m² in scotopic conditions by a Ganzfeld Q450 SC stimulator. Each stimulation was repeated 50 times, the individual responses for each eye were recorded, and an average response was also saved for each intensity using the RETIport software (Ronald Consult, Germany). Following this, the animal was exposed to a white background (25cd/m²) light for 2mins to generate photopic conditions. The animals were then exposed to white light flash stimulations of 3 and 10 cds/m² whilst maintaining the 25cd/m² background light. Each stimulation was repeated 50 times, individual responses for each eye were saved, and an average response generated for each intensity using the RETIport software (Ronald Consult, Germany).

4.4.2 Application of the Naka-Rushton equation to response curves

The response curves generated were examined using a semi-automated MATLAB (MathWorks, USA) script to identify, and quantify the amplitudes of the a-, b- and c-waves of each response. The amplitudes of each wave were analysed in a Microsoft Excel spreadsheet designed to apply the Naka-Rushton equation (Naka and Rushton, 1966) to the a-, b- and c-waves of electroretinogram responses. The Naka-Rushton equation was applied to the response amplitudes of the intensities shown in Table 5.

The Naka-Rushton equation is defined as:

$$R(L) = V_{max} \frac{L^n}{L^n + K^n}$$

R= response amplitude

V_{max}= theoretical maximum response of the retina

L= Luminance/ Stimulation intensity

K = ½ *V_{max}*

n= slope

Table 5: The Naka-Rushton equation was applied to the a, b and c waves.

ERG wave	Intensities that Naka-Rushton was applied to (cd-s/m ²)
<i>a</i>	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3
<i>b</i>	0.001, 0.003, 0.01, 0.03, 0.1
<i>c</i>	0.03, 0.1, 0.3, 1, 3

Once the equation had been applied to all curves, the V_{max} , K and n values were extracted and compared for differences between wild type and each degeneration severity category of FAM84B KO mice (Mild, Moderate and Severe).

4.4.3 Statistical analysis

A single factor ANOVA test was used to compare the V_{max} and K values for each retina were compared between the four groups (wild type, mild, moderate and severe). If the p-value returned was <0.05 , then a two-tailed Student's ttest was applied to all group comparisons. This allowed visualisation of which groups were significantly different from each other. If the returned p-value was <0.05 , then the Bonferroni correction was applied as a post-hoc test. The p-values were then expressed as; not significant or $p < 0.05$ depending on the significance level. The same methodology was applied to each age group (12, 30, 48 and 60 weeks of age).

4.5 Histology

4.5.1 Tissue and slide processing

Animals were euthanised by cervical dislocation, and heads were cut at the neck to detach them from the body. Heads were fixed in 40ml Modified Davidson's fixative (recipe shown in Table 6) for 48 hours at 4°C , then transferred to 70% ethanol for storage at 4°C . Eyes were excised from the head and embedded in paraffin blocks using Leica ASP6020/ EG1150H paraffin embedding station (Leica Microsystems, Germany). Sections of 5-8 μm were cut using a microtome (Leica RM2255, Leica Microsystems, Germany), and placed onto Superfrost® Plus slides (ThermoFisher Scientific, USA). Slides were left to dry on a heating pad (P-LAB, Czech Republic) at 50°C overnight. Slides were deparaffinised and rehydrated by immersing slides in a series of solutions using the protocol shown in Protocol 2. Slides were then stained with Harris' haematoxylin (SigmaAldrich, USA) and eosin (SigmaAldrich, USA) using an automated tissue processor (Leica ST5020/ CV5030, Leica Microsystems, Germany), mounted using Aquatex® (Merck Millipore, USA) and cover slips were placed on top (VWR, USA). Sections were viewed using a Brightfield microscope (Zeiss, Germany).

Table 6: Modified Davidson's fixative recipe

Reagent	Volume
37% formaldehyde (Penta, Czech Republic)	300ml
100% ethanol (VWR, USA)	150ml
Acetic acid (Penta, Czech Republic)	50ml
dH ₂ O	500ml
Total	1000ml

Protocol 2: Deparaffinisation and rehydration of tissue sections

Step	Reagent	Time
1	Xylene (VWR, USA)	10min
2	Xylene (VWR, USA)	5min
3	Isopropanol (Penta, Czech Republic)	5min
4	100% ethanol (VWR, USA)	5min
5	96% ethanol	5min
6	70% ethanol	5min
7	dH ₂ O	-

4.5.2 Cell nuclei counting

The number of nuclei in the GCL, INL and ONL was counted in haematoxylin and eosin stained retinal sections. Both eyes of 2 wild type and 2 homozygous FAM84B KO animals were chosen, and 2 images from each eye were analysed. Both groups contained one animal that was sacrificed at 42 weeks of age, and one animal sacrificed at 60 weeks of age. Images were measured and cropped appropriately using ZEN Blue (Carl Zeiss, Germany) software, so that a 200µm long stretch of retinal tissue could be analysed. Sections of retinal tissue 1mm away from the optic disc were chosen. Cell nuclei were counted manually using the cell counter plugin in ImageJ (National Institute of Health, USA) in the GCL, INL and ONL layers. Nuclei number was compared between genotypes and between age groups initially with a single factor ANOVA. If the calculated p-value was <0.05, a two-tailed Student's ttest was applied, followed by a Bonferroni post-hoc test. Significant p-values were expressed as "p<0.05".

4.6 Immuno-histochemistry

4.6.1 Preparation of slides for immunostaining

Slides were prepared as previously described in section 4.5.1. Following deparaffinisation and rehydration, heat-induced epitope retrieval was carried out by either cooking slides in HIER Citrate buffer pH 6 or 9 (Zytomed Systems, Germany) for 5min at 95°C using Declocking Chamber™ NxGen (Biocare Medical, USA), or cooking slides overnight at 60°C in UVP HB-1000 hybridisation oven (ThermoFisher Scientific, USA). Slides were allowed to cool to room temperature and washed 2x5mins in PBS. Endogenous peroxidase activity was blocked by immersing slides in a Coplin jar with the blocking solution described in Table 7 for 20min at room temperature. Slides were washed 2x5min in PBS. Unspecific antibody binding was blocked by applying 30µl of the solution described in Table 8 onto each tissue section, and incubating at room temperature for 20min.

Table 7: Endogenous peroxidase activity blocking solution

Reagent	Volume
Methanol (Penta, Czech Republic)	72ml
30% H ₂ O ₂ (Penta, Czech Republic)	8ml
Total	80ml

Table 8: Unspecific antibody binding blocking solution

Reagent	Volume
Albumin (P-LAB, Czech Republic)	0.2g
dH ₂ O	10ml

4.6.2 Detection of retinal cell markers

Retinal cell markers were detected on retinal tissue sections using the conditions summarised in Protocol 3. Primary antibodies were diluted in Antibody Diluent (Zytomed Solutions, Germany) according to their individual optimisation, where 50µl of solution was applied to each section. Sections were incubated with primary antibody at room temperature for 60min. Sections were washed 2x5min in PBS before exposure to secondary antibody for 30min at room temperature. Sections were washed again 2x5min in PBS. Chromogen detection was carried out by applying drops to the sections and incubating for 1-10 mins and checking under a microscope. Slides were counterstained using Harris' Haematoxylin (SigmaAldrich, USA) for 2mins at room temperature, then applying warm running tap water for 1-2min. Slides were mounted with Aquatex® (Merck Millipore, USA) and coverslips placed on top (VWR, USA). Sections were viewed using a Brightfield microscope and images were taken using AxioScan Icc 5 camera and Zen Blue software (Zeiss, Germany).

Protocol 3: Antibody conditions for detection of retinal cell markers in retinal tissue sections

Primary antibody					Secondary antibody		
Target	Supplier	Catalogue no.	Dilution	Antigen retrieval	Secondary antibody	Chromogen detection	Supplier
Anti-heme oxygenase 1	Abcam, (United Kingdom)	ab13243	1:250, 500, 750	pH 6,9	HRP-Polymer anti-Rabbit	AEC	Zytomed Solutions, Germany
Calretinin	Novus Biologicals, USA	NBP1-88220	1:600	pH 6			
CD31	Abcam, (United Kingdom)	ab28364	1:100	pH 9			
Collagen IV	Abcam, (United Kingdom)	ab6586	1:500	pH 9			
Fam84b	Novus Biologicals, USA	NBP2-47459	1:50	pH 9			
Ki-67	Thermo Fisher Scientific, USA	MA5-14520	1: 500	pH 6			
PKC-α	Novus Biologicals, USA	NBP1-87268	1:100	pH 9			

POU4F3	Novus Biologicals, USA	NBP1-88349	1:500	pH 9			
Rhodopsin	Novus Biologicals, USA	NBP2-55553	1:100	pH 9			
Tyrosine hydroxylase	Abcam, (United Kingdom)	ab112	1:500	pH 6			

4.6.3 Immunofluorescence of RPE cell marker

Slides were prepared as previously described in section 4.5.1. Following deparaffinisation and rehydration, heat-induced epitope retrieval was carried out by cooking slides in HIER Citrate buffer pH 6 or 9 (Zytomed Systems, Germany) at 60°C in UVP HB-1000 hybridisation oven (ThermoFisher Scientific, USA). Slides were allowed to cool to room temperature and washed 2x5mins in PBS. Unspecific antibody binding was blocked by applying 30µl of the solution described in Table 9 onto each tissue section, and incubating at room temperature for 60min. Sections were exposed to primary antibody, diluted in PBS 0.1% Tween20 (SigmaAldrich, USA) and 2% albumin (P-LAB, Czech Republic), for 2hrs at room temperature, washed 2x10min in PBS, and exposed to secondary antibody for 60min at room temperature. Dilutions were prepared as per described in Protocol 4. Slides were washed 3x10min in PBS and counter stained with DAPI (SigmaAldrich, USA). Slides were shielded from light to prevent bleaching, mounted in Aquatex® (Merck Millipore, USA) and viewed using a fluorescent microscope. Images were taken using Zen Blue software (Zeiss, Germany).

Table 9: Blocking solution for immunofluorescence detection

Reagent	Volume
Albumin (P-LAB, Czech Republic)	0.1g
Foetal bovine serum (UMG Media, Canada)	250µl
PBS 0.1% Tween20 (SigmaAldrich, USA)	4.75ml
Total	5ml

Protocol 4: Antibody conditions for immunofluorescence detection of RPE marker

Primary antibody					Secondary antibody			
Target	Supplier	Catalogue no.	Dilution	Antigen retrieval	Secondary antibody	Supplier	Catalogue no.	Dilution
RPE-65	Abcam, United Kingdom	ab231782	1:4000	pH 9	Anti-rabbit-Alexa488	ThermoFisher Scientific (USA)	A21206	1:200

4.6.4 Fam84b detection using tyramide amplification

Slides were prepared as previously described in section 4.6.1. The signal for Fam84b protein detection was amplified using Tyramide SuperBoost™ Kit with AlexaFluor™ Tyramides (Catalogue number B40923, ThermoFisher Scientific, USA), and the enclosed protocol was followed. Anti-Fam84b (NBP2-47459, Novus Biologicals, USA) was used at a dilution of 1:50. Slides were counterstained with DAPI (SigmaAldrich, USA), mounted with Dako Fluorescence Mounting Medium (HPST, Czech Republic) and viewed using a fluorescent microscope. Images were taken using ZEN Blue software (Zeiss, Germany).

4.7 *In situ* hybridisation

4.7.1 Riboprobe design and template amplification

Five PCR primer pairs were designed to amplify the region of cDNA to create the *in situ* anti-sense and sense probes. cDNA reverse transcribed from mouse eye, heart and uterus tissue was obtained from a colleague's cDNA library. The primer pairs were used to amplify the selected region of the FAM84B gene, and the PCR products (shown in Table 10) were assessed for specificity. PCR was carried out as a 50µl reaction via Protocol 5, using the melting temperatures described by the supplier of the primers (SigmaAldrich, USA). PCR products were run on a 1% agarose (SeaKem LE Agarose, East Port Life Science, Czech Republic) gel by gel electrophoresis. Gels were run at 60V until products were appropriately separated. Figure 12 shows the resulting gel. The PCR product amplified by primer pair 3 from mouse heart tissue was selected due to a strong band correlating to the expected product size (Figure 12, lane 12). This selected PCR product was purified using the QIAquick PCR purification kit (Qiagen, Germany), separated by gel electrophoresis, then the 1004bp band was excised from a gel using GeneJET gene extraction kit (ThermoFisher Scientific, USA). The PCR product was ligated into a pGEM-T easy vector (Promega, USA) and transformed into *E.coli* as per the enclosed protocol. X-gal plates were prepared by mixing 30µl IPTG and 30µl X-gal (20mg/ml) per plate. Transformed colonies were selected and grown overnight in LB broth with ampicillin (ThermoFisher Scientific, USA) at 37°C. Bacteria were either stored in LB Broth with 80% glycerol (P-LAB, Czech Republic) at -80°C, or the plasmids were isolated using QIAprep® Miniprep kit (Qiagen, Germany). Isolated plasmids were sent for sequencing to determine the insert orientation.

Table 10: Primer pairs used to amplify the selected genomic region of cDNA

Primer pair	Forward	Reverse	Product size
1	5'-ATCTACAATCCCGTCCCTCC-3'	5'-CATCGGTCAGGAAGCTGTTG-3'	951 bp
2	5'-GGATCTACAATCCCGTCCCTC-3'	5'-TAGGGCTCAGTGGCTTGTAG-3'	1197 bp
3	5'-AAGCTGGGTACAGGAAAGAC-3'	5'-AATGCACCACCTGGAAATTG-3'	1004 bp
4	5'-CTGGGTACAGGAAAGACCGGAG-3'	5'-CCGGACTGCACTTGTGAGC-3'	923 bp
5	5'-GGATCTACAATCCCGTCCCTC-3'	5'-CATCGGTCAGGAAGCTGTTG-3'	953 bp

Protocol 5: PCR protocol for amplification of prospective probe from cDNA

Step	Temperature (°C)	Time	
Denaturation	95	5min	
Denaturation	95	30sec	x42
Annealing	56	45sec	
Elongation	72	75sec	
Final Elongation	72	10min	
End	12	∞	

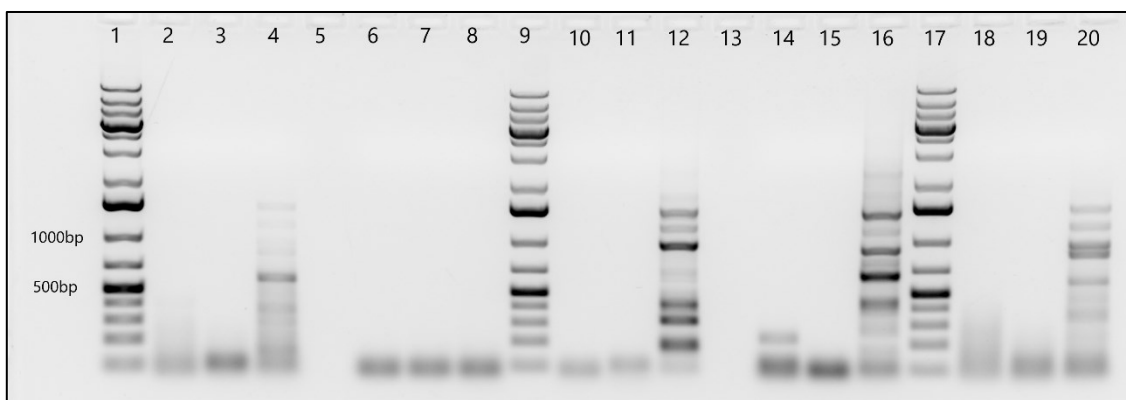


Figure 12: Amplified PCR products during preparation of *in situ* hybridisation probes. The primers described in Table 10 were used to amplify a region of the FAM84B gene in cDNA derived from mouse eye, heart and uterus tissue. PCR products were separated by gel electrophoresis. Lane 1, GeneRuler™ 1kb Plus(ThermoFisher, USA); 2, primer pair(PP) 1 with eye cDNA; 3, PP1 with uterus cDNA; 4, PP1 with heart cDNA; 5, empty; 6, PP2 with eye cDNA; 7, PP2 with uterus cDNA; 8, PP2 with heart cDNA; 9, GeneRuler™ 1kb Plus; 10, PP3 with eye cDNA; 11, PP3 with uterus cDNA; 12, PP3 with heart cDNA; 13, empty; 14, PP4 with eye cDNA; 15, PP4 with uterus cDNA; 16, PP4 with heart cDNA; 17, GeneRuler™ 1kb Plus; 18, PP5 with eye cDNA; 19, PP5 with uterus cDNA; 20, PP5 with heart cDNA. The PCR product amplified by PP3 with heart cDNA (lane 12) was used for further steps as an expected band of 1004bp was successfully detected.

4.7.2 Riboprobe synthesis

Transformed *E.coli* colonies were grown in LB broth with 50µg/ml ampicillin, overnight at 37°C with shaking. Plasmid DNA was then purified using HiSpeed Plasmid Midi Kit (Qiagen, Germany) and the enclosed protocol was followed. DNA concentration was measured using Nanodrop™ (ThermoFisher Scientific, USA) and stored at -20°C until use. Using sequencing results, samples from different colonies were selected to synthesise the anti-sense and sense RNA *in situ* hybridisation probes. Purified plasmids were linearised using reactions described in Table 11. The restriction reactions occurred at 37°C for 2hrs. Linearised DNA was processed using QIAquick PCR purification kit (Qiagen, Germany), the DNA concentration was determined and stored at -20°C.

Table 11: Restriction reactions for plasmid linearisation

	Reagent	Volume
For anti-sense probe:	DNA	4µl (11µg)
	SpeI (Life Technologies, USA)	5µl
	Tango buffer 10x (ThermoFisher Scientific, USA)	10µl
	dH ₂ O	81µl
	Total	100µl
For sense probe:	DNA	5µl (9.65µg)
	NcoI (Life Technologies, USA)	5µl
	Tango buffer 10x (ThermoFisher Scientific, USA)	10µl
	dH ₂ O	80µl
	Total	100µl

Linearised plasmids were transcribed to form the sense and anti-sense riboprobes from the DNA template, by setting up the reactions described in Table 12. The transcription reaction was allowed to

commence for 4hrs at 37°C. The Sp6 RNA polymerase (East Port Life Science, Czech Republic) was used to transcribe the sense riboprobe, and T7 RNA polymerase (East Port Life Science, Czech Republic) was used to transcribe the anti-sense riboprobe. This can be visualised via the pGEM-T easy vector (Promega, USA) map in Figure 13. Following transcription, riboprobes were purified using RNeasy Mini Kit (Qiagen, Germany), the RNA concentration determined using Nanodrop™ (ThermoFisher Scientific, USA), and then stored at -80°C.

Table 12: Reactions for transcription of sense and anti-sense riboprobes

	Reagent	Volume
For anti-sense probe:	5x Transcription buffer (Promega, USA)	4µl
	100mM DTT (Promega, USA)	2µl
	DIG RNA labelling mix (SigmaAldrich,USA)	2µl
	RNase OUT™ (Invitogen, USA)	1µl
	T7 RNA polymerase (EastPort Life Sciences, Czech Republic)	2µl
	DNA template	5µl (1µg)
	dH ₂ O	4µl
	Total	20µl
For sense probe:	5x Transcription buffer (Promega, USA)	4µl
	100mM DTT (Promega, USA)	2µl
	DIG RNA labelling mix (SigmaAldrich,USA)	2µl
	RNase OUT™ (Invitogen, USA)	1µl
	Sp6 RNA polymerase (EastPort Life Sciences, Czech Republic)	2µl
	DNA template	6µl (1µg)
	dH ₂ O	3µl
	Total	20µl

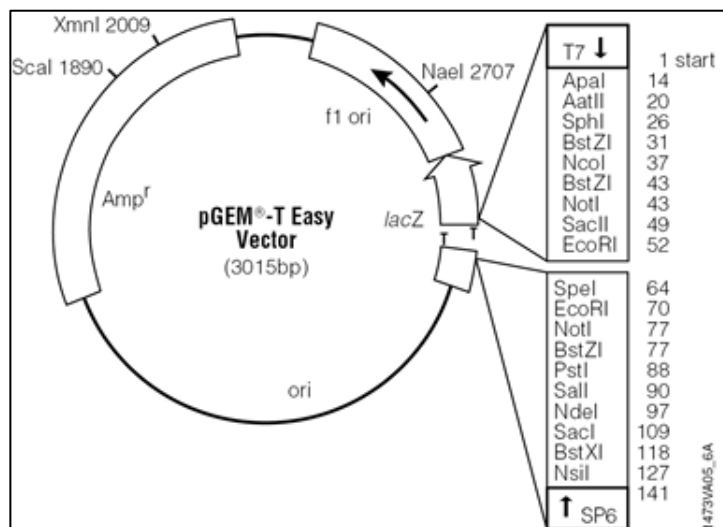


Figure 13: Vector used for amplification and transcription template of *Fam84b* riboprobes. pGEM®-T Easy Vector (Promega, USA) was used to create sense and anti-sense riboprobes. Plasmids were cleaved by *SpeI* and transcribed with T7 RNA polymerase to create anti-sense riboprobes. Plasmids were cleaved with *NcoI* and transcribed with Sp6 RNA polymerase to create sense riboprobes.

4.7.3 Fam84b mRNA localisation in eye tissue sections

Paraffin-embedded mouse eye tissue sections were processed as previously described in section 4.5.1, in an RNase free environment. Following tissue section rehydration, slides were processed using Protocol 6. Probes were diluted in hybridisation buffer (Table 15) in concentrations of 1, 2 and 3ng/ml (Protocol 6, Step 15). Sections were then viewed using a Brightfield microscope (Zeiss, Germany).

Protocol 6: RNA in situ hybridisation

Step	Task	Time
1	Immerse slides in Xylene (VWR, USA)	2x 10min
2	Immerse slides in 100% EtOH (VWR, USA)	2x 5min
3	Immerse slides in 70% EtOH (VWR, USA)	2x 5min
4	Wash slides in PBS	2x 10min
5	Immerse slides in 3% H ₂ O ₂ (Penta, Czech Republic)	10min
6	Wash slides in PBS	2x 5min
7	Expose slides to 10µg/ml Proteinase K (P-LAB, Czech Republic) diluted in PBS	10min
8	Expose slides to 2mg/ml Glycine (Biotech, Sweden) diluted in PBS	10min
9	Wash slides in PBS	5min
10	Fix tissue in 4% paraformaldehyde (preparation described in Table 13)	20min
11	Wash slides in PBS	3x 5min
12	Immerse slides in Acetylation solution (preparation described in Table 14)	10min
13	Wash slides in PBS	3x 5min
14	Immerse slides in hybridisation buffer (preparation described in Table 15)	1hr
15	Dilute probe to appropriate concentration in hybridisation buffer, and heat to 80°C.	3min
16	Cover slides with 100µl probe mix, apply HybriSlip™ (SigmaAldrich, USA), and place into humid slide box	
17	Incubate at 70°C	Overnight
18	Dip slides in 5xSSC (Life Technologies, USA) solution at 70°C to remove HybriSlip™	
19	Immerse slides in 5xSSC with 50% Formamide and 0.1% Tween-20 at 70°C	5x 30min
20	Cool slides to room temperature	~45min
21	Wash slides in TBS (preparation described in Table 16)	4x 5min
22	Dissolve Blocking Reagent (SigmaAldrich, USA) in TBS and apply blocking solution to slides	1hr
23	Apply 1µl/5ml anti-DIG antibody/ DIG nucleic acid detection kit (SigmaAldrich, USA) to slides	
24	Incubate at 4°C	Overnight
25	Wash slides in TBS at room temperature	4x 5min
26	Immerse slides in NTMT solution (preparation described in Table 17)	2x 5min
27	Prepare and apply BM-purple (SigmaAldrich, USA) solution to slides until optimal staining	-
28	Wash slides in PBS	2x 5min
29	Fix tissue in 4% paraformaldehyde	10min
30	Wash slides in PBS	2x 10min
31	Mount slides with Aquatex® (Merck Millipore, USA) and place cover slips (VWR, USA).	

Table 13: Preparation of 4% paraformaldehyde solution

Reagent	Volume
Paraformaldehyde (P-LAB, Czech Republic)	40g
10x PBS	100ml
4M NaOH (P-LAB, Czech Republic)	0.5ml
dH ₂ O	895.5ml
Total	1000ml
Heat to 60°C and mix until dissolved. Filter into storage tubes.	

Table 14: Preparation of Acetylation Solution for in situ hybridisation

Reagent	Volume
Triethanolamine (P-LAB, Czech Republic)	795µl
Acetic Anhydride (SigmaAldrich, USA)	150µl
dH ₂ O	59.95ml
Total	60ml

Table 15: Preparation of Hybridisation buffer for in situ hybridisation

Reagent	Volume
50% formamide (SigmaAldrich, USA)	50ml
5xSSC pH7 (Life Technologies, USA)	25ml
100x Denhardt's solution (ThermoFisher Scientific, USA)	1ml
10% Tween-20 (SigmaAldrich, USA)	1ml
tRNA 50µg/ml (SigmaAldrich, USA)	500µl
Heparin 50µg/ml (SigmaAldrich, USA)	100µl
UltraPure™ Salmon Sperm DNA Solution (Life Technologies, USA) 10mg/ml	500µl
dH ₂ O	21.9ml
Total	100ml

Table 16: Preparation of Tris-buffered saline (TBS)

Reagent	Volume
1M Tris-HCl pH7.6 (SigmaAldrich, USA)	50ml
5M NaCl (SigmaAldrich, USA)	15ml
dH ₂ O	435ml
Total	500ml

Table 17: Preparation of NTMT solution for in situ hybridisation

Reagent	Volume
1M Tris-HCl (SigmaAldrich, USA) pH9.6	20ml
5M NaCl (SigmaAldrich, USA)	4ml
1M MgCl ₂ (SigmaAldrich, USA)	1ml
Tween-20 (SigmaAldrich, USA)	200µl
dH ₂ O	175ml
Total	200ml

4.8 Preparation of cell lysates

4.8.1 hTERT-RPE1 lysate

hTERT RPE-1 (ATCC® CRL-4000™) cells were used for subsequent western blot and co-immunoprecipitation experiments. Cell cultures were maintained in media and sub-cultured as recommended in the supplier's guidelines. Cells were allowed to grow to confluency in a 10cm² dish and then scraped and lysed in RIPA buffer (Table 18). hTERT-RPE1 cell lysates were prepared as described in Table 19, then sonicated using SonoPlus (Bandelin, Germany) and stored at -20°C until use.

Table 18: RIPA buffer recipe for cell lysates

Reagent	Volume
150mM NaCl (SigmaAldrich, USA)	0.87g
Nonidet™ P-40 Substitute (SigmaAldrich, USA)	1ml
Sodium deoxycholate (SigmaAldrich, USA)	0.5g
SDS pellets 99% (P-LAB, Czech Republic)	1g
1mM EDTA (P-LAB, Czech Republic)	5ml
50mM Tris-HCl pH8 (Biotech, Sweden)	5ml
dH ₂ O	89ml
Total	100ml

Table 19: hTERT-RPE1 cell lysate preparation

Reagent	Volume
Confluent hTERT-RPE1 cells	10cm ² dish
RIPA buffer (see Table 18)	120μl
cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail (SigmaAldrich, USA). 1 tablet dissolved in 1ml dH ₂ O	15μl
PhosSTOP™ (SigmaAldrich, USA). 1 tablet dissolved in 1ml dH ₂ O	15μl
Total	150μl

4.8.2 Mouse retinal cell lysates

Mice were euthanised by cervical dislocation. Retinal lysates were prepared by using an adapted version of a protocol published by Skeie, Tsang and Mahajan, (2011). Mouse eyes were stabilised by gently holding the optic nerve with forceps, a microsurgical blade was used to make an incision in the cornea from limbus to limbus. Leaking anterior chamber fluid was absorbed using a cotton pad. Another set of forceps was used to pinch the eye just behind the lens, partially closed and then pulled away from the optic nerve to eviscerate the lens. The retinal tissue was eviscerated by carefully pulling the forceps that were stabilising the eye forward from the optic nerve towards the cut in the cornea. The yellow-orange vascularised tissue of the retina and the lens were then placed in microfuge tubes to create the lysates described in Table 20. Retinal and lens tissue was lysed in RIPA buffer (Table 18). 8 retinas (4 animals, 2 ♂ and 2 ♀) were pooled together for the wild type and FAM84B KO retinal lysates. 8 lenses (4 animals, 2 ♂ and 2 ♀) were pooled together for the wild type and FAM84B KO lens lysates. Tissue lysates were ruptured in Tissue Lyser II (Qiagen, Germany) for 3mins, then inverted for 30min at 4°C. Finally, tissue lysates were centrifuged at 12,000rpm at 4°C for 20min and sonicated using SonoPlus (Bandelin, Germany) then stored at -20°C until use.

Table 20: Preparation of mouse retinal/ lens cell lysate

Reagent	Volume
Mouse retinal/ lens tissue	from 8 eyes
RIPA buffer (see Table 18)	320µl
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (SigmaAldrich, USA). 1 tablet dissolved in 1ml dH ₂ O	40µl
PhosSTOP™ (SigmaAldrich, USA). 1 tablet dissolved in 1ml dH ₂ O	40µl
Total	400µl

4.9 SDS-PAGE and Western blot

4.9.1 Experimental procedure

hTERT-RPE1 and mouse retinal cell lysates were prepared as described in sections 4.8.1 and 4.8.2 respectively, and underwent SDS-PAGE and western blot analysis of their protein content. Lysate total protein concentration was measured using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) and its enclosed protocol, and then determined by Epoch™ microplate spectrophotometer (BioTek, USA). Proteins were separated using a 1.5mm 10% polyacrylamide gel prepared as described in Table 21. Lysates were prepared so that 40ug of protein was loaded into the wells of the gel, with appropriate amounts of Laemmli buffer (SigmaAldrich, USA) and lysis buffer. Samples were denatured at 95°C for 5mins prior to SDS-PAGE. PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific, USA) was used as a reference for protein molecular weight. Proteins were then transferred onto Nitrocellulose Blotting Membrane (GE Healthcare Life Sciences, USA). Successful transfer of protein was confirmed with brief Ponceau S (SigmaAldrich, USA) staining. Running and transfer buffers were prepared as per the laboratory standards.

Table 21: Preparation of polyacrylamide gel for SDS-PAGE

Reagent	Running gel	Stacking gel
dH ₂ O	5160µl	2580µl
1.5M Tris HCl pH8.8	3400µl	-
0.5M Tris HCl pH6.8	-	1700µl
10% SDS (P-LAB, Czech Republic)	133µl	67µl
Polyacrylamide (P-LAB, Czech Republic)	4440µl	2200µl
10% APS (SigmaAldrich, USA)	133µl	67µl
TEMED (SigmaAldrich, USA)	13µl	7µl
Total	13279µl	6540µl

4.9.2 Detection of Fam84b in a human RPE cell line and mouse retina lysates

Membranes for Fam84b detection were blocked in the solution described in Table 22 for 30min at room temperature. Membranes were exposed to anti-Fam84b (NBP2-47459, Novus Biologicals, USA) diluted 1:1000 in PBS +0.1%Tween-20 +1%Milk, overnight at 4°C. Membranes were then exposed to Goat anti-rabbit-HRP secondary antibody (Abcam, United Kingdom) diluted 1:10,000 in PBS +0.1%Tween-20 +1%Milk, for 1hr at room temperature. All washing of membranes occurred in PBS+0.1%Tween-20. Peroxidase activity and visualisation of stained proteins were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, USA) and Detection – ChemiDoc MP Imaging system (BioRad, USA). A number of exposure images were obtained for optimal viewing. Membranes were stripped using Restore™ Stripper Buffer (ThermoFisher Scientific, USA) for 15min, and exposed to anti-β-tubulin (Abcam, United Kingdom) diluted 1:1000 in PBS +0.1%Tween-20 +1%Milk, overnight at 4°C. Finally, they were then re-exposed to the secondary antibody and detection was performed as described above.

Table 22: Blocking solution for Fam84b western blot detection

Reagent	Volume
PBS	49.95ml
Milk powder (P-Lab, Czech Republic)	5g
Tween-20 (SigmaAldrich, USA)	0.05ml
Total	50ml

4.9.3 Detection of activated Ras downstream effectors in mouse retina lysates

Membranes for phosphorylated Raf-1, MEK1 and ERK1 detection were blocked in the solution described in Table 23 for 30min at room temperature. Membranes were exposed to anti-Raf1 phosphoS338 antibody (ab51042, Abcam, United Kingdom), anti-MEK1 phosphoS298 antibody (ab96379, Abcam, United Kingdom), and anti-ERK1 phosphoY204 antibody (ab131438, Abcam, United Kingdom), diluted 1:1000 in PBS +0.1%Tween-20 +1%Milk, overnight at 4°C. Membranes were then exposed to Goat anti-rabbit-HRP secondary antibody (Abcam, United Kingdom) diluted 1:10,000 in TBS +0.1%Tween-20 +1%Albumin, for 1hr at room temperature. All washing of membranes occurred in TBS+0.1%Tween-20. Peroxidase activity and visualisation of stained proteins were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, USA) and Detection – ChemiDoc MP Imaging system (BioRad, USA). A number of exposure images were obtained for optimal viewing. Membranes were stripped using Restore™ Stripper Buffer (ThermoFisher Scientific, USA) for 15min, and exposed to anti-β-tubulin (Abcam, United Kingdom) diluted 1:1000 in PBS +0.1%Tween-20 +1%Milk, overnight at 4°C. Finally, they were re-exposed to the secondary antibody and detection was performed as described above.

Table 23: Blocking solution for phosphorylated Ras effector western blot detection

Reagent	Volume
TBS (as described in Table 16)	99.9ml
Tween-20 (SigmaAldrich, USA)	0.1ml
Albumin (P-LAB, Czech Republic)	5g
Total	100ml

4.9.4 Quantification of Ras effector phosphorylation in mouse retina lysates

Western blots described in section 4.9.3 were completed in triplicate. The intensities of bands corresponding to phosphorylated Raf-1, MEK1 and ERK1 were compared between the retinal lysates of

wild type and FAM84B KO mice. This was achieved by the use of ImageJ (National Institute of Health, USA). Band intensity was quantified, then normalised to the wild type band intensity for each antibody. These values were then normalised to the intensity of the β -tubulin loading control bands of the corresponding blot lane. This gave rise to an intensity value which represented the fold difference in phosphorylated Raf-1, MEK1 and ERK1 between wild type and FAM84B KO retinal lysates. Calculations were completed in Microsoft Excel.

4.10 Co-immunoprecipitation

4.10.1 Immunoprecipitation of Fam84b binding partners

The hTERT-RPE1 cell line (ATCC® CRL-4000™) was used for Fam84b co-immunoprecipitation experiments. Cells were prepared as previously described in section 4.8.1, but lysed in 200 μ l 1% Nonidet™ P40 Substitute (ThermoFisher Scientific, USA) per 10cm² dish instead. During the time of writing this thesis work, this protocol was still undergoing optimisation, but shows the how results included in this work was performed. 50 μ l of Dynabeads™ Protein G for Immunoprecipitation (ThermoFisher Scientific, USA) were used per sample, and washed twice in PBS+ 0.1%Tween-20. anti-Fam84b (NBP2-47459, Novus Biologicals, USA) antibody diluted 1:100 in PBS +0.1%Tween-20 was incubated with the Dynabeads™ for 30min at 4°C with inversion. Negative control samples without anti-Fam84b, and anti-GFP (SigmaAldrich, USA) instead were also set up. The supernatant was removed using a magnet, and the bead-antibody complex washed twice with PBS +0.1%Tween-20. The bead-antibody complex was then washed with 1% NP-40 before the addition of 200 μ l hTERT-RPE1 cell lysate per sample, and incubation at 4°C with inversion for 1hr. The supernatants of each sample (flow-through) was transferred to a new tube via a magnet, and the Dynabeads™ were washed in triplicate with 1% NP-40. Protein bound to the Dynabeads™ was eluted in triplicate by adding 20 μ l Elution buffer (preparation described in Table 24), heating to 95°C for 5min, and removing the supernatant (elution).

Table 24: Elution buffer for co-immunoprecipitation

Reagent	Volume
dH ₂ O	10 μ l
Nonidet™ P40 Substitute (ThermoFisher Scientific, USA)	0.1 μ l
Laemmli buffer (SigmaAldrich, USA)	8 μ l
20x DTT (Promega, USA)	2 μ l

4.10.2 Western blot analysis of Fam84b co-immunoprecipitation

Elutions and flow-through samples obtained were separated by SDS-PAGE and western blot was performed as described in sections 4.9.1 and 4.9.2, for detection of the presence of Fam84b in these samples.

4.10.3 Mass spectrometry

5 μ l of Dynabeads™ from each sample were sent to an associate lab in BIOCEV (Vestec, Czech Republic) to perform mass spectrometry directed identification of the proteins detected in the samples. Figure 34 was produced by removing the proteins that had a higher intensity in the beads only sample than anti-Fam84b and anti-GFP samples, and then comparing the differences in intensity between these samples.

5 RESULTS

5.1 FAM84B KO mice were evaluated via the IMPC pipeline

FAM84B KO mice were evaluated using the standardised screens of the IMPC pipeline at CCP. Significant differences were reported in the retinal structure of FAM84B KO mice when compared to non-mutated C57Bl/6NCrl animals ($p < 0.00001$). Some minor corneal and lens opacities were also reported, however opacities in the structures of the anterior segment can often be detected in anaesthetised mice without any related phenotype. Other ocular parameters assessed; corneal morphology, corneal opacity, lens morphology, lens opacity, optic disc morphology, ocular blood vessel structure, ocular blood vessel patterning, were all reported as “normal” or not significantly different when compared to non-mutated control animals. Figure 14 provides a summary of the statistical output of all the IMPC screens performed on this cohort of animals. Statistically significant differences between wild type and FAM84B KO animals were also observed in Immunology screens, where whole blood samples show altered percentages of B1B cells ($p < 0.00001$), B2B cells ($p < 0.00001$), NK cell subsets ($p < 0.0005$) and T cell subsets ($p < 0.0005$). In addition, the Gross Pathology screen unveiled differences in FAM84B KO mouse heart pathology ($p < 0.0005$). Nevertheless, the potential phenotype described in the posterior segment of FAM84B KO mouse eyes sparked interest for this thesis work, and will be the focus. The findings in the Immunology and Gross Pathology screens will be available to trigger the expansion of a wider FAM84B KO mouse project in the future.

Age (weeks)	4-16	9			10	11	12	13	14			15	16							
Procedure	Body Weight	Open Field	Grip Strength	SHIRPA and dysmorphology	Acoustic Startle/ PPI	Light-Dark Box	Calorimetry	Electrocardiogram	Echocardiogram	IPGTT	ABR	X-ray	Body Composition	Eye Morphology	Biochemistry	Haematology	Immunology	Heart Weight	Organ Weight	Gross Pathology
Fam84b																				

Figure 14: Summary of significant differences observed in performed IMPC standardised tests. FAM84B KO animals were evaluated using the standard IMPC phenotyping tests at the Czech Centre for Phenogenomics, Vestec, Czech Republic. Each screen consists of many parameters. Significant differences were unveiled between Fam84b KO and non-mutated control animals whilst assessing eye morphology, percentage of immune cells in blood, and gross pathology. Grey, no parameters were significantly different between wild type and Fam84b KO animals. The colour scheme represents the significance level of all the parameters combined within that screen. Red corresponds to more, and yellow less significant. See main text for p-value alphas. PPI, Pre-pulse Inhibition; IPGTT, Intraperitoneal Glucose Tolerance Test; ABR, Auditory Brainstem Response.

5.2 Visualisation of FAM84B KO mouse retinas

FAM84B KO mice and non-mutated controls were examined via SD-OCT, initially at the IMPC-directed 16 weeks of age. At first glance, KO animals displayed patches of disorganisation of retinal cells and deformation of retinal layers. The deformation of the retina was concentrated around the photoreceptor and RPE layers, which formed bow-shaped structures facing towards, and invading, the inner layers of the retina (Figure 15B, 15C). These structures bore similarities to drusen, which are observed in AMD pathology. The “patches” of retinal disorganisation were always specifically localised to the superior retina when cross-referenced with the corresponding fundus images. Signs of retinal detachment from Bruch’s membrane was also apparent. Deformation of the retinal nerve fibre layer (RNFL) and ganglion cell layer (GCL) was also prominent in some animals (Figure 15C). The retina of one animal also displayed a break in the photoreceptor layer (Figure 15D). These observations of the initial SD-OCT examinations pointed towards the characteristics of a retinal disease, featuring aspects of the early signs of potential photoreceptor and RPE layer degeneration. Wild-type retinas on the other hand displayed well-defined layering, and no sign of pathological damage or malformation (Figure 15A).

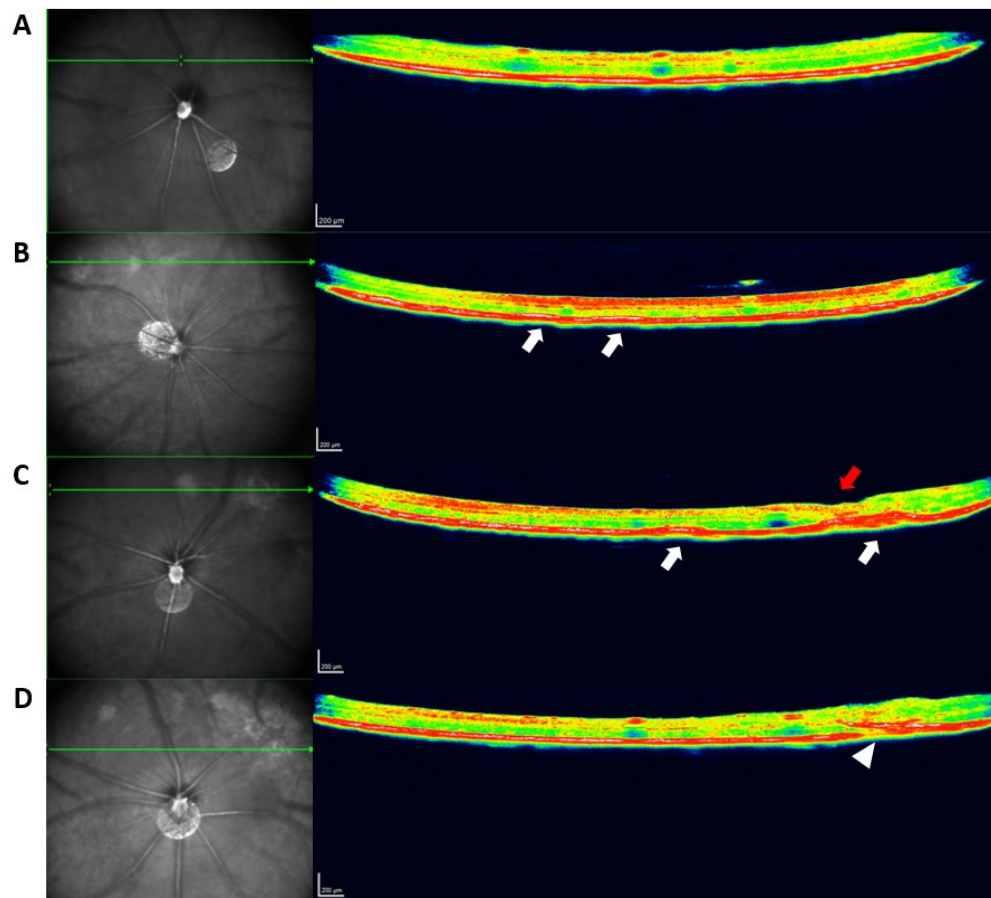


Figure 15: Visualising the retinal phenotype in FAM84B KO mice by SD-OCT. (A) Fundus image (left) and its corresponding b-scan cross section (right) of a wild type mouse at 16 weeks of age. No sign of retinal pathology was observed in wild type animals. (B, C, D) Example fundus images and corresponding b-scan cross section of FAM84B KO mouse retinas. (B) Photoreceptor and RPE layers displaying mild “bowing” (white arrows). (C) More severe bow-shaped morphology of the photoreceptor and RPE layers, with deformation of the RNFL and GCL layers (red arrow). (D) Area of apparent disruption of the photoreceptor layer (white arrowhead).

Next, we asked whether FAM84B KO animals displayed this obscure retinal morphology in a stable manner, or if it was indeed a genetically induced retinopathy, that would become more severe over time. FAM84B KO animals and non-mutated controls were examined every 6 weeks from 6- to 60-weeks of age using SD-OCT to investigate this, and all affected areas of each KO eye were quantified using the HRA+OCT Spectralis® PLUS (Heidelberg Engineering, Germany) software. All KO animals in the youngest age group displayed these areas of bow-shaped retinal layer dysmorphology, but covered a smaller area ($0.46 \pm 0.06 \text{ mm}^2$) than that of animals in higher age groups, such as the 60-week age group where KO eyes showed an average area of damage spanning $1.81 \pm 0.51 \text{ mm}^2$ of the retinal surface. This may mean that the deformation of Fam84b-deprived retinas could begin during embryonic development, or from birth. Table 25 shows the average area that the retinal damage covers in the eyes of each age group. Overall, the data shows that as KO animals age, this obscure outer retinal morphology spreads outwards and invades adjacent tissue, leading to an increase of damage across the retinal surface area. In the 30-week age group, a peak is seen due to two animals presenting eyes with a particularly severe and widespread phenotype, with damage covering 3.77 mm^2 and 5.64 mm^2 of their total retinal surface area. These animals did not appear in the higher age groups. However, the overall pattern observed was an increase in surface area of retinal damage with age. Here, 68.7% of FAM84B KO eyes measured displayed at least a 10% increase in retinal damage surface area when compared to their previous scan 6 weeks before, and 31.3% of eyes presented at least a 30% increase in measured damage area over a 6-week period. Supplementary Table 1 contains the surface area of retinal damage in all measured FAM84B KO retinas.

Age (weeks)	Mean Retinal Damage $\pm$ Standard Error (mm^2)	n
6	0.46 ± 0.06	8
12	0.68 ± 0.09	6
18	1.06 ± 0.17	14
24	1.25 ± 0.34	7
30	1.66 ± 0.38	15
36	1.45 ± 0.53	17
42	1.37 ± 0.33	17
48	1.30 ± 0.32	18
54	1.42 ± 0.42	12
60	1.81 ± 0.51	12

Table 25: Mean retinal damage of FAM84B KO animals. Table showing the mean retinal damage of animals lacking the Fam84b protein transcript. As animals age, the surface area of retina that is covered by abnormal retinal morphology increases. Quantification was achieved by SD-OCT (Spectralis®, Heidelberg Engineering) and marking the affected areas using b-scans and the corresponding fundus image for each eye. n, number of eyes measured.

Figures 16A and 16B show examples of fundus images that display the same animals tracked over a period of 24 weeks, where affected areas expand outwards towards adjacent tissue over time. Figure 16A shows the left eye of a KO animal, which possessed abnormal retinal morphology that covered an area of 0.44mm^2 at 6 weeks of age, rising up to 2.67mm^2 coverage at 30 weeks of age. Figure 16B shows the right eye of a KO animal tracked at older ages, displaying abnormal retinal morphology covering 3.04mm^2 of the retina at 36 weeks of age, increasing to 6.42mm^2 at 60 weeks of age. On closer observation, it was clear that this abnormal morphology specifically started and localised to the superior-temporal area of the retinal surface. In addition, the damage observed in KO retinas was not always symmetrical between the left and right eyes of a given animal. It was unpredictable whether an animal presented a more severely affected left or right eye. This can be visualised when comparing Figures 16B and 16C, which presents both eyes of the same animal, where the right eye displayed an increasingly severe phenotype over time, whereas the left eye still presents observable damage to the retina, but remained relatively mild and did not significantly worsen over time. It was also possible for an animal to demonstrate similarly affected symmetry between eyes. In comparison, non-mutated control animals consistently showed no sign of this pathology across all age groups measured, with well-defined retinal layer boundaries and a lack of any malformation of the inner or outer retina.

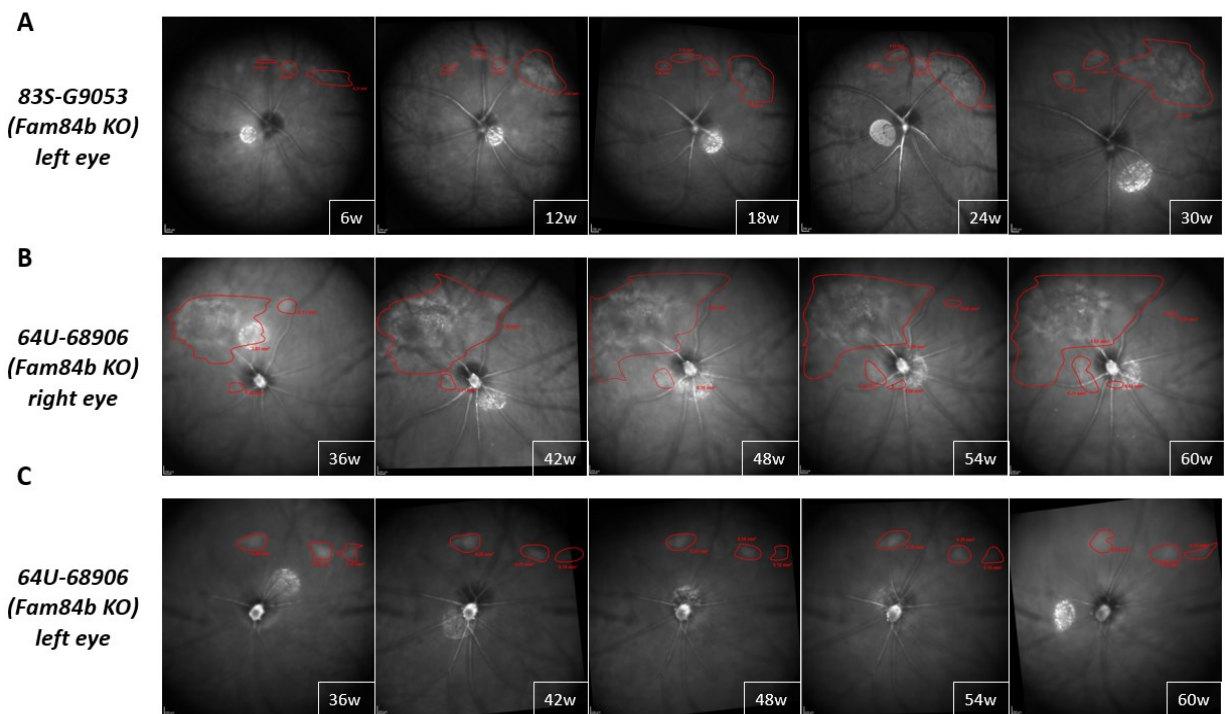


Figure 16: Fundus images tracking FAM84B KO animals retinal damage over time. (A) Example of retinal damage quantification of the left eye of a FAM84B KO animal from 6 to 30 weeks of age. During this time, the abnormal retinal morphology increased in surface area from 0.44mm^2 at 6 weeks of age, to 2.67mm^2 at 30 weeks of age. (B) Quantification of the right eye of another KO animal tracked from 36 to 60 weeks of age. Measurements revealed that this eye's surface area of damage increased from 3.04mm^2 to 6.42mm^2 over a period of 24 weeks. (C) Fundus images of the left eye of the same KO animal in (B). Comparing the two eyes, the damage to the left eye is present but remains relatively stable over the period of 24 weeks, whereas the right eye experiences increased damage over time. The left eye presented 0.56mm^2 surface area of damage at 36 weeks, and 0.63mm^2 at 60 weeks of age. Images were obtained using Spectralis® PLUS (Heidelberg Engineering, Germany), and borders of degenerated areas were marked on fundus images (in red) from their corresponding SD-OCT b-scans. Refer to methods section 4.3.3 for more information.

On observation of the OCT b-scans from each age group, it was also clear that with increased age, the disorganisation of the retinal layers not only spreads outwards, but also inwards. The disruption of cells in the outer retina layers was mild in animals of the younger age groups, but with increasing age, the patches of bow-shaped morphology of the outer retina started to occupy areas designated to the inner retinal neurones. This sparks the beginning of the deformation of the RNFL and GCL, where pressure from the outer layer primes the complete buckling of the retinal tissue. Figure 17 visualises this, which compares consecutive b-scans of a FAM84B KO and a non-mutated wild type retina. In this particular example, the structural changes in the retinal layers can be observed, as this dynamic phenotype develops from an area that displays only a bow-shaped outer retina at 36 weeks of age, to an area with increasingly disordered inner and outer retinal layering at 60 weeks of age. From 54 weeks of age, the buckling and deformation of the RNFL and GCL becomes apparent. Aside from these abnormal areas, the rest of the retinal tissue displays neat and well-defined layer morphology comparable to that of wild-type retinas. It is important to state that not every KO animal adheres to the exact same time frame of degeneration as the example in Figure 17. Some animals measured displayed a severely damaged retina either earlier or later in life. All KO animals displayed retinal damage, but the number and severity of patches with retinal damage, and their potency to invade the surrounding tissue varied with each KO eye. Unravelling the role

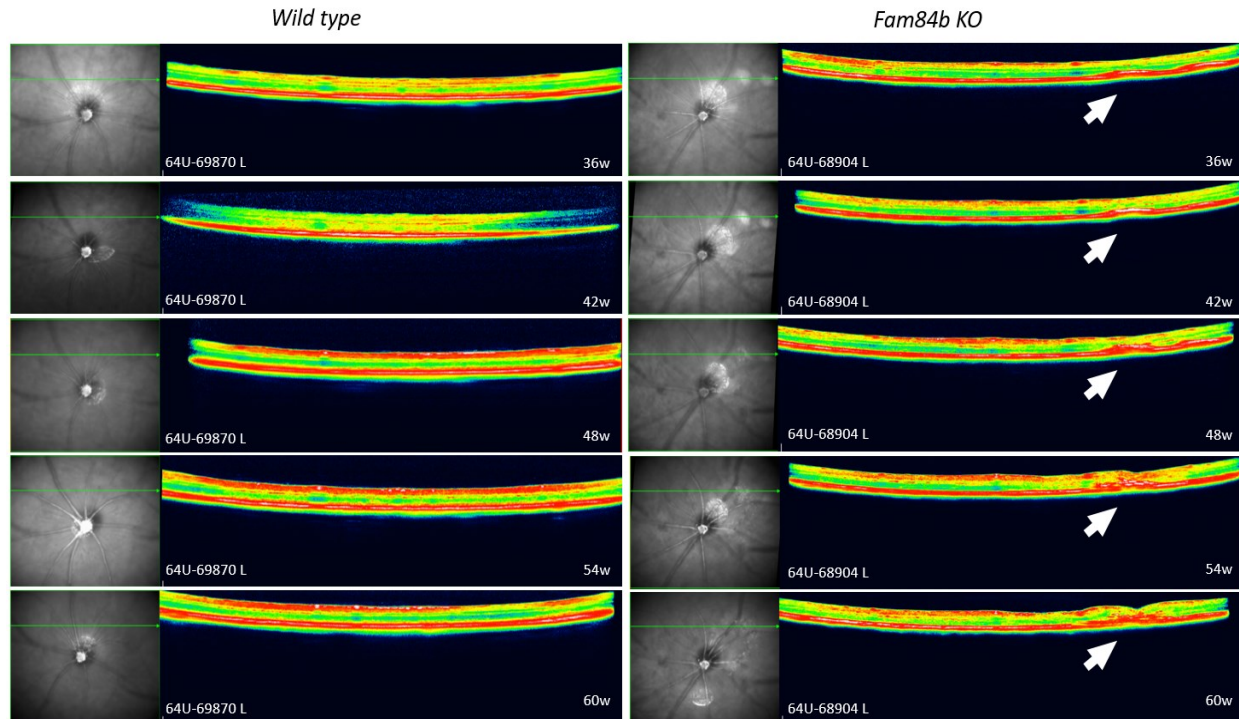


Figure 17: Comparison of consecutive OCT b-scans between wild type and FAM84B KO retinas. OCT scanning was carried out for animals 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 weeks of age. Examples in this figure show images of retinas at 36-60 weeks of age. The corresponding fundus image is shown on the left of the b-scan. The green line on the fundus image designates the position on the retina that the b-scan is displaying. Left; OCT b-scan of the left eye of a non-mutated control animal. Wild type retinas show well-defined retinal layers and no sign of deformation of retinal cells across all age groups. Right; OCT b-scan of the left eye of a FAM84B KO animal, aged 36-60 weeks of age. The bow-shaped morphology (shown by the white arrows) of the outer retinal layers can be observed in the 36 week old retina. As the animal ages, deformation of the layers begins to invade the space designated for the inner retina around 48 weeks of age. At 54 weeks of age, this animal's retinal nerve fibre layer (RNFL) and ganglion cell layer (GCL) starts to become buckled and deformed. This is just one example of the phenotype that is present in FAM84B KO retinas. Animals can present this phenotype in a range of severities and time-scales. Retinal damage starts at the outer retina, before spreading to the inner retinas and adjacent retinal tissue.

Fam84b plays in homeostasis of the retina will surely shed light on the varying potency of the phenotype observed in FAM84B KO mice.

5.3 FAM84B KO mice display reduced retinal thickness

A key characteristic of retinal pathologies is the occurrence of changes in retinal thickness. This can be an important factor in determining whether and observed phenotype is degenerative, which could stem from the deterioration of specific cell-types or layers of the retina. The next essential question was whether FAM84B KO retinas not only showed retinal disorganisation, but degeneration too. The total thickness, inner retina thickness, and outer retina thickness of KO and non-mutated retinas was quantified in mice aged 12, 24, 36, 48 and 60 weeks of age using The Iowa Reference Algorithms (Retinal Image Analysis Lab, Iowa Institute for Biomedical Imaging, Iowa City, IA, USA) (Abramoff, Garvin and Sonka, 2010; Kang et al., 2006; Garvin et al., 2009). Figure 18A shows the total, inner and outer retina boundaries and the quantified retinal regions measurements were taken from. Figure 18B shows a comparison of the total retinal thickness between KO and non-mutated retinas, where FAM84B KO retinas were significantly thinner at each age group (Bonferroni adjusted $p < 0.0001$) compared to wild types. This implies that FAM84B KO retinas do not only possess areas of deformation as seen from OCT b-scan analyses, but the whole retinal tissue is either not assembled correctly during development, or undergoing early-onset degeneration. No significant differences were found between the thicknesses of wild type or KO retinas of different age groups. This may suggest that overall thinning of the whole retina does not continue as KO animals age, at least up until the age of 60 weeks.

Next, to identify which retinal layers were potentially affected, the thickness of the inner and outer retinal layers was quantified, to determine whether thinning has occurred in the photosensitive outer retina or the signal processing neurones of the inner retina. Figure 19A shows the comparison of the inner retina thickness between KO and non-mutated controls. KO retinas displayed significantly thinner inner retinas in all age groups (Bonferroni adjusted, $p < 0.0001$), and no significant differences were found between age groups. Figure 19B shows the outer retina thickness comparison between FAM84B KO and non-mutated controls. Here, the outer retinas of KO animals were all significantly thinner than that of wild types, at 12 weeks of age (Bonferroni adjusted, $p < 0.001$) and at 24, 36, 48 and 60 weeks of age (Bonferroni adjusted, $p < 0.0001$). These data show that absence of the Fam84b protein contributes to the thinning of both the inner and outer retinal layers. Interestingly, the measurements of KO animals in the 60 weeks of age group revealed significantly thicker outer retinas compared to; 24 week old KO animals (Bonferroni adjusted, $p < 0.001$); 36 week old KO animals (Bonferroni adjusted, $p < 0.05$); and 48 week old KO animals (Bonferroni adjusted, $p < 0.01$). However, no significant differences in outer retina thickness were found between KO animals of 12 and 60 weeks of age. This could be explained by a gradual thinning of the outer retina during the first months of life, but as the areas of retinal dysmorphology start to spread to surrounding retinal tissue and invade the inner retina, the outer retina thickness significantly increases once more due to degenerative scarring.

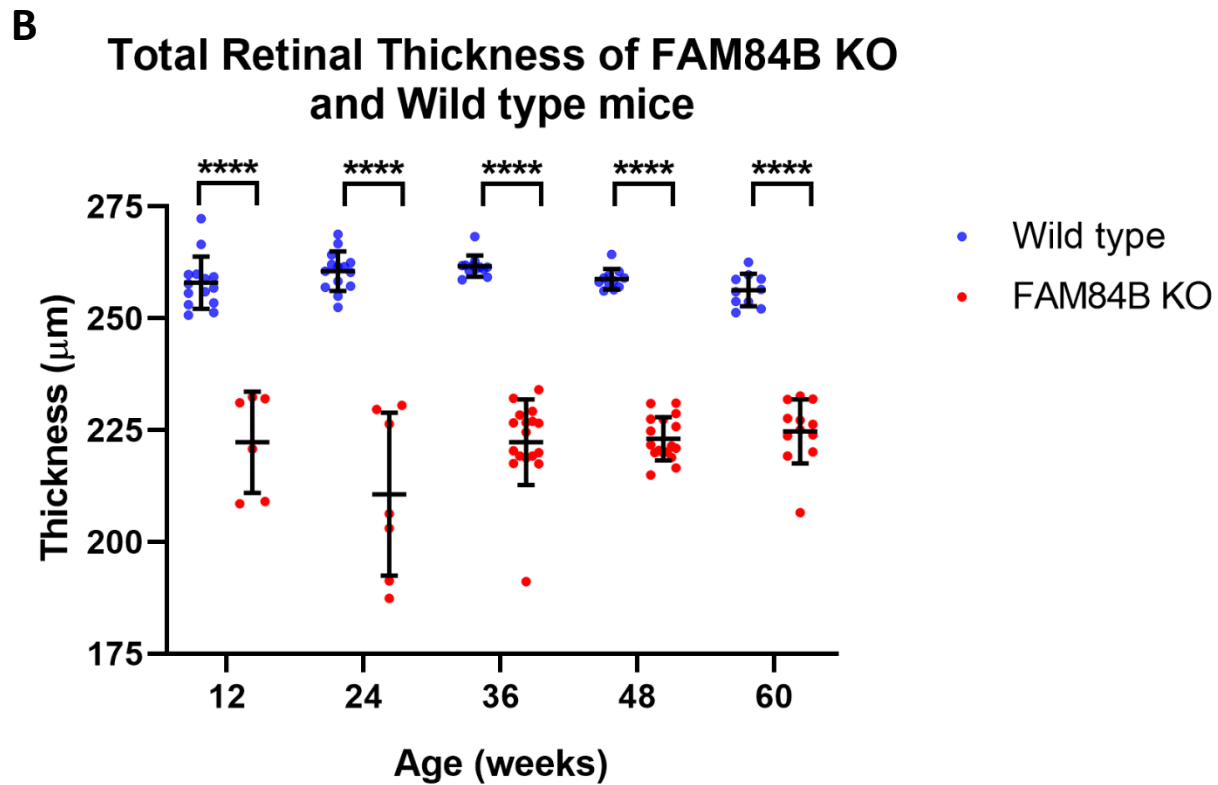
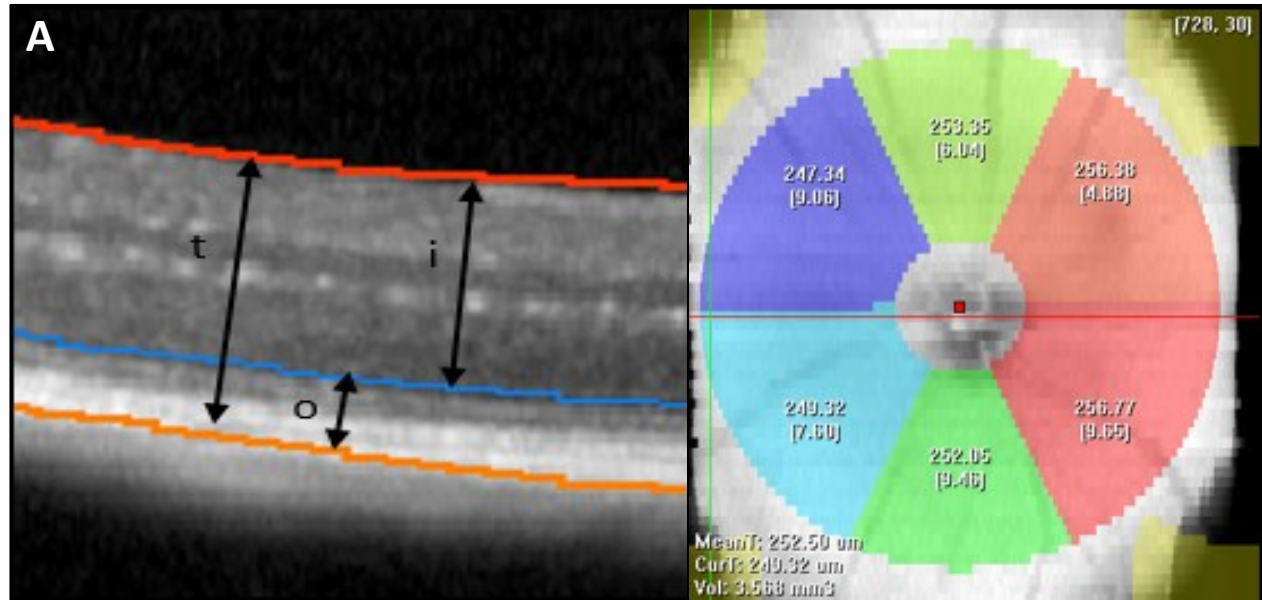


Figure 18: Quantification of retinal thickness in FAM84B KO and wild type mice. (A) OCT b-scans of FAM84B KO and wild type mice were analysed using the OCTExplorer software and The Iowa Reference Algorithms (Retinal Image Analysis Lab, Iowa Institute for Biomedical Imaging, Iowa City, IA, USA), and the thickness of total, inner and outer retinal layers were measured (left). The regions of the retinas included in the analysis are shown by the coloured segmented circle on the right. The region surrounding the optic disc and the peripheral retina were not included in the thickness analysis. t, total retina; i, inner retina; o, outer retina. The red square at the centre of the fundus image corresponds to the centre of the optic nerve head. (B) Total retinal thickness comparison between FAM84B KO and wild type retinas. KO animals displayed significantly thinner retinas at 12, 24, 36, 48 and 60 weeks of age (Bonferroni adjusted, $p < 0.0001$). The total retinal thickness was defined as the distance from the inner limiting membrane (ILM) to the outer boundary of the retinal pigmented epithelium (ORPE). “****”, $p < 0.0001$. Error bars show standard deviation.

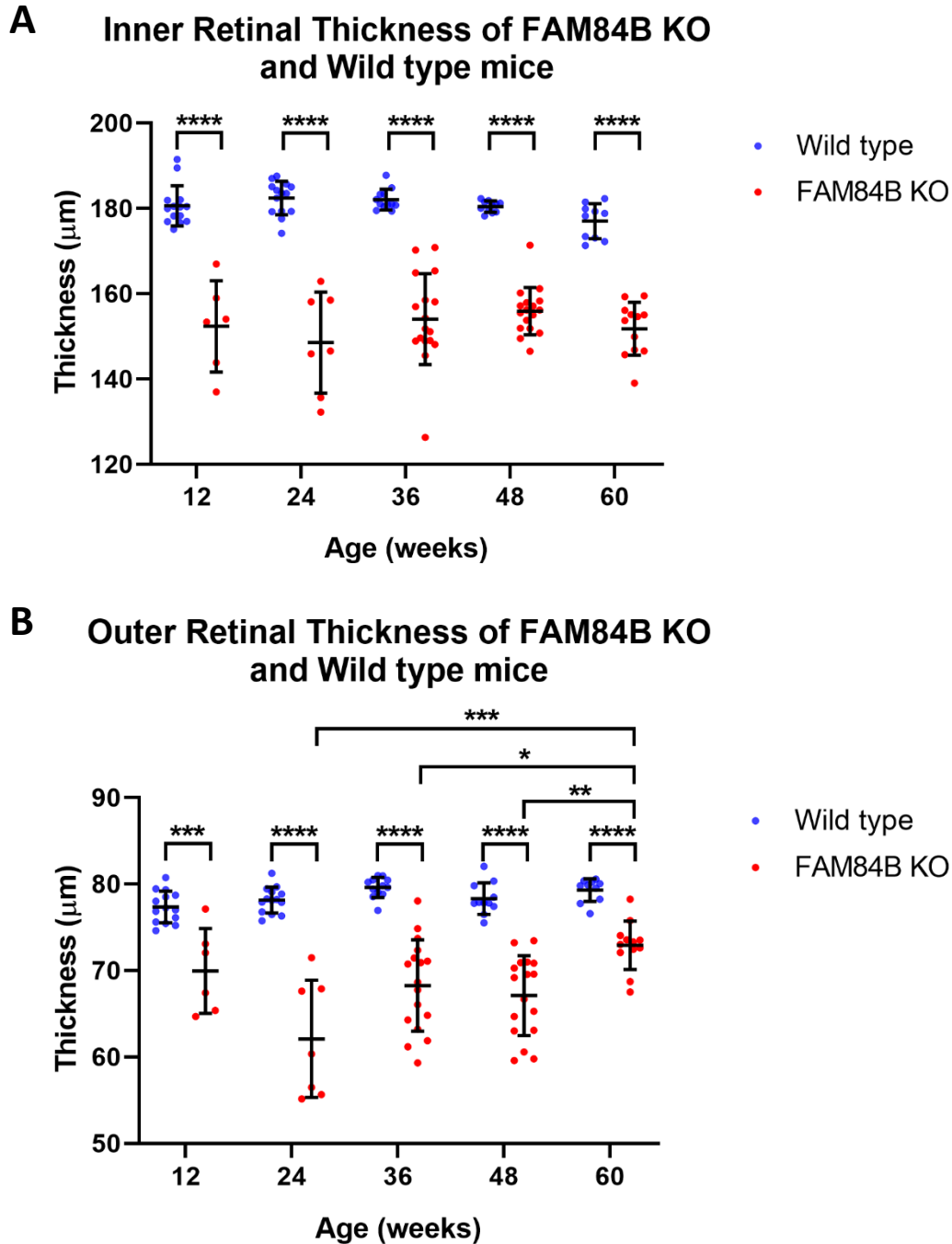


Figure 19: Quantification of inner and outer retinal thickness in FAM84B KO and wild type mice. (A) Inner retinal thickness comparison between FAM84B KO and wild type retinas. KO animals displayed a significantly thinner inner retina at all age points (Bonferroni adjusted, $p < 0.0001$). (B) Outer retinal thickness comparison between FAM84B KO and wild type retinas. KO animals displayed a significantly thinner outer retina than wild types at 12 weeks of age (Bonferroni adjusted, $p < 0.001$) and at 24, 36, 48, 60 weeks of age (Bonferroni adjusted, $p < 0.0001$). 60 week old KO animals also revealed a significantly thicker outer retina compared to KO animals aged; 24 weeks (Bonferroni adjusted, $p < 0.001$), 36 weeks (Bonferroni adjusted, $p < 0.05$), and 48 weeks (Bonferroni adjusted, $p < 0.01$). The inner retinal thickness was defined as the distance from the ILM to the outer boundary of the outer nuclear layer (oONL). The outer retinal thickness was defined as the distance from the oONL to oRPE. “*”, $p < 0.05$; “***”, $p < 0.01$; “****”, $p < 0.001$; “*****”, $p < 0.0001$. Error bars show standard deviation.

To investigate this further, the average thickness for each retinal layer in FAM84B KO and wild type animals was determined. The mean thickness of each retinal layer for the measured wild type and FAM84B KO mice is summarised in Supplementary Tables 2 and 3. Figure 20 visualises a comparison of the most notable and dynamic changes in the outer nuclear layer (ONL) of FAM84B KO retinas compared to wild types. Here, it was observed that slight reductions in wild type ONL thickness occur as animals age; which may relate to natural retinal ageing or the propensity of C57Bl/6N mice sub-strains to experience age-related retinal degeneration. More importantly, FAM84B KO retinas displayed a significantly reduced ONL thickness in all measured age groups. KO retinas also exhibited much higher variation in their ONL thickness, which complements the high variation in potency of the phenotype observed in OCT retinal cross-sections. Dynamic ONL thickness changes were also observed when comparing age groups. ONL thickness reduced on average between the ages of 12 and 24 weeks, suggesting a degenerative phase of the phenotype. Then an overall increase in ONL thickness occurs between 24 and 48 weeks. This likely correlates to a phase in which most animals experience increased retinal disorganisation in the outer retina, and defining the ONL boundaries becomes increasingly difficult because of this. Finally, at 60 weeks animals displayed a lower ONL thickness compared to 48 weeks old, where a degradative phase returns.

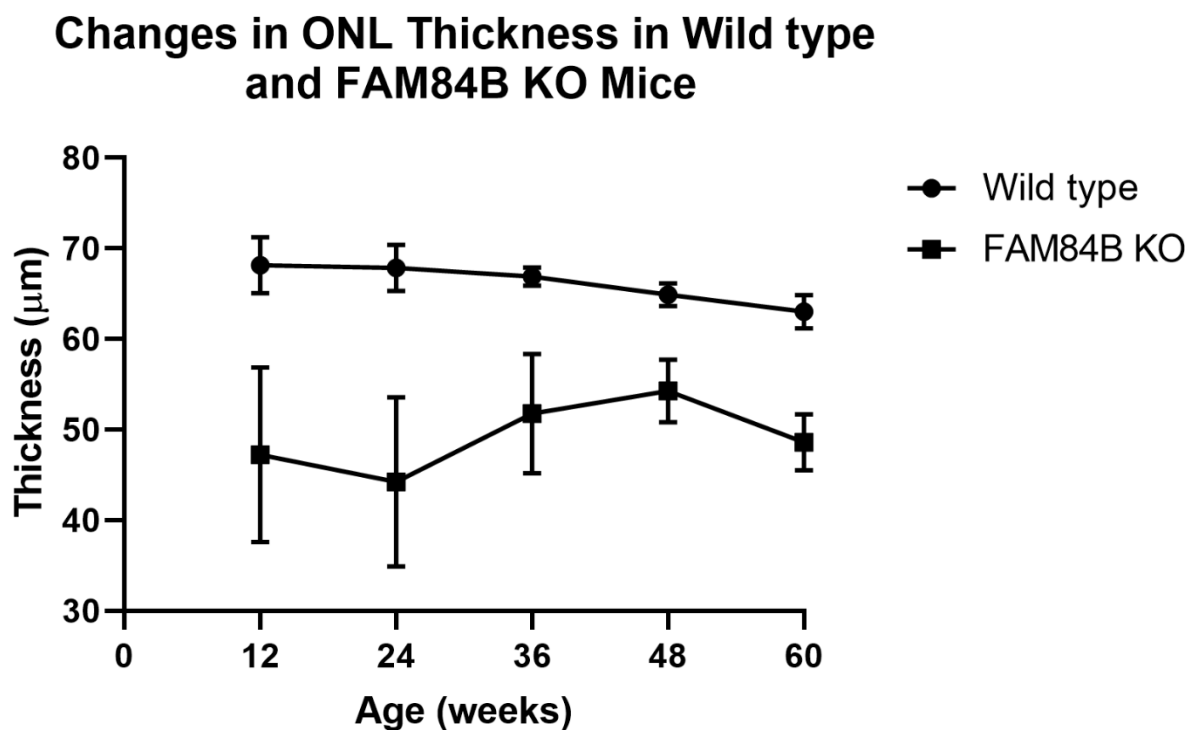


Figure 20: Comparison of outer nuclear layer (ONL) thickness between FAM84B KO and wild type mouse retinas. Wild type retinas showed a slow reduction in ONL thickness with increasing age. FAM84B KO retinas presented significantly reduced ONL thickness at 12, 24, 36, 48 and 60 weeks of age compared to wild types. Dynamic changes in ONL thickness were observed in Fam84b KO retinas, with overall reductions between 12-24 weeks and 48-60 weeks of age. Conversely, overall increases in ONL thickness were observed between 24-48 weeks of age. Retinas were scanned using Spectralis® PLUS (Heidelberg Engineering, Germany) and thickness measurements were obtained using the OCTExplorer software and The Iowa Reference Algorithms (Retinal Image Analysis Lab, Iowa Institute for Biomedical Imaging, Iowa City, IA, USA). Error bars show standard deviation.

5.4 Histological analysis of FAM84B KO retinas

Although OCT imaging allows repeat measurements to track morphological changes in the mouse retina, its resolution limits the ability to clearly identify and observe abnormalities at the cellular level. Histological analysis of FAM84B KO retinas was employed to visualise the degenerative phenotype with improved clarity. Figure 21 displays examples of the typical morphology observed in wild type and FAM84B KO retinas. Figures 21A and 21B show wild type retinal sections stained with haematoxylin and eosin, which display healthy retinal morphology with cell nuclei organised within well-defined layer borders. This is prominent in both the inferior and superior retina. Conversely, retinal layering in FAM84B KO retinas is not as well defined in the inferior retina, especially evident in the ONL (Figure 21C). Here, the varying thickness of the ONL is apparent where photoreceptor nuclei do not adhere to a consistent and distinct border, also occupying areas devoted to the inner photoreceptor segments below and the outer plexiform layer above. During OCT measurements, the most degenerative characteristics of FAM84B KO retinas were found in the superior retina. This was also echoed in histological staining. Figure 21D shows an example of an area of extreme degeneration, and severe retinal cell disorganisation and

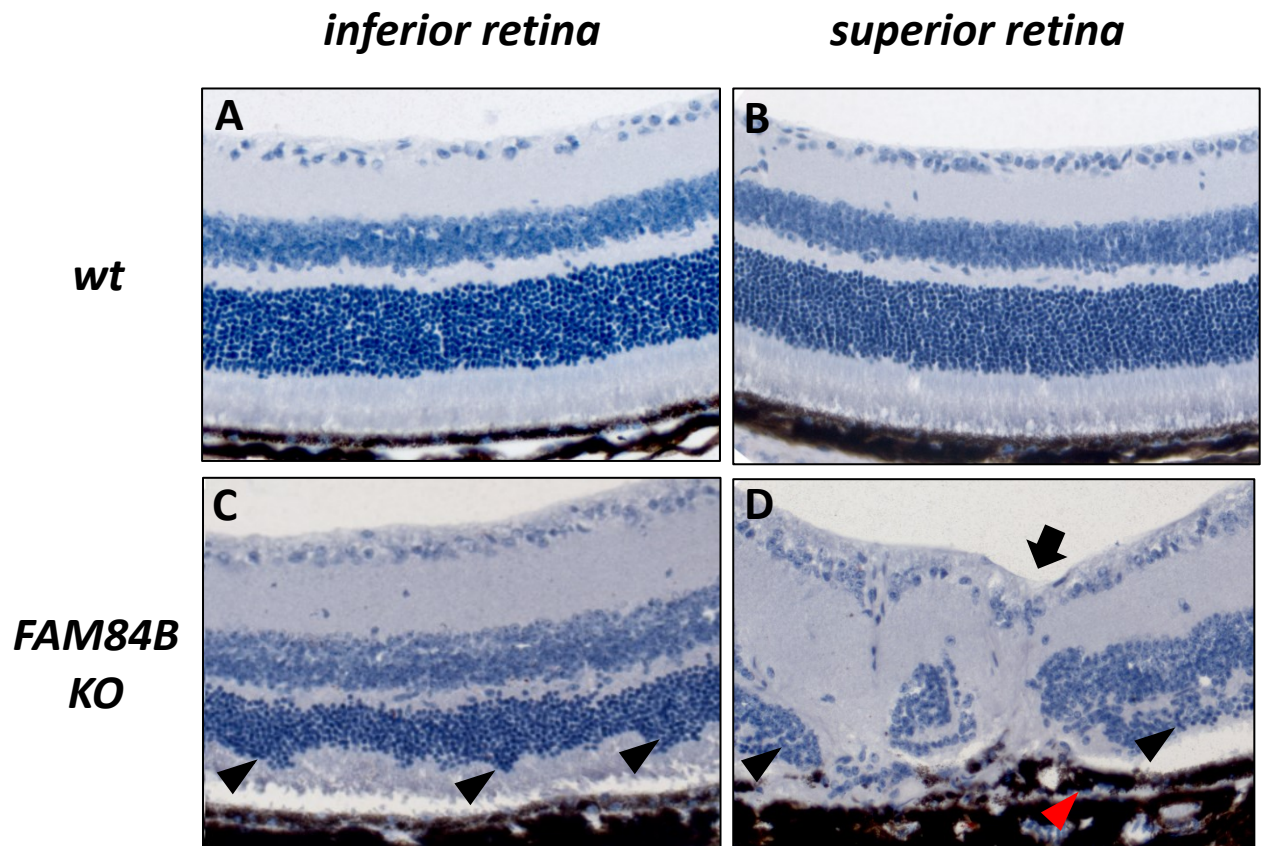
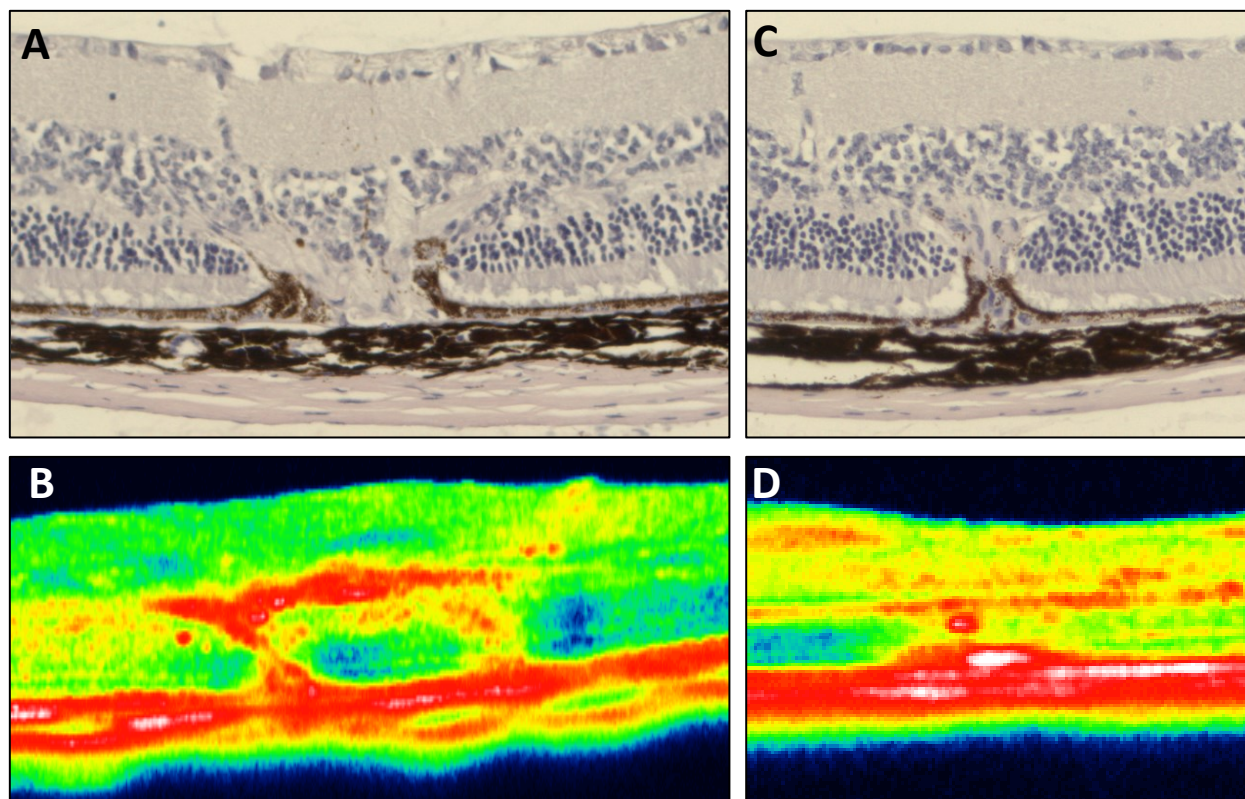


Figure 21: Histological sections of wild type and FAM84B KO retinas. The inferior and superior retinas of wild type and FAM84B KO retinas were compared. (A+B) Wild type retinas showed well defined retinal layering, and no evidence of degeneration. (C) FAM84B KO inferior retinas typically displayed sub-normal morphology, with a varying ONL thickness and mislocation of photoreceptor cell nuclei in the OPL and area for the photoreceptor segments, shown by black arrowheads. (D) Example of an area of severe degeneration observed in the superior retina of a FAM84B KO animal. Clear retinal layering is abolished with no visible boundaries, and all nuclei appear very disorganised. Pigmented layers are also mislocalised in areas normally dedicated to photoreceptors (red arrowhead). The structure of the retina is also deformed, especially around the RNFL and GCL shown by a black arrow.

deformation of the retinal macro-structure. Normal layering in these areas is completely abolished, and it is not possible to identify the cell populations in specifically assigned layers. This extends from the GCL to the RPE layer, where even pigmented layers exhibit mislocation. Areas of the retina demonstrating this dysmorphology in Figure 21D was only present in the superior retina. Areas of the superior retina that did not display this morphology resembled the morphology of Figure 21C.

Histological analysis has permitted a high-resolution view of FAM84B KO retinal morphology, ensuring a more accurate description of what is occurring at the cellular level. To emphasise the phenotype's potential potency and severity, Figure 22 shows comparative paraffin-embedded sections of FAM84B KO retinas and their matching OCT b-scan taken before sacrifice. Figures 22A and 22B display retinal areas beginning to show signs of degeneration at 16 weeks of age. Here, mislocation of RPE cells is prominent, deviating from its usual monolayer appearance. In addition, there is a presence of outer nuclear layer cellular projections, leading to contact between the inner and outer nuclear layers. This can also be reliably viewed via the corresponding cross-sections from OCT b-scans (Figures 22B, 22D). Figure 22E demonstrates an example of FAM84B KO retinal morphology from an animal 42 weeks of age. Degeneration has progressed so far that pigmented cells occupy a large part of the outer retina. Again, there is no visible retinal layering, cells were very disorganised, and the structure of the retinal tissue is deformed. The OCT b-scan of same area of this retina is shown for comparison in Figure 22F. These images display the dynamic characteristics of FAM84B KO retinas when ageing of the animals is permitted.



↓ ageing

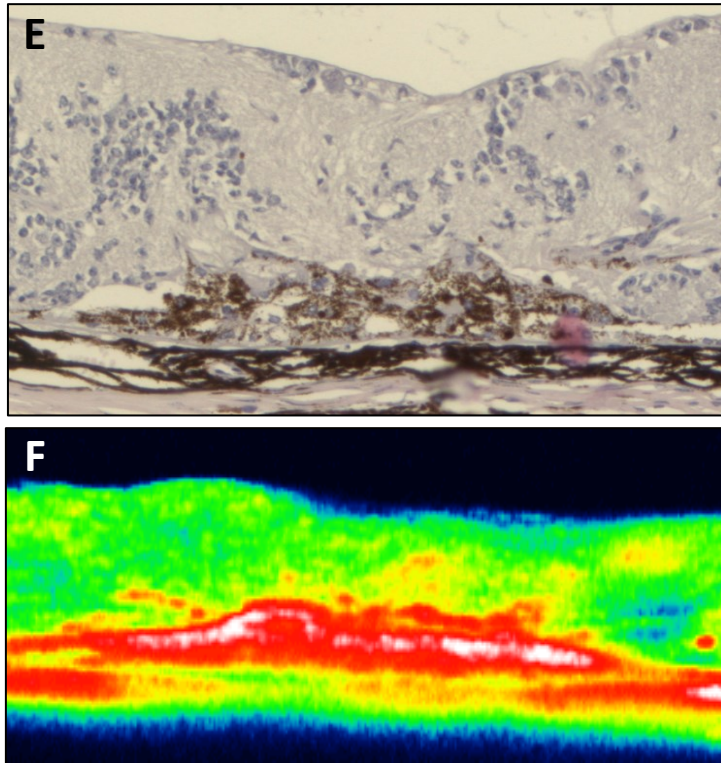


Figure 22: Comparison of FAM84B KO retina images attained by paraffin-embedded sections and Optical Coherence Tomography. (A, C) Paraffin-embedded sections of a 16 week old FAM84B KO retina displaying degeneration. Mislocation of a part of the RPE monolayer, outer nuclear layer cellular projections, and contact between the inner and outer nuclear layer is prominent. (B) OCT b-scan cross section of the same area of the retinas in (A). The cellular projections can be seen spanning across the outer retina. (D) OCT b-scan corresponding to the same area of the retina in (C). (E) Paraffin-embedded section of a 42 week old FAM84B KO retina with severe degenerative characteristics. Widespread mislocation of pigmented cells has occurred, with no identifiable retinal layers. Cell nuclei are also very disorganised, with buckling of the overall retinal structure. (F) Corresponding OCT b-scan to the same area of the retina as (E). Sections were stained with haematoxylin and eosin.

Following paraffin-embedded histological staining observations of retinas, we asked whether FAM84B KO and wild types possessed a comparable number of cells within their retinas. To explore this, the nuclei of the ganglion cell layer (GCL), inner nuclear layer (INL), and the ONL were counted across a 200 μ m span of haematoxylin and eosin stained retinal sections. Measurements were taken 1mm away from the optic disc, and not in areas of severe degeneration due to the difficulty in identifying individual layers in these retinal regions. Figure 23A and 23B show images of a wild type and FAM84B KO retina in which the GCL, INL and ONL nuclei were counted, respectively. Retinas of animals aged 42 and 60 weeks were used to potentially identify any age-related differences. There were no significant differences found between the GCL nuclei counts between wild type and FAM84B KO retinas, nor were any differences observed between the two age groups (Figure 24A). Similarly, there were no significant differences found between the INL nuclei counts between wild type and FAM84B KO retinas, however, the number of nuclei in the INL of 60 week old FAM84B KO retinas was significantly higher (Bonferroni adjusted, $p < 0.05$) than that of KO animals 48 weeks of age (Figure 24B). FAM84B KO retinas also possessed a significantly lower number of nuclei in the ONL compared to wild types (Bonferroni adjusted, $p < 0.05$), at both 42 and 60 weeks of age (Figure 24C). This may suggest that the photoreceptors of FAM84B KO retinas are undergoing cellular death, or a reduced number are established during retinal development. This is a question that would be important to answer in the future.

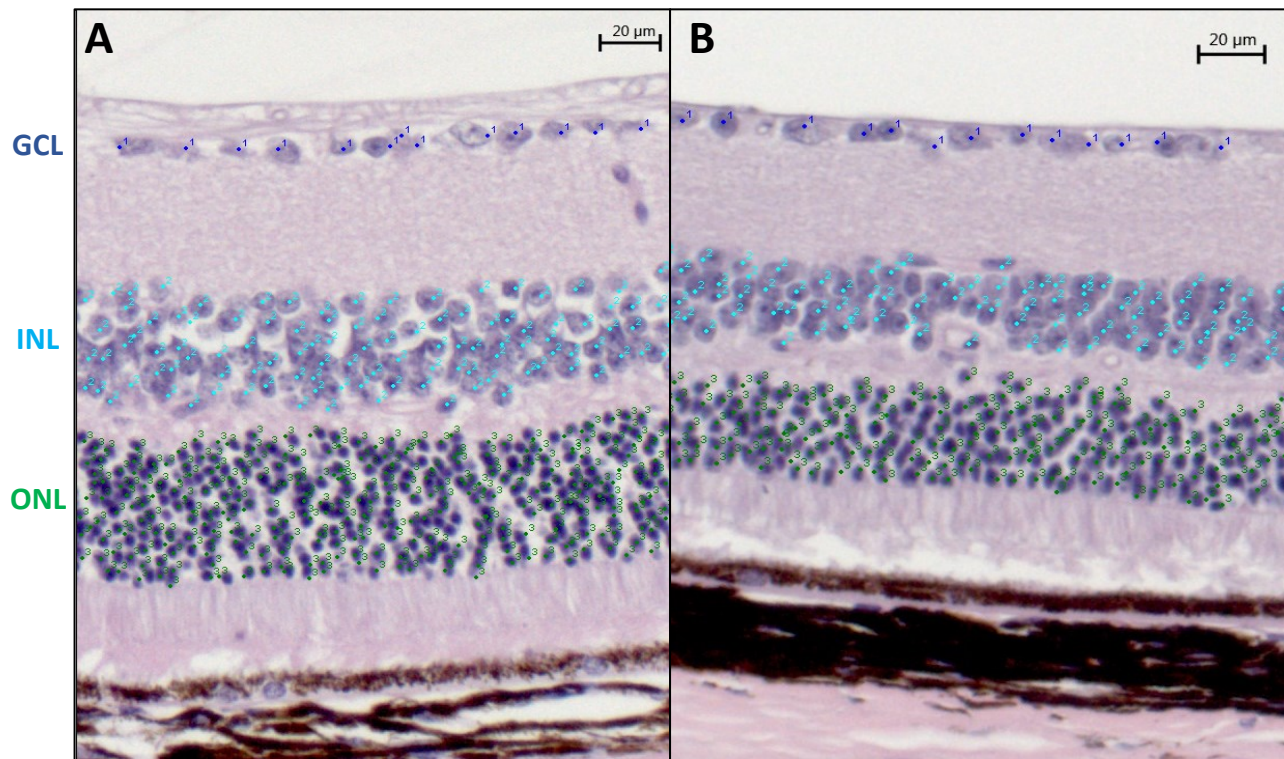
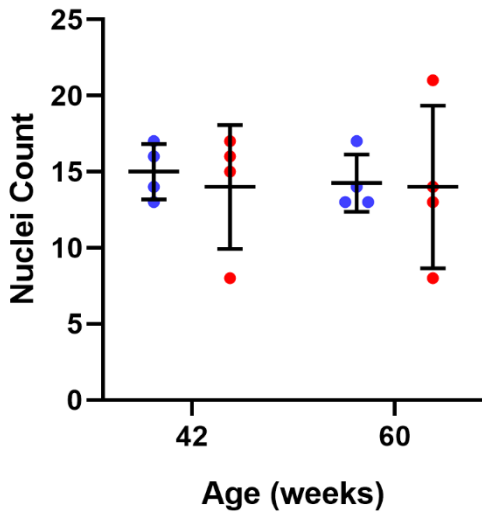
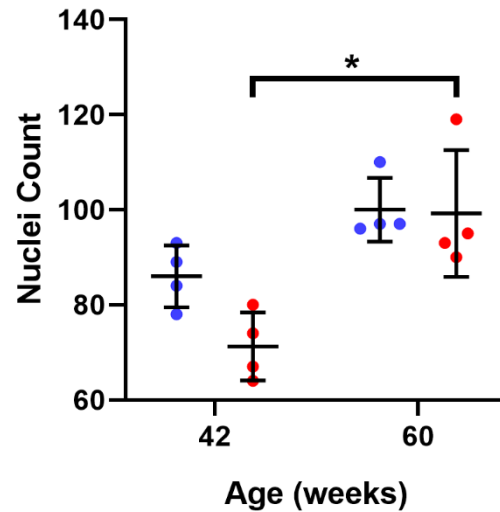


Figure 23: Nuclei counting in the GCL, INL and ONL of wild type and *Fam84b* KO retinas. The number of nuclei in three retinal layers were counted in a 200µm length of retinal tissue. Areas 1mm away from optic disc were used for nuclei quantification. Retinas from animals aged 42 and 60 weeks of age were used for comparison. (A) Example image of a wild type retina in which the nuclei were counted. (B) Example image of a *FAM84B* KO retina in which the nuclei were counted. GCL, ganglion cell layer, INL, inner nuclear layer; ONL, outer nuclear layer. Paraffin-embedded sections of mouse retinas were stained with haematoxylin and eosin to visualise cell nuclei. Nuclei counting was performed using ImageJ.

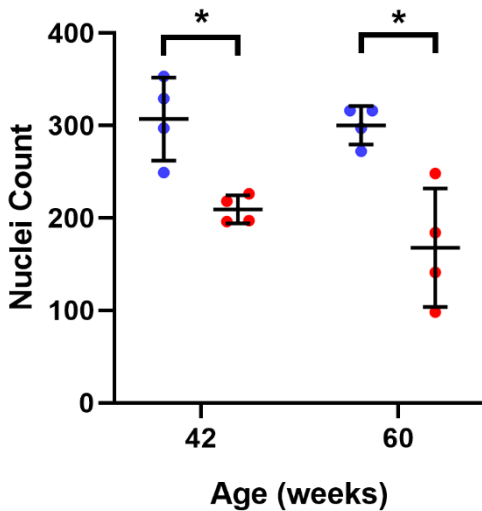
A GCL Nuclei Count in Wild type and FAM84B KO Retinas



B INL Nuclei Count in Wild type and FAM84B KO Retinas



C ONL Nuclei Count in Wild type and FAM84B KO Retinas



• Wild type • KO

Figure 24: Nuclei count comparison between GCL, INL and ONL of wild type and Fam84b KO retinas. (A) No significant differences were found between the number of nuclei in the GCL of wild type and FAM84B KO retinas. (B) No significant differences were found between the number of nuclei in the INL of wild type and Fam84b KO retinas. Yet, FAM84B KO animals did show a significantly higher number of nuclei in the INL at 60 weeks of age, when compared to 48 weeks of age. (C) FAM84B KO retinas displayed a significantly lower number of nuclei in the ONL, at both 42 and 60 weeks of age. KO, knock out; GCL, ganglion cell layer, INL, inner nuclear layer; ONL, outer nuclear layer. Paraffin-embedded sections of mouse retinas were stained with haematoxylin and eosin to visualise cell nuclei. Error bars show standard deviation. “*”, $p < 0.05$.

5.5 Immuno-histochemical detection of standard retinal neurone markers

It has been demonstrated that FAM84B KO retinas possess severe cellular disorganisation, and due to this, attempts to identify retinal cell populations via standard haematoxylin and eosin staining is increasingly problematic. The next question asked was whether FAM84B KO retinas comprise all the standard cells and neurones required to give rise to a fully functional and healthy retinal tissue. This was investigated by the means of immunohistochemistry, to distinguish the localisation of proteins expressed by specific retinal neurone populations. This approach allowed the comparison of wild type retinas with FAM84B KO retinas displaying areas of mild and severe degeneration.

Firstly, the presence of Rhodopsin, a light-sensitive G-protein coupled receptor responsible for light detection in rod cells, was examined. Wild type retinas neatly expressed Rhodopsin in the outer segments of the photoreceptors, as expected (Figure 25A). The same was also true for FAM84B KO in areas that only display mild degeneration (Figure 25B). However, Figure 25C visualises that areas of FAM84B KO retinas that express severe degeneration, lacked rhodopsin staining. This suggests that there is lack of the photosensitive pigment, and that severely degenerated regions of KO retinas are incapable of responding to light via this mechanism.

Next, the distribution of Calretinin was observed and compared between wild type and FAM84B KO retinas. Calretinin is a calcium-binding protein important for the excitability of neurones, and is a useful maker of retinal amacrine and ganglion cells (Chidlow et al., 2011). Calretinin was detected to localise to specific sub-types of ganglion cells in the GCL, and amacrine cells in the INL of wild type retinas. Additionally, 3 layers of terminals were positively stained in the inner plexiform layer (IPL) (Figure 25D). FAM84B KO retinas displaying mild degeneration also mimicked this pattern (Figure 25E). Conversely in regions of severe degeneration, an altered arrangement was seen. The anti-Calretinin antibody successfully stained its target protein, however due to the condition of the region, the distribution of amacrine and ganglion cells was highly disorganised (Figure 25F). An interesting observation in these sections is that the layers of terminals in the IPL still stain positively, proposing that the synaptic connections between the processing neurones of the inner retina may remain intact.

Following this, Protein kinase C- α (PKC- α) was stained on retinal sections in order to determine the integrity of bipolar cells, and comparisons between wild type and FAM84B KO retinas were made. Figure 25G illustrates the labelling of bipolar cells within wild type retinas by PKC- α , where their terminals lie in the inner and outer plexiform layers. This was also the case in FAM84B KO retinas that displayed mild degeneration (Figure 25H). However, PKC- α labelling of severely degenerated retinal areas revealed the presence of bipolar cells, but similarly with Calretinin labelling showed complete disorganisation and reduced integrity of these essential retinal neurones (Figure 25I).

Tyrosine hydroxylase, a putative marker of dopaminergic amacrine cells (Chidlow et al., 2011), was also labelled and compared between Fam84b genotypes. Once again, positive staining was established in all groups, but wild type (Figure 25J) and mildly degenerated FAM84B KO retinas (Figure 25H) displayed well-ordered labelling of dopaminergic amacrine cell bodies in the INL, and their processes at the IPL-INL border. Cell bodies of amacrine cells were not detected within regions of severely degenerated retinas. Staining correlating with their processes displayed a random patterning, disrupted by the deformed and unlayered regions of KO retinas (Figure 25L).

Finally, the integrity of the RPE monolayer is an important aspect of retinal maintenance and function, and was inspected in FAM84B KO retinas. This time, an immunofluorescence approach was applied to reduce the impact of melanin on the visibility of positive labelling. An antibody against RPE-65, an important enzyme involved in the recycling of photosensitive pigments, was used to tag this monolayer. Wild type retinas displayed strong and consistent staining of the RPE, positioned below the photoreceptor outer segments as expected (Figure 25M). FAM84B KO retinas possessing mild degeneration displayed fragmented staining of the RPE layer (Figure 25N), suggesting that there are certain zones of the RPE that are not fully functional. RPE-65 staining was not detected in areas of severe degeneration (Figure 25O), and DAPI staining again reiterates that disorganisation of the retina is most likely affiliated with this loss of RPE labelling. The lack of RPE-65 labelling in severely degenerated FAM84B KO retinas also implies that these areas would be incapable of reprocessing pigments to a photosensitive state, and are therefore rendered dysfunctional.

Ki-67 is a commonly used immuno-histochemical marker that is expressed in cells during the active stages of the cell cycle (G_1 , S, G_2 , M), but is absent in resting cells (G_0). Therefore, to understand whether the described phenotype is related to dysregulated cell division, wild type and FAM84B KO retinal sections were exposed to an anti-Ki-67 antibody, to detect any actively dividing cells. As Fam84b shares a sequence similarity with the family of HRASLS mentioned previously, this approach was employed to shed light on whether the lack of Fam84b in retinal tissue leads to aberrant regulation of cell proliferation. Ki-67 was not detected in wild type retinas (Figure 26A) as expected, due to the consensus that cells in the rodent adult retina are terminally differentiated. FAM84B KO retinas with mild (Figure 26B) or severe degeneration (Figure 26C) also showed no sign of Ki-67 expression. Figure 26D displays that actively dividing cells expressing Ki-67 are present in the spleen. The lack of expression in FAM84B KO retinas suggests that the phenotype is not due to the over-proliferation of cells in the retina. This could be confirmed further with EDU pulsing, and detection of the incorporated bases into the DNA of, if any, dividing cells. However, the reduced retinal thickness and nuclei count of FAM84B KOs potentially argues for a reduced capacity to divide during retinal development.

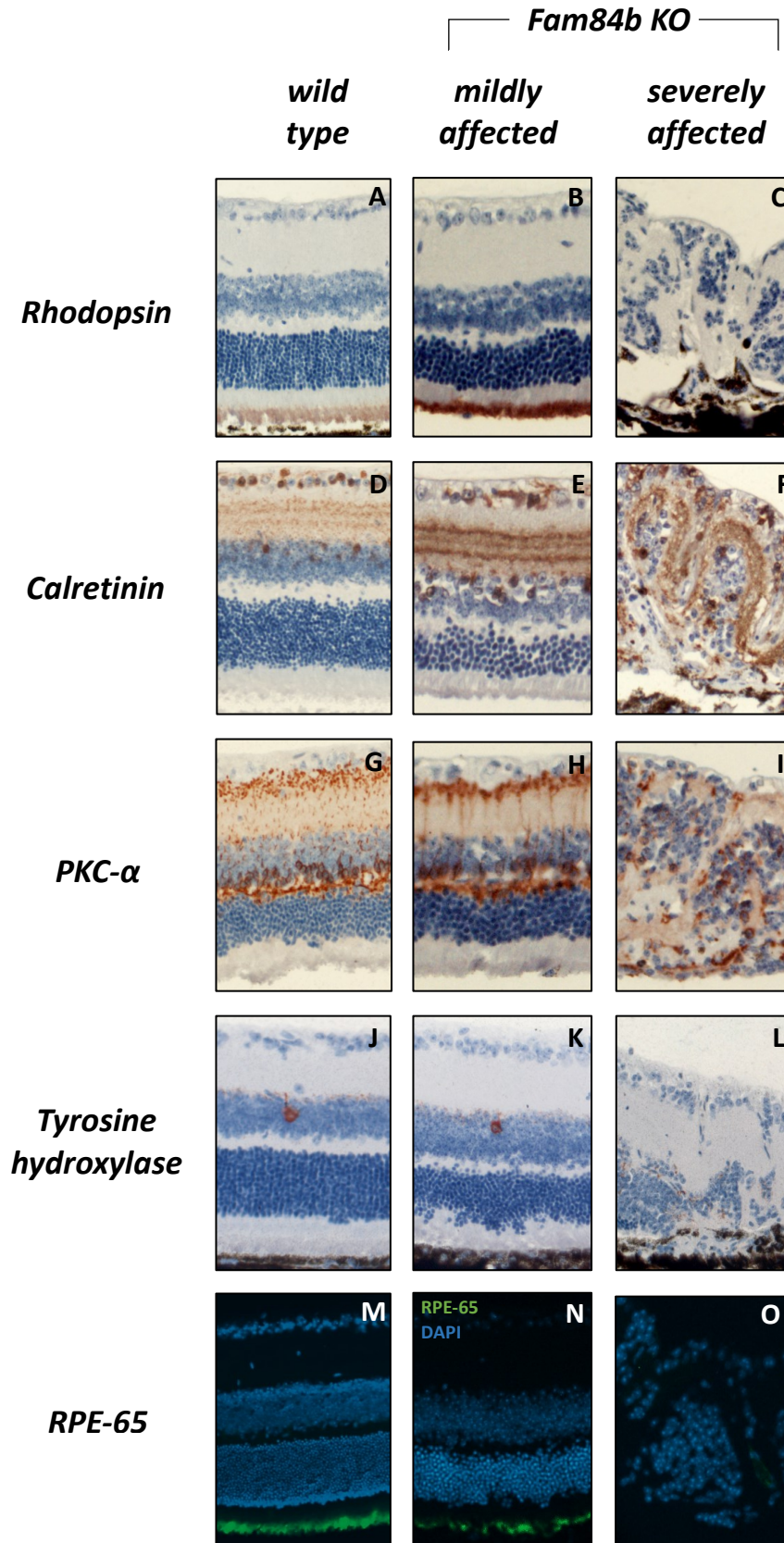


Figure 25: Immuno-histochemistry of retinal cell markers. Different retinal cells were labelled using antibodies against proteins known to be expressed in specific cell populations. This approach was used to investigate the integrity of the retinal neurones in FAM84B KO retinas in comparison to wild types. (A, B, C) Photoreceptor inner segments labelled by anti-Rhodopsin. Rhodopsin was not detected in severely degenerated Fam84b KO retinas (C). (D, E, F) Ganglion and amacrine cells labelled with anti-Calretinin. 3 layers of terminals are visible in the IPL (D, E), which are completely jumbled in severely affected areas of Fam84b KO retinas (F). (G, H, I) Bipolar cells and their processes labelled by anti-PKC- α . Bipolar cell processes terminate in the IPL and OPL in wild type and mildly degenerated retinas (G, H). Bipolar cells are highly disorganised in severely degenerated FAM84B KO retinas. (J, K, L) Dopaminergic amacrine putatively labelled by anti-tyrosine hydroxylase. Amacrine cell bodies sit in the INL, and their processes at the IPL-INL border (J, K). Processes are arranged in a disorderly fashion in severely degenerated Fam84b KO retinas. (M, N, O) The retinal pigmented epithelium labelled with anti-RPE-65. The RPE appears as a monolayer of cells between the photoreceptor and choroid layers (M). Mildly degenerated FAM84B KO retinas display a fragmented RPE-65 signal compared to wild type (N). Areas of severe degeneration possess a lack of RPE-65 staining (O).

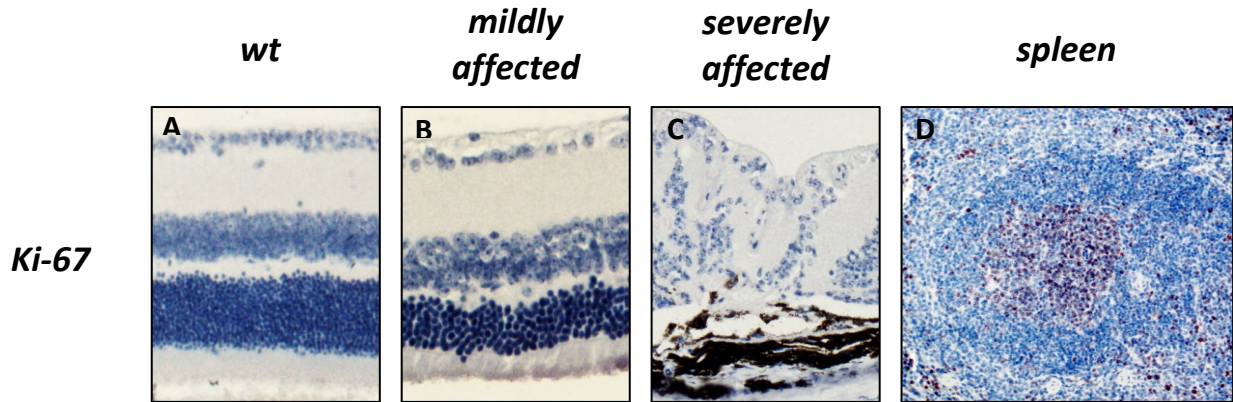


Figure 26: Wild type and FAM84B KO retinas do not express Ki-67. Paraffin-embedded eyes were sectioned and stained with anti-Ki-67 to detect cells that are in an active stage of the cell cycle (G_1 , S, G_2 , M). Ki-67 was not detected to be expressed in wild type retinas (A), FAM84B KO retinas displaying mild degeneration (B) or severe degeneration (C). (D) Sections of wild type spleen were used as a positive control, and Ki-67 is shown to be expressed in the white pulp of mouse spleen. Sections were counter-stained with haematoxylin.

5.6 The functionality of FAM84B KO retinas was assessed via electroretinography

Considering FAM84B KO animals display the abnormal retinal morphology shown above, a vital question asked was how efficient these eyes are at efficiently processing light stimulation. A detailed assessment of the mouse ERG was carried out to evaluate this. The responses elicited from exposure to varying light stimulations were gathered for each eye of wild type and FAM84B KO animals. Animals were measured at 18, 30, 48 and 60 weeks of age to recognise any age-related changes in retinal efficiency over time. For analysis purposes, the Naka-Rushton equation (Naka and Rushton, 1966) was fit to a-, b- and c-waves to extract the predicted maximum response (V_{max}), and the light intensity required to elicit half the maximum response (K) for each retina. These variables were then compared between mice. An example S-shaped curve produced by applying the Naka-Rushton equation to the a-wave amplitude responses of a wild type mouse can be seen in Figure 27. It shows how the V_{max} , K and n values can be extracted from the curve; V_{max} is the amplitude in which the sigmoidal curve is at plateau; K is intensity of light which elicits half the V_{max} ; and n represents the steepness of the slope. Reductions in V_{max} , and increases in K are associated with retinal diseases (Johnson et al., 2019).

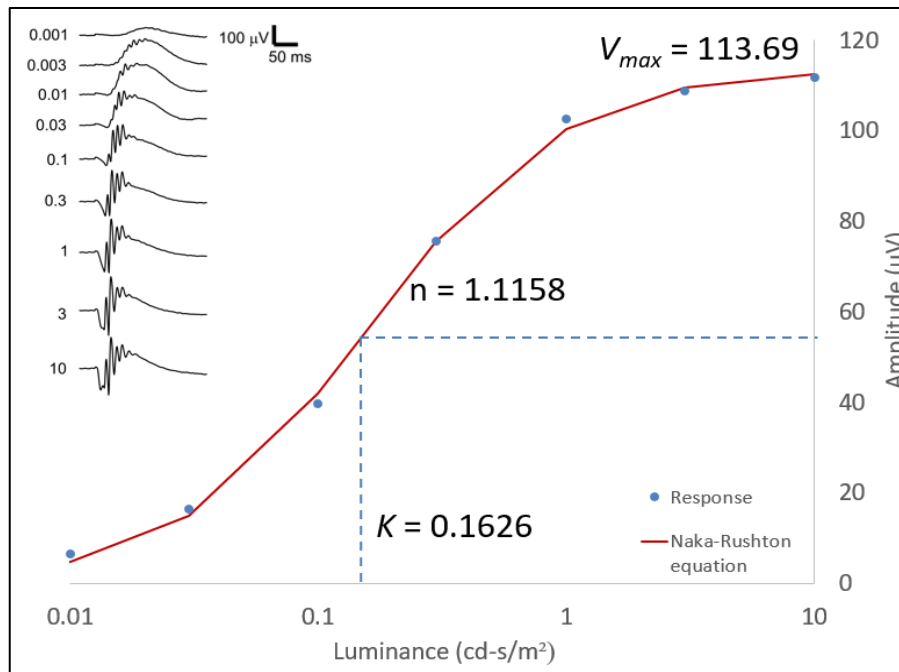
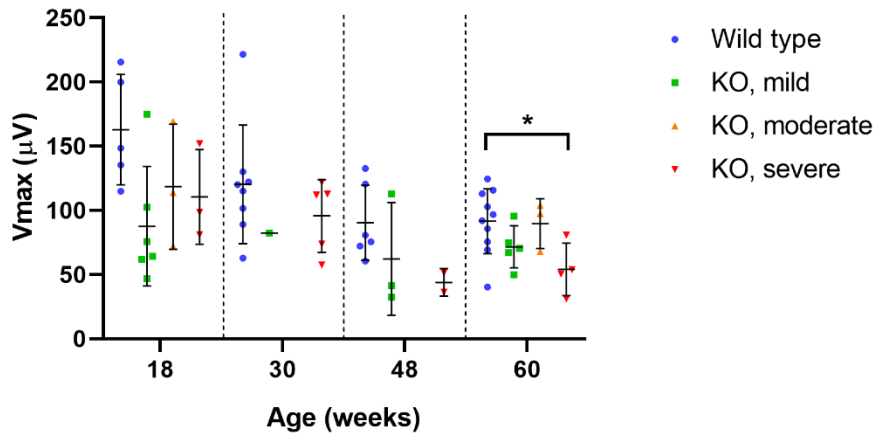


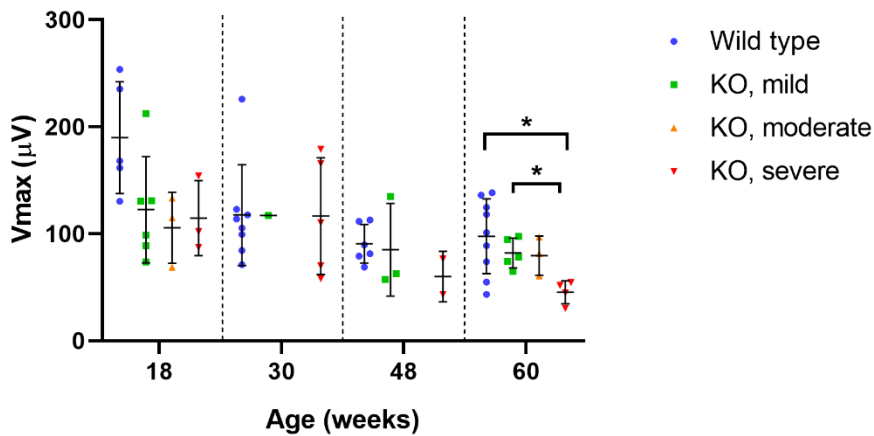
Figure 27: Fitting of the Naka-Rushton equation to the mouse ERG. An example sigmoidal curve produced when applying the Naka-Rushton equation to wild type a-wave amplitudes. Animals were exposed to light flashes with increasing brightness from 0.001 to 10 cd-s/m² and amplitudes were extrapolated. The V_{max} corresponds to the maximum predicted response of the retina, and is drawn from the amplitude at which the sigmoidal curve is at plateau. The K value corresponds to the light intensity at which the amplitude is half the V_{max} . Finally, the value of n corresponds to the steepness of the slope during its growth phase.

First, the V_{max} of a-wave amplitudes was examined for differences between wild type and the FAM84B KO retinas with varying presentation of the phenotype. FAM84B KO animals were divided into groups based on the amount of retinal degeneration observed during the corresponding OCT assessments, the grading criteria is outlined in Table 4 in section 4.3.4. Initial observations revealed that the a-wave V_{max} of all mice declined with increasing age, likely attributed to the natural ageing of mouse retinas. No significant differences were detected between groups at 18, 30 or 48 weeks of age (Figure 28A). However, at 60 weeks of age, a-wave V_{max} of the severely degenerated group were significantly lower than the wild type group (Bonferroni adjusted, $p < 0.05$). No significant differences were found between K values of wild type or FAM84B KO retinas in any age group (data not shown). As the a-wave corresponds to the summed hyperpolarisation of photoreceptors, a reduced V_{max} suggests that there is either a reduced response of photoreceptors to light, or a reduced number of photoreceptors in severely degenerated FAM84B KO retinas at 60 weeks of age. Furthermore, as no significant differences were observed between K values, the sensitivity of the photoreceptors that are present in KOs, seem to harbour a similar sensitivity to light as wild types.

A Comparison of a-wave V_{max} between wild type and FAM84B KO mice



B Comparison of b-wave V_{max} between wild type and FAM84B KO mice



C Comparison of c-wave V_{max} between wild type and FAM84B KO mice

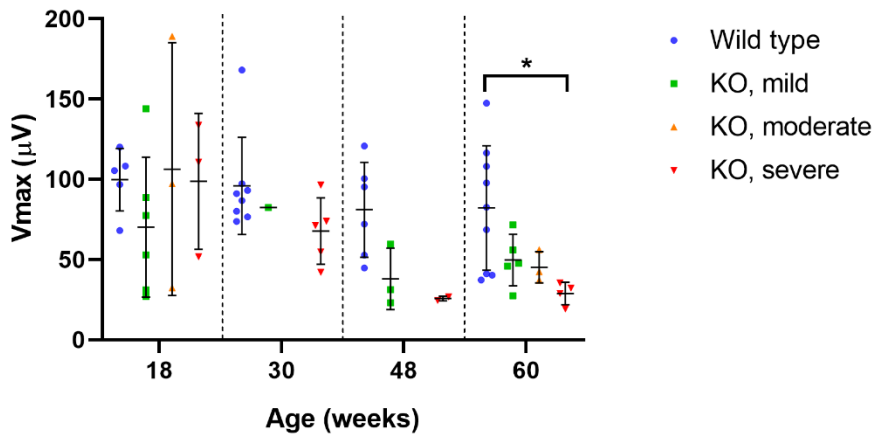


Figure 28: V_{max} comparison of wild type and FAM84B KO mice across four age groups. The Naka-Rushton equation was fit to the response amplitudes of wild type and FAM84B KO retina responses to varying light intensities. The V_{max} was extracted for a, b, and c waves of the ERG and compared between wild types and KO groups presenting varying severity of degeneration. (A) At 60 weeks of age, retinas in the severely damaged group displayed significantly lower a-wave V_{max} values compared to wild types (Bonferroni adjusted, $p < 0.05$). (B) Severely damaged retinas displayed significantly lower b-wave V_{max} compared to wild types and KO retinas in the mild degeneration group at 60 weeks of age. (C) KO retinas with severe degeneration display a significantly lower c-wave V_{max} compared to wild types at 60 weeks of age. “*”, $p < 0.05$. Error bars show standard deviation.

Next, analysis of the V_{max} for the b-wave exhibited a similar pattern; no significant differences were found until the age of 60 weeks. However, this time the severe degeneration group exhibited significantly lower b-wave V_{max} values than wild types, and the mild degeneration group as well (Figure 28B) (Bonferroni adjusted, $p < 0.05$). No significant differences were observed between K values (data not shown). This finding suggests that not only do severely affected FAM84B KO retinas demonstrate a reduced response capacity compared to wild types, but also reinforces that there is great variability associated with the presentation of this phenotype. It also may propose that Fam84b's role in retinal homeostasis ties in with an underlying mechanism that would influence its potency in KO animals. On the contrary, the amplitude of the b-wave is thought to be the collective activity of bi-polar cells and other processing inter-neurons. This means that the b-wave amplitude is functionally linked with the activity of the photoreceptors, or a-wave. Therefore, we could predict that the lower a-wave V_{max} values at 60 weeks of age in the severe degeneration group would result in the equivalent effect in b-wave V_{max} . Finally, V_{max} of the c-wave also revealed the severe degeneration group displayed significantly lower C-wave V_{max} at 60 weeks of age compared to wild type animals (Figure 28C) (Bonferroni adjusted, $p < 0.05$). No significant differences were observed between c-wave K values (data not shown).

5.7 Fam84b is expressed in the wild type mouse retina

To verify that the observed degeneration and degrading functionality of FAM84B KO retinas is due to the lack of a functional Fam84b protein, it was important to prove that Fam84b is typically expressed in the rodent retina. Wild type and FAM84B KO mice were sacrificed, and their retinas eviscerated to obtain retinal cell lysates. The retinal lysates were subjected to western blot analysis to detect the presence of Fam84b, where wild type retinal lysates revealed a band corresponding to the 42kDa size of Fam84b. This band was not present in KO retinal lysate samples. Lysates of mouse lenses were also incorporated into this analysis, in which Fam84b was not detected in wild type or KO lenses. The human RPE cell line h-TERT RPE1 (ATCC® CRL-4000™) also tested positively for Fam84b expression (Figure 29A).

To discover Fam84b's localisation within the mouse retina, immunohistochemistry was performed with an anti-Fam84b antibody (Novus Biologicals, USA). Fam84b was detected in the GCL, which looked to localise either to the endothelial cells of blood vessels residing in the GCL, or a subpopulation of ganglion cells. Fam84b signal was also detected in the OPL, speckled across the entire length of the wild type retina (Figure 29B, left). To increase the signal associated with moderately expressed protein in the retina, tyramide amplification paired with immunofluorescence staining was carried out. Detection of Fam84b was maintained in the GCL and OPL, with successful amplification of signal in both layers. Fam84b expression was also observed in the photoreceptor and RPE layers, coinciding with the positive expression of Fam84b in the h-TERT RPE1 cell line (Figure 29B, right).

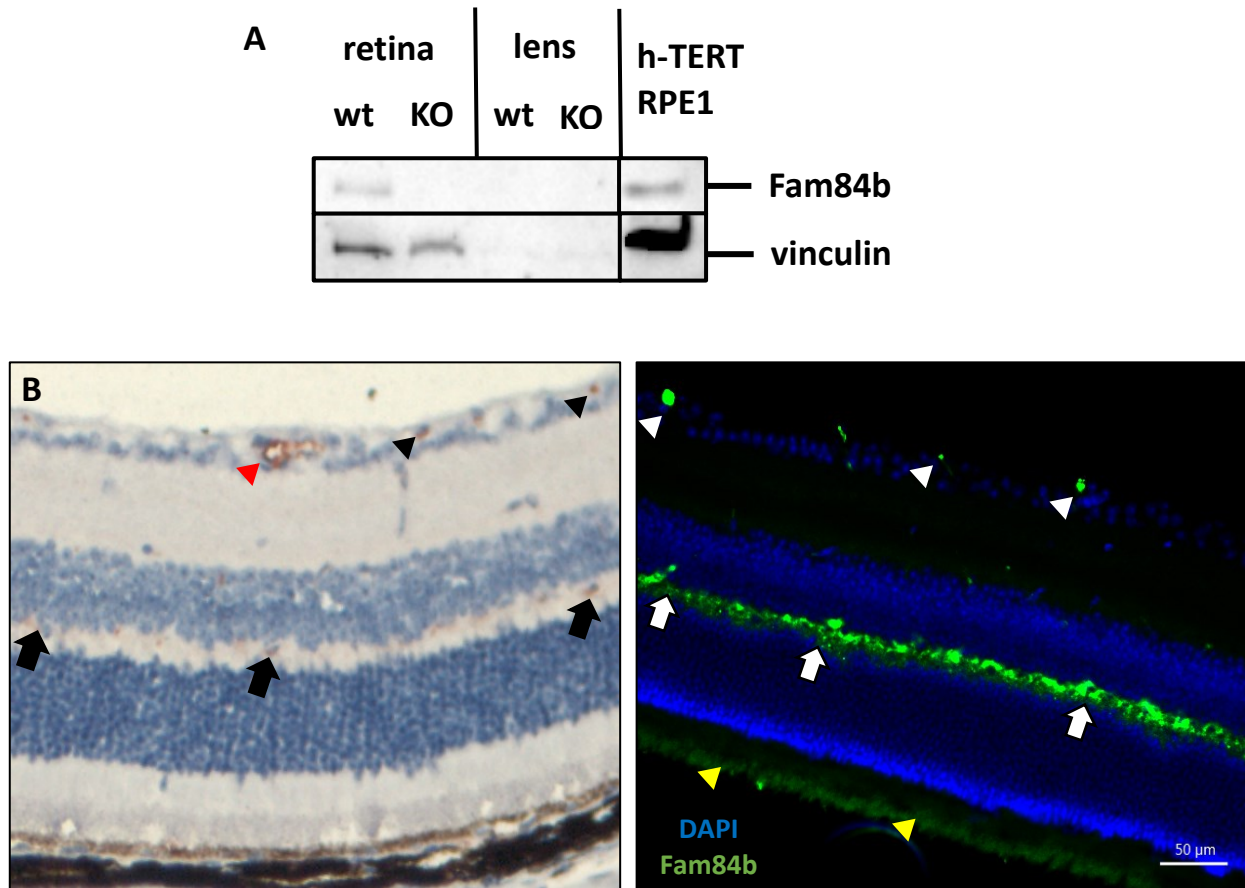


Figure 29: Fam84b is expressed in wild type mouse retinas. (A) Wild type and FAM84B KO retinal and lens lysates were subjected to western blot analysis to detect the expression of Fam84b. Wild type retinas express Fam84b, but wild type lenses do not. Neither FAM84B KO retinas nor both lens lysates showed positive expression of Fam84b. The human cell line h-TERT RPE1 was also shown to express Fam84b. The cytoskeletal protein vinculin was used as a loading control detected by anti-vinculin. Predicted band sizes; Fam84b - 42kDa, vinculin - 117kDa. (B) Immunohistochemistry was performed with anti-Fam84b to detect the localisation of Fam84b within the wild type mouse retina. Left, classical immunohistochemistry detected Fam84b expression in the GCL, in blood vessel endothelial cells (red arrowheads), and either in in endothelial cells or a sub-type of ganglion cells (black arrowheads). Fam84b expression was also speckled in the OPL (black arrows). Right, signal was amplified via a tyramide amplification kit (ThermoFisher Scientific, USA). Fam84b was detected in the GCL (white arrowheads), and strongly in the OPL (white arrows). Signal was also detected in the photoreceptor and RPE layers (yellow arrowheads). Retinal sections were counterstained with DAPI.

During these staining experiments, the retina was not the only structure in which positive Fam84b expression was observed. Cross-striated muscle (Figure 30A), peripheral nerves (Figure 30B) and lacrimal glands (Figure 30C) all showed positive expression of the Fam84b protein, when compared to the negative control sections that were not exposed to the anti-Fam84b antibody (Figures 30D, 30E and 30F, respectively). This shows that Fam84b may perform a role that it not strictly specific to retinal tissue. Nevertheless, it's possible that the varying phenotype severity that FAM84B KO animals display is linked to its action in these tissues. However, this thesis work has not investigated potential changes in the function of striated muscle, nor the peripheral nervous system or exocrine systems of FAM84B KO animals. This would be an interesting avenue to explore in the future.

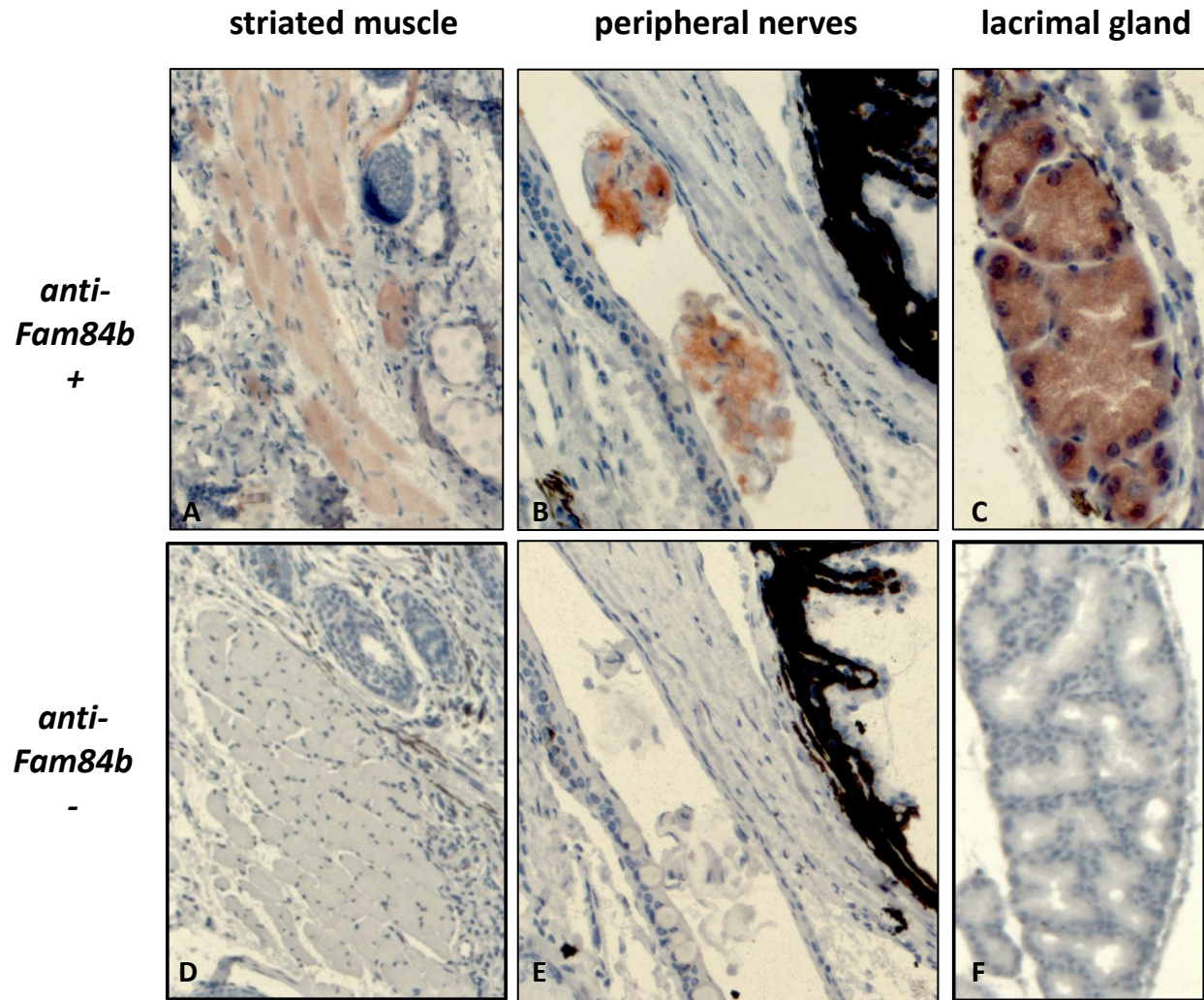


Figure 30: Fam84b expression is not limited to the retina. Immunohistochemistry also revealed Fam84b expression in structures other than the retina. Sections of wild type retinas also included tissue surrounding the eye, which were exposed to anti-Fam84b and counterstained with haematoxylin. (A) Expression of Fam84b was observed in cross-striated muscle when compared to sections not exposed to anti-Fam84b in (D). Nerves of the peripheral nervous system were found to express Fam84b (B), compared to a negative control section in (E). Lacrimal gland of the exocrine system displayed strong positive staining for Fam84b (C), negative control shown in (F).

Next, anti-sense and sense RNA probes were designed in order to bind to and discover the localisation of Fam84b mRNA in the wild type mouse retina, by means of *in situ* hybridisation. Fam84b mRNA showed widespread expression in wild type retinal tissue, including strong signal in striated muscle. (Figure 31A). Fam84b mRNA was localised to the GCL, IPL, OPL and the photoreceptor layers (Figure 31B), as well as the pigmented layers of the RPE and choroid (Figure 31C, 31D).

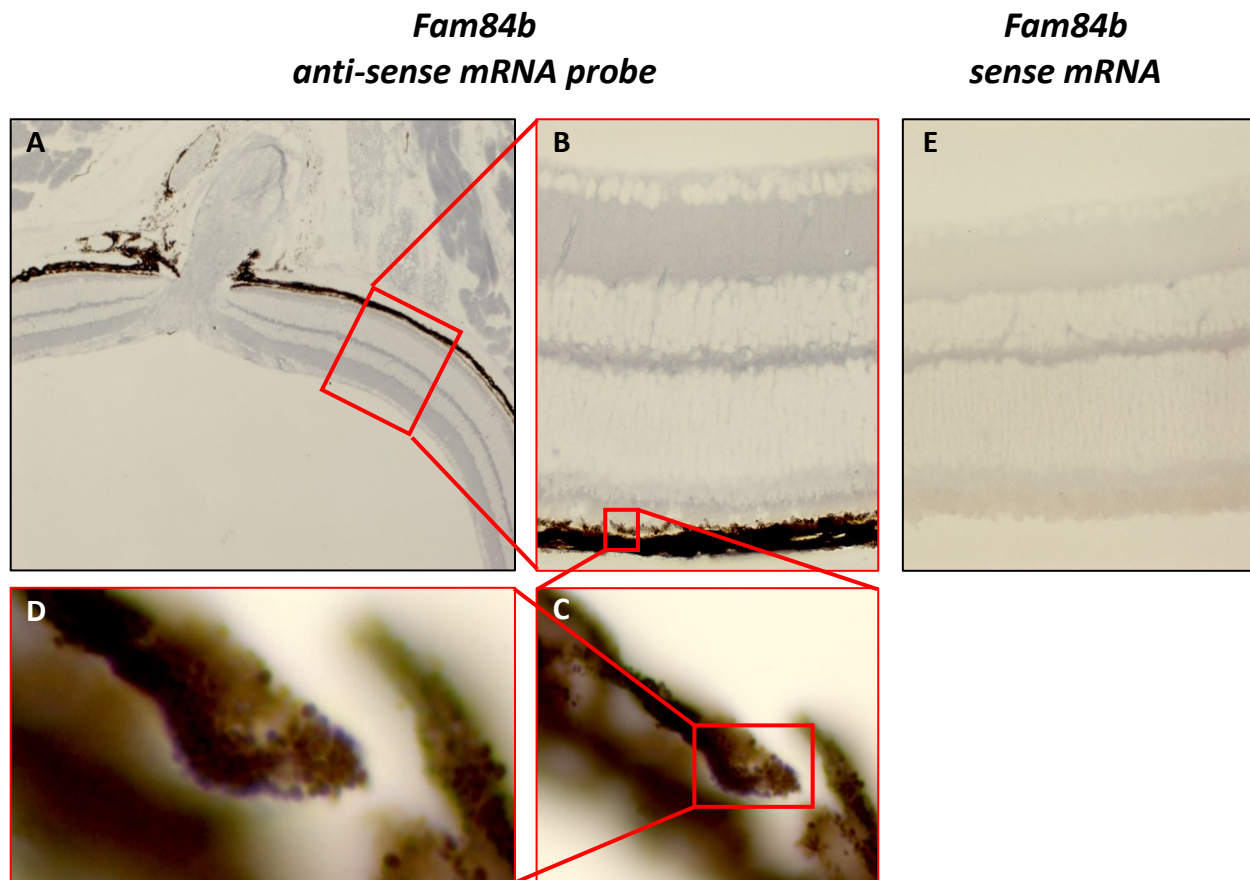


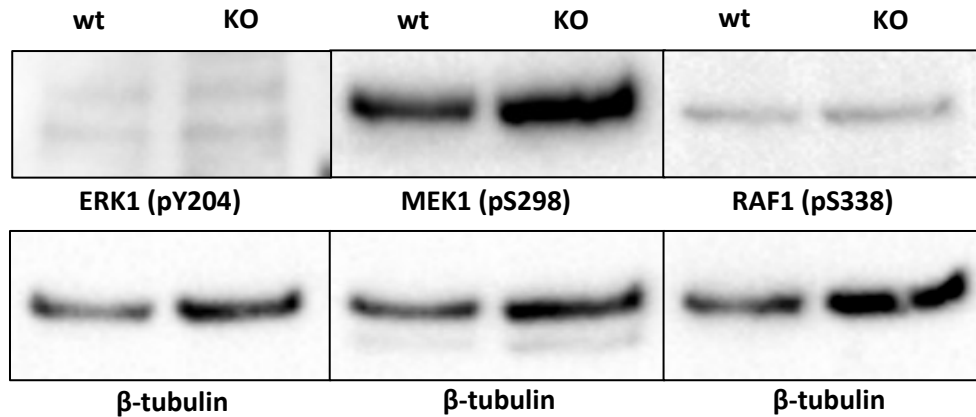
Figure 31: Localisation of Fam84b mRNA in wild type mouse retinas via *in situ* hybridisation. Fam84b mRNA sense and anti-sense probes were designed and synthesised to confirm immunohistochemistry experiments shown previously. (A) 200x magnification of wild type retina stained with anti-sense Fam84b mRNA probe. Widespread detection of Fam84b mRNA was visible in retinal tissue. (B) 400x magnification focused on a section of the retina. Detection of Fam84b mRNA is seen in the GCL, IPL OPL and photoreceptor layers, as well as axons in between. (C) 1000x magnification of a section of the RPE layer displaying positive expression of Fam84b mRNA. (D) Enlarged image of purple-stained RPE nuclei seen in image C. (E) 400x magnification of a section of retina stained with a Fam84b mRNA sense probe.

5.8 Assessing changes in the Ras-ERK pathway of FAM84B KO retinas

Fam84b displays a sequence homology to a family of HRAS-like suppressors, in which the catalytic domain of HRASLS is amongst the sequence shared. To investigate whether Fam84b also possesses a function that affects Ras signalling, the activity of the canonical Ras-Erk pathway was assessed by western blot analysis. The levels of phosphorylated Raf-1, MEK1 and ERK1 were compared between wild type and Fam84b KO retinal lysates, to monitor any potential alterations in Ras function.

There are numerous post-translational phosphorylation events that can occur on the kinases of this cascade, due to the large amount of signalling input, cross-talk and cascade regulation associated with Ras function. However, the antibodies for western blot experiments were chosen to bind the kinases in their actively signalling form. Phosphorylation of serine 338 on Raf-1 is not only important step for its dimerisation and activation at the plasma membrane, but also for its interaction with the substrate MEK1 (Matallanas et al., 2011). This residue is an important Raf-1 phosphoregulatory site, and its phosphorylation status has been described to be directly stimulated by Ras, where if substituted, Raf-1 activation diminishes (Chong, Lee and Guan, 2001). Growth factor stimulation and integrin activation is also known to lead to the phosphorylation of S338 by Pak family kinases. Raf-1 phosphorylates its prime substrate MEK1 on residues S217 and S221 leading to its increased activity. Yet, a strengthened interaction between Raf-1, MEK1 and ERK2 is achieved by phosphorylation on MEK1 at S298, by Rac-effector kinase PAK1 (Matallanas et al., 2011). This is a key event in order to identify whether the kinases are in close proximity to actively signal. The MEK kinases hold dual-specificity, to phosphorylate both threonine and tyrosine residues on their substrates. In the case of ERK1, both T202 and Y204 must be phosphorylated in order for it to activate its downstream effectors.

Comparisons of the levels of phosphorylated ERK1 at Y204, MEK1 at S298 and Raf-1 at S338 in wild type and FAM84B KO retinal lysates were performed in triplicate. The bands corresponding to the molecular weights of the three kinases were normalised to a loading control, β -tubulin, and the band intensities of each sample were compared (Figure 32A). ERK1 (pY204) was consistently detected at reduced levels in Fam84b KO retinal lysates, with a mean fold change of 0.64 ± 0.08 . MEK1 (pS298) levels showed slight inconsistencies where one attempt saw a 1.08-fold increase, and the other two repeats displayed reductions of 0.83 and 0.87-fold in FAM84B KO retina pMEK1 levels, when compared to wild type retinal lysates. This resulted in a mean fold change of 0.93 ± 0.06 . Similarly, with Raf-1 (pS338), attempts saw fold increases of 1.1 and 1.29, and a decrease of 0.82 in FAM84B KO lysates. The mean fold change for pRaf-1 (S338) in FAM84B KO lysates compared to wild type was 1.08 ± 0.11 (Figure 32B).

A**B**

Ras Effector Activation in FAM84B KO Retinas Compared to Wild type

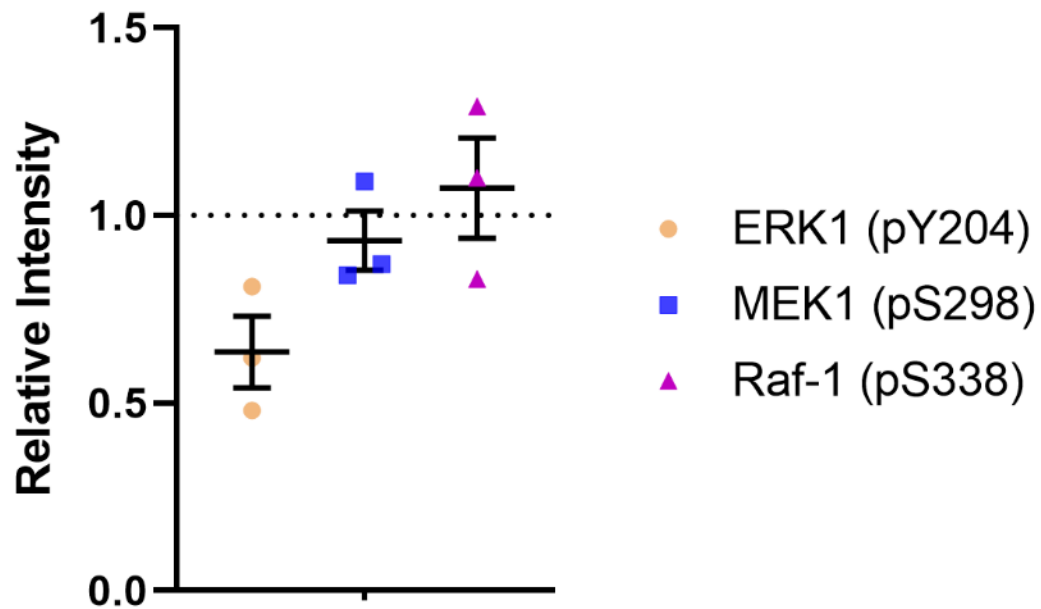


Figure 32: Detection of alterations in Ras-ERK pathway activation. Comparative western blot analysis to measure the levels of phosphorylated kinases downstream of Ras in wild type and FAM84B KO mouse retinal lysates. Anti-ERK1 (pY204), anti-MEK1 (pS298) and anti-Raf1 (pS338) antibodies were chosen to detect the levels of endogenous kinase activity. (A) Example of bands detected corresponding to the molecular weights of the three kinases. Bands were normalised to levels of β-tubulin, and band intensity was compared between wild type and FAM84B KO retinal lysates. (B) Plot to show the detected pERK1, pMEK1 and pRaf-1 fold changes in FAM84B KO retinas compared to wild type. Blots were completed in triplicate. Levels of ERK1 (pY204) were consistently lower in KO lysates compared to wild type. MEK1 (pS298) and Raf-1 (pS338) displayed minimal or inconsistent differences. Error bars show standard error. The dotted line shows a relative intensity of 1, and represents the level of active kinases measured in wild type lysates, active kinase levels of FAM84B KO lysates were compared to this. Error bars show mean fold change ± standard error.

5.9 Detection of proteins that specifically interact with Fam84b

To further unravel Fam84b's potential role in retinal homeostasis, co-immunoprecipitation was performed in order to discover its interacting protein partners. At the time of writing this thesis, the coimmunoprecipitation experiments were undergoing optimisation. Lysates of the cell line hTERT-RPE1 were chosen due to their positive expression of Fam84b as shown in Figure 29. Anti-Fam84b (Novus Biologicals) was conjugated to Protein G Dynabeads™ (ThermoFisher Scientific, USA), cell lysates were exposed to the bead-Ab complex, and then eluted. hTERT-RPE1 cell lysates exposed to beads that had not been conjugated to anti-Fam84b acted as a negative control. Before eluting, beads were sent for mass spectrometry identification of proteins bound to the bead-anti-Fam84b-Fam84b complex. Cell lysates, eluted samples and flow through samples were loaded on SDS-PAGE, and western blot was carried out in order to visualise whether the co-immunoprecipitation was successful. Anti-Fam84b was used as the primary antibody for detection on blots.

A band corresponding to the molecular weight of Fam84b (42kDa) was observed in hTERT-RPE1 lysate, the eluted sample, and the flow-through, indicating a successful binding of Fam84b to the bead-Ab complex (Figure 33A). An intense band ~50kDa was also detected in the elution sample, this could possibly represent a post-translationally modified Fam84b. In addition, a band corresponding to ~25kDa was also detected in elution samples, which most likely is attributed to an unspecific reaction caused by the IgG light chain. Following this, samples were run on SDS-PAGE again in order to excise four bands of interest to be characterised by mass spectrometry (Figure 33B). Mass spectrometry identified the presence of GRIPAP1 in the bead eluted sample, which was not detected in bead samples not conjugated to anti-Fam84b. This proposes that Fam84b specifically interacts with GRIPAP1 in hTERT-RPE1 cells. Figure 34 visualises the mass spectrometry results, comparing the intensity of proteins detected in the anti-Fam84b sample and the anti-GFP control sample.

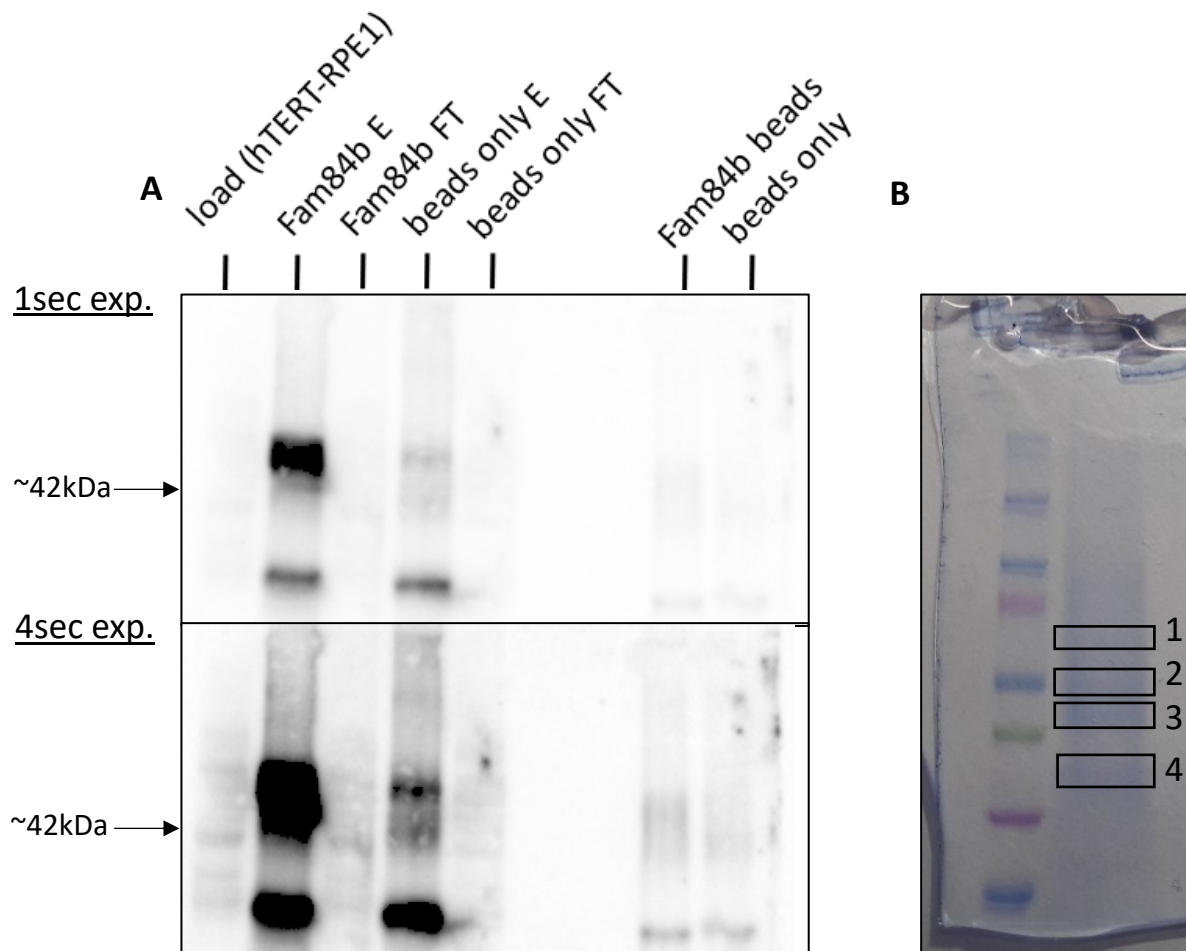


Figure 33: Co-immunoprecipitation of Fam84b in hTERT-RPE1 cells. To discover Fam84b's interacting protein partners, Fam84b was co-immunoprecipitated in hTERT-RPE1 cells. (A) Blot showing the resulting separation of hTERT-RPE1 cell lysate (load), eluate from beads conjugated to anti-Fam84b (Fam84b E), flow-through from sample with beads conjugated to anti-Fam84b (Fam84b FT), eluate from beads not conjugated to anti-Fam84b (beads only E), flow-through from sample with beads not conjugated to anti-Fam84b (beads only FT). Beads were sent for protein identification via mass spectrometry. The remaining beads after triple elution were also ran on SDS-PAGE. Two exposures (exp.) are shown for clear visibility. A band corresponding to the molecular weight of Fam84b (~42kDa) was present in load, Fam84b E and Fam84b FT lanes. A high intensity band ~50kDa was also observed in the Fam84b E sample. ~25kDa unspecific bands were observed in both elution samples. (B) Samples were run again on SDS-PAGE so that bands of interest could be excised for mass spectrometry identification. 1, ~60kDa. 2, ~50kDa, corresponded with high intensity band observed in A. 3, ~40kDa, corresponds with proposed Fam84b band in A. 4, ~30kDa. However, Fam84b was not detected in any of the excised bands by mass spectrometry.

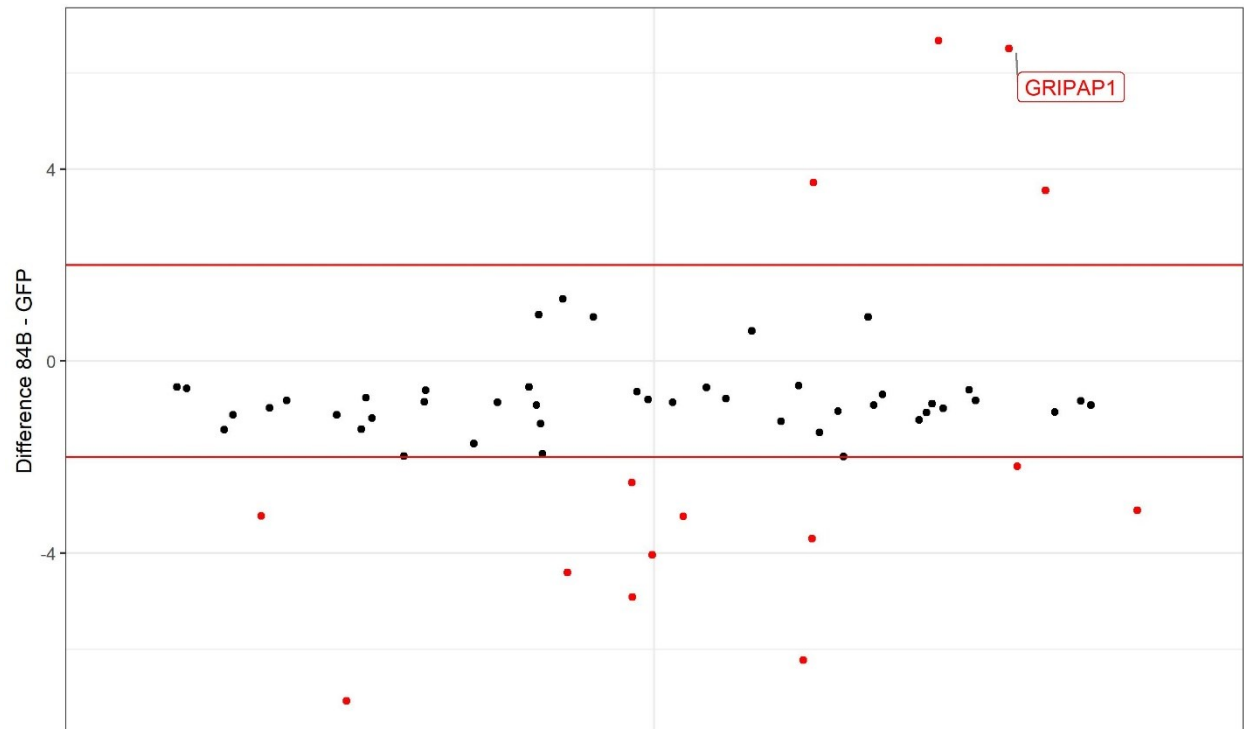


Figure 34: Detected protein intensity differences between co-immunoprecipitation samples. Mass spectrometry was used to detect proteins bound to the antibody-bead complexes. The graph shows the differences between the protein intensities detected in hTERT-RPE1 cell lysates exposed to anti-Fam84b and anti-GFP. Proteins which possessed an absolute difference of more than 2 are highlighted in red. GRIPAP1 was of particular interest and is also highlighted, which presents a novel binding partner for Fam84b. Proteins that had a higher intensity in the negative control sample compared to samples exposed to anti-Fam84b and anti-GFP were removed from the analysis.

6 DISCUSSION

This thesis work has presented a FAM84B KO mouse model and characterised a prominent phenotype comprising of dynamic intraretinal abnormalities. This phenotype was initially observed in a standardised IMPC-directed KO cohort, and efforts to further understand the morphology and functionality of FAM84B KO retinas have been implemented via secondary experiments.

6.1 Comparison of FAM84B KO retinas and known retinal pathologies

Comparisons were drawn between the phenotype observed in FAM84B KO retinas and the clinical presentation of known retinal diseases, as it's possible that FAM84B is a risk gene for an already characterised retinal pathology in humans. SD-OCT scans of FAM84B KO retinas revealed areas of bulging in the outer retinal layers, stemming from the RPE and photoreceptor layers (Figure 15). These areas generally became more degenerated with age (Figure 17), and possessed a similar bow-shaped morphology that is seen in the presence of soft drusen in dry-AMD. This is interesting as Holliday et al., (2013) identified an association between SNP rs11986011 and early-stage AMD in a cohort of European descent. This SNP lies within an intergenic region of 8q24.21 near to the FAM84B locus, and could represent a regulatory element affecting the expression of *Fam84b*, and therefore vulnerability to AMD. Although SNP rs11986011 was detected in low frequency within the cohort, it was strongly correlated with early AMD. Soft drusen carry an accumulation of lipid and protein material between the RPE and Bruch's membrane, and the pressure from this amassing material can lead to localised detachments of the RPE with no initial loss in visual acuity (Wojtkowski et al., 2009). Although visual acuity was not assessed in this work, FAM84B KO retinas did not show significantly lower response amplitudes in their ERG assessments until 60 weeks of age (Figure 28A-C).

SD-OCT images alone would point a diagnosis in the direction of dry-AMD in FAM84B KO animals, as layers of the outer retina also displayed elevated deformation with age, possibly corresponding to an increase in drusenoid material over time. To fully investigate a potential case of dry-AMD in these animals, fluorescein angiography would be a valuable technique to assess the presence of drusen, considering fluorescein binds to larger and more fragile soft drusen (Friedman et al., 2012). This method could also be repeated in order to observe age related changes in the growth and stability of potential drusen. Drusenoid lipid build up imposes stress on the RPE in AMD, most likely by disrupting its fluid transport efficiency. This, alongside the already high levels of oxidative stress in the retina, leads to RPE loss initially, and then RPE proliferation in an attempt to recover (Bowes Rickman et al., 2013). This could be an explanation for the increased amount of displaced pigmented material observed in FAM84B KO retinal histological sections (Figures 21D, 22E), but is contradicted by the lack of RPE-65 immunostaining in areas of severe degeneration (Figure 25O). On observation of FAM84B KO retinal histological sections at higher resolution, there doesn't seem to be any obvious pockets of fluid material underneath the RPE layer. This raises a question whether this characteristic of the phenotype really does correspond to dry-AMD, but then again human and mouse AMD may present themselves differently due to the lack of a macula in rodents, and interspecies variation.

Wet-AMD is characterised by choroidal neovascularisation (Seth, Sigler and Adelman, 2011), and histological sections of degenerated FAM84B KO retinas reveal cellular projections that link the inner and outer nuclear layers. These projections seem to appear in areas in which the RPE layer broken or displaced (Figure 22A, 22C). It is possible that these cellular projections are newly sprouted blood vessels from the choroid layer, and may represent cases of choroidal neovascularisation. Preliminary attempts to immunohistochemically stain blood vessels in the mouse retina to investigate this unfortunately failed, but it would be interesting to consider the prospect that FAM84B KO retinas experience the outer retinal invasion of choroidal blood vessels. Figures 21D and 22E show examples of severely degenerated KO retinas of older animals, in both images pigment is displaced in the outer retinal layers, and may represent pigments synthesised by mature melanocytes of potentially invading choroidal vessels. Detection of endogenous activated VEGF levels may also shed light as to whether we are dealing with a wet-AMD phenotype (Hsu et al., 2016). Areas of severe degeneration in FAM84B KO retinas show very disorganised cell nuclei localisation, and a complete absence of retinal layering. This may suggest these areas suffer from changes in cell polarity or regulation of migration.

Patients suffering from RP typically present perivascular deposits of bone spicule pigment in the mid-periphery of their fundi. Here, after photoreceptor death RPE cells detach from Bruch's membrane and migrate towards the inner retina, likely due to their affinity to vasculature (Li, Possin and Milam, 1995). Proliferation of pigmented cells may also contribute to the continued retinal degeneration seen in RP. The retinal degeneration of KO retinas in Figures 21D and 22E may also represent an RP-related phenotype, as irregular pigment localisation and cell nuclei displacement is apparent. The lack of RPE-65 staining in areas of severely degenerated FAM84B KO retinas (Figure 25O) could indicate proliferation of melanin alone, and not fully functional RPE epithelial cells. On the other hand, the lack of Ki-67 staining in KO retinas (Figure 22B, 22C) supports that the observed retinal degeneration is not due to any actively dividing cells. To possibly rule out any AMD- or RP-related pigment proliferation, we may look to pulse mice with BrdU (bromodeoxyuridine) for its incorporation into dividing cells, and detection after S-phase (Yahaya, McLachlan and Collie, 2013). This should support Ki-67 staining results shown above, and any uncertainties surrounding whether the phenotype is due to unwarranted proliferation would be brought into line.

6.2 FAM84B KO retinal morphology and function

FAM84B KO retinas were significantly thinner than non-mutated retinas at all age groups measured (Figure 18B), where dynamic changes in the ONL thickness were observed (Figure 20). This means that atrophy of the retina must be occurring in FAM84B KO retinas. The significant differences observed in the thickness of KO outer retinas between age groups was particularly interesting as this highlights that alterations are occurring mainly in the outer retina, which is backed up via our SD-OCT and histology morphology assessments, and is a key characteristic in retinal diseases such as AMD and RP. The differences observed in the thickness of the ONL between age groups are difficult to interpret without further experiments focused on the cell division cycle or migratory properties of these retinal cells. An initial degradative phase up to 24 weeks old may represent photoreceptor nuclei loss. This is an aspect of the phenotype that should be confirmed by TUNEL analysis, in order to detect whether the thinning of KO retinas is due to cells undergoing apoptosis. Animals aged 24-48 weeks saw ONL thickness increases,

which could correlate with the increased retinal disorganisation or migration of cells into the ONL of FAM84B KO animals. On the contrary, the same animals were not tracked from 6-60 weeks old, and rather individual animals were tracked for differing periods of time, meaning that they were included in only certain age groups. Due to the variation in the presentation of the phenotype between animals, this could act as a source of these great changes seen between age groups. Further, 67.8% of FAM84B KO eyes that received follow-up SD-OCT scans presented >10% increase in the surface area of retinal degeneration compared to their previous scan. As FAM84B KO retinas presented more degeneration with age, it became increasingly difficult to define retinal layer borders in these areas. We believe that our definition of retinal borders in these highly degenerated areas of KO retinas was completed as accurately as possible, and that these areas should be included in retinal layer thickness measurements to emphasise the occurring dynamic changes. But inevitably, there is a possibility for error within these complex border definitions due to the resolution of SD-OCT.

Although most FAM84B KO eyes presented a phenotype that progressively worsens with age, there was still a great amount of variability in how quickly eyes displayed severe degeneration, and also whether this occurred bilaterally. KO eyes displayed consistent reduction in total retinal thickness compared to wild types (Figure 18B), but the severe degeneration showed no obvious stimulation pattern. At this stage, we cannot rule out that the phenotype is a developmental issue, as KO animals at 6 weeks of age also showed mild degeneration of their retinas. An assessment of embryonic eye development in these KO mice might be a vital to further this project in the future. Nevertheless, Fam84b may function within a mechanism that contributes to the variability in phenotype presentation, such as a stress-combatting response; as the retina is vulnerable to oxidative stress due to high oxygen consumption, and prolonged exposure to light (one aspect of AMD pathology comprehensively reviewed in Bowes Rickman et al., 2013).

During ERG assessments of wild type and FAM84B KO mice, only significant differences were observed at 60 weeks of age. This approach measured the whole-field ERG of wild type and KO mice, meaning that the summed activity of the whole retina is assessed. The detected phenotype has been observed to localise to only parts of the retina, where it generally increases in severity and spreads to adjacent retinal tissue with age. The ERG data achieved coincide with this, as younger animals tend to manifest less severe degeneration, and only at an older age the KO retinas have succumbed to enough degeneration to detect differences in response amplitudes (Figures 28A, 28B, 28C). Before this occurs, the less affected regions of KO retinas appear to function as well as healthy wild type retinal tissue. It would be interesting to measure the electrical responses of selected areas of these KO retinas, in a multi-focal ERG approach. We would then be able to correlate the mildly and severely degenerated regions to their response to light, and deduce the local functionality of these retinal neurones. This would be helpful to realise whether the severely disorganised bipolar, amacrine and ganglion cells seen in FAM84B KO retina immuno-histochemical staining (Figure 25D-L) can still contribute to the eye's responses to light stimuli. The whole-field ERG method used in this work to examine the functionality of mouse retinas is effective for discussing its maximum capacity. However, the vital question would be is how relevant is this in the everyday visual capabilities of these mice? Morphological imaging, and ERG paired with an optometry approach, to determine visual acuity, would be a more complete assessment of visual competency. Data from these combined methodologies would also be more reliably extrapolated to humans as ultimately, our aim is to increase our understanding of retinal homeostasis in order to more effectively manage and treat its pathologies.

6.3 A new angle to study Fam84b activity and function

So far, Fam84b has only received research attention in the context of cancer, where it has been implicated in breast (Adam et al., 2003), oesophageal (Hsu et al., 2015; Huang et al., 2006), and prostate (Jiang et al., 2019; Lin et al., 2017; Wong et al., 2017) malignancies. These reports all described increases in Fam84b expression in cell lines or tissues derived from the stated cancer types. Unfortunately, the mystery surrounding Fam84b's function is yet to be unravelled. Furthermore, we are unable to pinpoint whether its increased expression is; driving tumourigenesis and metastasis in these tissues, a cellular strategy to counteract an already existing cancerous growth, or a by-product of dysregulated gene expression in these tumours. Two of these possibilities have been addressed somewhat. Hsu et al., (2015) displayed that knockdown of Fam84b delayed the growth of ESCC xenograft tumours, which would naturally point the finger at Fam84b as a perpetrator in the progression of these malignancies. Work by Huang et al., (2006) and Wong et al., (2017) investigated the co-amplification of FAM84B and the oncogene MYC at 8q24. Their experiments reported that Fam84b can be specifically overexpressed without MYC amplification, which suggests that Fam84b overexpression in some cancers is not tied to duplication or altered transcription of MYC. Nevertheless, tumour biology is complex, and although the oncogenic potential of MYC is implicated in many cancers, it is not the only tumour-driving process. Our results displayed a reduced level of ERK1 (pY204) in FAM84B KO mouse retinas (Figure 32B), which again proposes Fam84b can influence oncogenic signalling, but until a function for Fam84b is revealed, it will remain unclear whether it is acting for or against tumour progression.

The focus of this thesis work was the retinal degeneration detected in FAM84B KO mice, and our results show that Fam84b is highly attributed to retinal pathology in C57Bl/6NCrl mice. To our knowledge this is the first FAM84B KO mouse model to be studied, and the first time Fam84b has been linked to a role in retinal homeostasis. This work will offer a different angle to study this uncharacterised protein. Figures 29A and 29B show that Fam84b is expressed in the retinas of wild type mice, more specifically localising to the GCL, OPL and photoreceptor/RPE layers, and its absence leads to a degenerative retinal phenotype. Yet, Fam84b's function is still unknown.

6.4 Fam84b protein structure and function considerations

Fam84b's primary sequence aligns with the catalytic domain of HRALS family of enzymes, and also shares homology with LRAT (Jiang et al., 2019). The latter of these plays an important role in recycling visual chromophores to continue vision, and its homology to Fam84b may partly explain a retinal phenotype in FAM84B KO mice. Mutations in LRAT lead to reduced levels of functional pigment, night-blindness and morphological abnormalities in the RPE and photoreceptor layers (Dev Borman et al., 2012). The HRALS and LRAT enzymes utilise a Cys-His-His catalytic triad (Jiang et al., 2019; Shinohara et al., 2011) in order to carry out their function, but we must assume that Fam84b's secondary and tertiary structure would fold in a way for these residues to meet and create the proposed Ser-His-His catalytic triad. The crystal structures of HRALS and LRAT are available, but not for Fam84b yet. It's possible that the homologous domain folds in an altered conformation to give rise to a different function and binding partners for Fam84b. It is also conceivable that the cysteine residue present seven amino acids downstream of

Fam84b's proposed catalytic serine at S202 could create a Cys-His-His catalytic triad that exists in these LRAT-related enzymes. Nevertheless, Fam84b was identified to not possess PLA/AT activity (Shinohara et al., 2011), which interferes with this suggestion. Wang et al., (2014) observed the HRASLS3-mediated acylation and reduction of Ras-GTP and its downstream effectors. We observed a reduced level of ERK1 (pY204) in FAM84B KO mouse retinal lysates (Figure 32B) when investigating the potential effect of Fam84b on the Ras-ERK signalling pathway in the rodent retina. This presents a promising association for Fam84b functionality on Ras activity too, and suggests Fam84b could act to oppose HRASLS3 function, possibly as a competitive inhibitor. However, we observed no real changes in the levels of active Raf-1 (p338) and MEK1 (p298) between wild type and FAM84B KO retinal lysates; working against this theory as if Fam84b acts on Ras directly, we may expect to see changes in the levels of all downstream Ras effectors. These results instead suggest that Fam84b activity could affect MEK1 activity, rendering it unable to phosphorylate and activate ERK1, or it possesses a mechanism that reduces ERK1 activity via some cross-talk mechanism. These western blot analyses were performed in triplicate, giving rise to technical replicates as the same retinal lysates were tested for the amount of downstream Ras effectors. It would also be essential to also provide biological replicates, to confirm that the reduced levels of active pERK1 in KO lysates is also present in subsequent FAM84B KO animals.

ERK1/2 activity has a strong impact on the regulation of retinal function, as it has been previously shown that conditional KO of ERK1 and ERK2 in mouse RPE cells leads to; thinning of the ONL/INL, reduced ERG response amplitudes, localised structural damage in the retina, and a loss of photoreceptor cell markers (Pyakurel et al., 2017). All of these characteristics are observed in FAM84B KO retinas (Figures 19B, 28, 21D, 25C, respectively) likely as a result of the detected decreased activity of ERK1 (Figure 32B). Further, the work by Pyakurel et al., (2017) expresses evidence for ERK1/2's ability to regulate the expression of RPE65, another protein that is irregularly expressed in FAM84B KO mice (Figures 25N, 25O).

We revealed that the h-TERT RPE1 cell line expresses Fam84b (Figure 29A), an interesting future experiment may be to knockdown Fam84b in these cells using RNAi, and stimulate the cell line with growth factors in order to activate the Ras-ERK pathway. With this approach, we may be able to monitor the pathway's activity more closely as a direct result of Fam84b absence, removing any unknown effects resulting from the current *in vivo* approach. If I were to repeat these experiments, I would also include detection of the unphosphorylated downstream Ras kinases for comparison.

6.5 Fam84b may provide a link between MAPK pathways

We implemented co-immunoprecipitation of Fam84b in order to discover its interacting protein partners. This revealed a specific interaction with GRASP-1, aka. GRIPAP1, which was detected as attached to anti-Fam84b fused beads via mass spectrometry. Although the co-immunoprecipitation experiments were still undergoing optimisation at the current time of writing this thesis work, an interaction between Fam84b and GRIPAP1 might neatly explain the retinal degeneration, diminishing responses to light and reduced levels of active ERK1 observed in FAM84B KO retinas. GRIPAP1 is a neuronally-enriched scaffold protein for the JNK signalling pathway following its cleavage by caspase-3, and possesses the ability to interact with the second tier MAP2Ks MEKK1 and JNK1, leading to their activation (Ye et al., 2007). Its N-terminus also expresses homology to the catalytic domain of GEFs for the Ras family of GTPases, in which GRIPAP1

was demonstrated to possess RasGEF activity in vitro (Ye et al., 2000). This information elegantly links a function for Fam84b in a MAPK signalling pathway downstream of Ras, and also places it operating in a neural tissue such as the retina.

JNK pathways are explicitly linked to cellular stress, and are activated in cells experiencing infection, inflammation, oxidative stress, DNA damage or osmotic stress. The transcription of many genes involved in stress-adaptation or directing the cell towards an apoptotic fate is the outcome of JNK pathways, in a contextual manner. Zeke et al., (2016) provides a comprehensive review of JNK pathway mechanics, and their numerous influences in cellular homeostasis. The impact of JNK pathways in retinal disease, however, is one of particular interest. Du et al., (2012) showed that JNK1 activity is enhanced in a laser-induced AMD mouse model. Here, following disruption of Bruch's membrane c-jun phosphorylation is increased in tissue presenting choroidal neovascularisation. In the same study, JNK1 deficient mice displayed fewer markers of oxidative stress, reduced VEGF expression and reduced inflammatory cell infiltration after photocoagulation, when compared to mice expressing JNK1. Additionally, the presence of apoptotic cells in the retina was significantly lower in the JNK1 deficient AMD mouse models, suggesting that the activation of JNK pathways is a substantial driver of the symptoms presented in an AMD phenotype. Dysregulation of MAPKs is associated with AMD, with their activation following UV-induced damage, increased hydrogen peroxide concentration or decreased autophagy efficiency with ageing, where persistent activation of ERK1/2 or JNK pathways leads to retinal cell death (Kyosseva, 2016).

There is considerable crosstalk between MAPK pathways. ERK is able to trigger the suppression of JNK activity through dephosphorylation by DUSP4/16. JNK can retaliate, by inducing DUSP2 to dephosphorylate ERK and p38, and even enforce a positive feedback loop by phosphorylating its own MAP3Ks, MLK and ASK (Fey et al., 2012). Disruption of these pathways may lead to further chaos attributed to these crosstalk mechanisms and affect the activity of apoptotic factors such as BIM_{EL}, whose potency has been shown to be controlled by both ERK1/2 and JNK1 activity, in not only retinal development, but degeneration too (Donovan, Doonan and Cotter, 2010). As FAM84B KO mice display reduced activation of ERK1 in their retinas (Figure 32B), it would be surely worth delving into whether this reduction in ERK1 activity is due to a crosstalk mechanism dependent on Fam84b's interaction with GRIPAP1 in the future. This is conceivable, as we detected no significant changes in the phosphorylation status of Raf-1 and MEK1 between wild type and FAM84B KO retinas (Figure 32B).

Although this work has yet to tie down a function for Fam84b, its homology to LRAT could also spring a potential link with JNK signalling. Inefficient clearance or conversion of all-*trans* retinal can prove toxic for photoreceptors. Liao et al., (2020) recently overexposed a mouse-derived photoreceptor cell line to all-*trans* retinal, and revealed increased JNK activity, leading to their apoptosis through heightened levels of Bak and cytoplasmic cytochrome c. KO of JNK1 and JNK2 alleviated this effect. Subsequently, we could still ponder over whether Fam84b may yet still play a role in ocular retinoid management like its cousin LRAT, where FAM84B KO retinas accumulate all-*trans* retinal, leading to JNK-mediated apoptosis of photoreceptors, followed by the suppression of ERK1 activity.

MAPK dysregulation does not only apply to pathologies in the outer retina such as AMD, as the activity of JNK2/3 has also been implicated in mouse models of glaucoma, in which ganglion cells and the optic nerve undergo an apoptotic fate instead (Fernandes et al., 2012). In addition, the JNK pathway also serves a role in retinal development, as a lack of either of the MAP2Ks, MKK4 or MKK7, leads to a decreased number of ganglion cells in the adult mouse retina, alongside some minor retinal structural changes. Despite this,

added protection from optic nerve injury is observed. Double KO of MKK4 and MKK7 was shown to lead to retinal dysgenesis, displaying morphology such as optic nerve hypoplasia (Syc-Mazurek et al., 2019), reiterating the importance of the JNK pathways in retinal development and degeneration. Nevertheless, as FAM84B KO mice do not seem to present any pathologies in the GCL or morphology of the optic nerve, Fam84b may interact with GRIPAP1 in a complex that affects JNK1 activity only.

Care must be taken with our co-immunoprecipitation results however, as this method has only been performed once in this way so far, and should be confirmed in triplicate to fully establish a true interaction between Fam84b and GRIPAP1. Additionally, Fam84b was not detected in the bead complex, nor in the excised SDS-PAGE (Figure 33B) bands during mass spectrometry analysis, which suggests that Fam84b was not co-immunoprecipitated at all. This is inconsistent with the western blot detection of Fam84b in the same immunoprecipitated samples, which revealed a band corresponding the 42kDa molecular weight of Fam84b (Figure 33A) in hTERT-RPE1 cell lysates, the eluted anti-Fam84b bound material, and in the corresponding flow through samples. Yet, it is clear that these co-immunoprecipitation experiments will need to be optimised and completed before robust claims for new interactions with Fam84b are made.

In the meantime, we can discuss other proteins that Fam84b has been reported to interact with in the literature. The pioneering Adam et al., (2003) identified specific binding between Fam84b and α -catenin, a protein known for its importance in regulating cell-cell adhesion. With our results showing that FAM84B KO retinas possess retinal abnormalities, this interaction has shown relevance since mutations in the CTNNA1 gene lead to RPE dysmorphology and ONL thinning in humans and *Ctnna1^{tvrm5}* mice. Histological sections of *Ctnna1^{tvrm5}* retinas also reveal pigment displacement and duplication around the RPE and photoreceptor regions (Saksens et al., 2015), presenting similarities to our FAM84B KO retinal sections. Mutations in CTNNA1 and FAM84B KO mice could represent disruption of the same retinal homeostasis mechanism, leading to reduced cell-cell adhesion and neuronal disorganisation. This is intriguing, as α -catenin not only links adherens junctions to the cytoskeleton, but also possesses a link to Ras-ERK activation, where keratinocytes devoid of α -catenin experience sustained Ras-ERK signalling (Vasioukhin et al., 2001).

6.6 Review of thesis aims

The first aim of this thesis work was to fully characterise the retinal phenotype unveiled in FAM84B KO mouse retinas. SD-OCT retinal cross sections obtained at numerous animal ages were extensively evaluated and tracked for dynamic retinal alterations. This was paired with histological sectioning and immunostaining in order to assess the morphology and differences in neurone architecture of wild type and FAM84B KO mice. Our best efforts to compare and contrast these observed retinal features within the scope of this work was also made with studies of known retinal diseases from the literature. Retinal function was also assessed via ERG, with the overall conclusion that FAM84B KO mice display a degenerative phenotype in relation to morphology and light sensitivity, which worsens with ageing.

The next aim was to determine the expression of Fam84b within retinal tissue. To our knowledge, this is the first study focusing on Fam84b's role within the retina, and our immuno-staining and *in situ* hybridisation experiments were designed to fulfil this aim. Our immuno-staining results show promise, and were able to specifically place the Fam84b expression in particular layers of the neural retina, and in

specific structures surrounding the eye. However, it would be interesting to further determine the precise cells or neurones that are expressing Fam84b, as it would possibly shed more light on this protein's function.

The third aim was to find clues about the function of Fam84b by discovering its interacting protein partners. Co-immunoprecipitation was utilised for this, and although these experiments were not yet fully optimised, a potential interaction with GRIPAP1 proposes the start of a viable and interesting story associated with Fam84b's role within the retina. Future experiments on this project will begin with refining and improving the work started in this thesis project.

The final aim was to analyse the effect of FAM84B KO on a Ras-MAPK signalling pathway in the retina. Phosphorylated kinase effectors downstream of Ras were detected, quantified and compared between retinal lysates from wild type and FAM84B KO mice. Ras acts as signalling node on the plasma membrane of eukaryotic cells and can influence many cell fates. This thesis work only analysed one branch of Ras effectors, but did manage to discover alterations in the activity of ERK1, a kinase that has been shown previously to play a part in retinal development and degeneration. Further, the fact that the phosphorylation status of effectors upstream of ERK1 are comparable between mice with and without Fam84b expression suggests that Fam84b's function may not directly elicit its effect on the Ras-ERK pathway specifically, but possibly in some crosstalk or feedback mechanism. Analysis of the activity of other important MAPK pathways, such as the JNK pathway, would be important to get the bottom of the mystery surrounding Fam84b's role in retinal homeostasis.

6.7 Review of thesis hypotheses

The first hypothesis tested was whether KO of FAM84B leads to retinal degeneration in C57Bl/6NCrl mice. This work began with the standardised phenotype screening for the worldwide IMPC project. From these initial tests, it was clear that Fam84b must play some part in the efficient functioning of the retina. As we developed and carried out further experiments with a range of animals at differing ages, a strong correlation between the absence of Fam84b and presence of retinal degeneration was apparent. It's true that Fam84b must not be essential for retinal function, as KO retinas seem to behave close to that of wild types during their youth, but start to suffer from retinal damage and reduced retinal efficiency later in life. Taken together, we would accept this hypothesis, KO FAM84B mouse retinas do experience retinal degeneration.

The second hypothesis investigated was whether Fam84b reduces Ras-associated signalling in the normal retina. This hypothesis was devised based upon Fam84b's homology to the HRASLS family, which have been shown to reduce Ras-associated signalling. Our results have displayed that FAM84B KO mouse retinas actually show a reduction in ERK1 activity, but no real difference in Raf-1 or MEK1 activity. As mentioned previously, this work only measured a small part of the vast potential of Ras-associated signalling, but from the results obtained here we would reject this hypothesis. This is because Fam84b absence delivered a different effect than expected in retinal tissue. Nevertheless, from these experiments we could not decipher whether Fam84b may have conflicting effect on another aspect of Ras-associated signalling, and further investigation would be needed in this area.

7 CONCLUSION

This thesis work has presented a FAM84B KO mouse model and analysed the resulting retinal phenotype, which shares similarities to retinal pathologies such as AMD and RP. However, it's still unclear where Fam84b fits into the normal functioning of the retina. The results of this thesis have displayed that Fam84b's absence disrupts retinal morphology and function, and disturbs Ras-ERK signalling in the C57Bl/6NCrl mouse retina. This could be through a mechanism related to Fam84b's homology to HRASLS or LRAT, or its potential ability to link MAPK pathways. MAPK signalling is a simple three-tier cascade on the surface, but its complex regulation is yet to be unravelled in full and Fam84b may very well play a decisive role within this field. More work is still to be done regarding this elusive protein, yet here on out, new strategies to study it can now be implemented via its role in retinal homeostasis.

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9 SUPPLEMENTARY FIGURES

ID	Sex	FAM84B Genotype	Age (weeks)	Eye	Area (mm ²)								Total (mm ²)	RNFL/GCL deformation	Damage Category
					#1	#2	#3	#4	#5	#6	#7	#8			
64S-B7583	m	hom	42	R	3.89	0.42							4.31	yes	severe
64S-B7583	m	hom	42	L	0.31	0.07	0.36						0.74	no	mild
64U-F1575	f	hom	36	R	0.12	0.09	0.12						0.33	no	mild
64U-F1575	f	hom	36	L	2.38								2.38	yes	severe
64U-F1575	f	hom	42	R	0.02	0.04	0.14	0.18					0.38	no	mild
64U-F1575	f	hom	42	L	2.7	0.04							2.74	yes	severe
64U-F1575	f	hom	48	R	0.03	0.01	0.13	0.23					0.4	no	mild
64U-F1575	f	hom	48	L	0.02	2.96							2.98	yes	severe
64U-F1575	f	hom	54	R	0.11	0.16	0.2						0.47	no	mild
64U-F1575	f	hom	54	L	0.05	2.8							2.85	yes	severe
64U-F1575	f	hom	60	R	0.06	0.12	0.19						0.37	no	mild
64U-F1575	f	hom	60	L	3.26								3.26	yes	severe
64U-71441	m	hom	36	R	0.3	0.26							0.56	no	mild
64U-71441	m	hom	36	L	9.26								9.26	yes	severe
64U-71443	f	hom	36	R	0.37								0.37	yes	moderate
64U-71443	f	hom	36	L	1.1								1.1	yes	moderate
64U-71443	f	hom	42	R	0.51								0.51	yes	moderate
64U-71443	f	hom	42	L	1.38								1.38	yes	moderate
64U-71443	f	hom	48	R	0.81								0.81	yes	moderate
64U-71443	f	hom	48	L	2.07								2.07	yes	severe
64U-71443	f	hom	54	R	0.95	0.03							0.98	yes	moderate
64U-71443	f	hom	54	L	1.98								1.98	yes	severe
64U-71443	f	hom	60	R	1.02	0.06							1.08	yes	moderate
64U-71443	f	hom	60	L	2.66								2.66	yes	severe
64U-71444	f	hom	36	R	0.7								0.7	yes	moderate
64U-71444	f	hom	36	L	0.37								0.37	yes	moderate
64U-71444	f	hom	42	R	0.77								0.77	yes	moderate
64U-71444	f	hom	42	L	0.6								0.6	yes	moderate
64U-71444	f	hom	48	R	0.75								0.75	yes	moderate
64U-71444	f	hom	48	L	0.77								0.77	yes	moderate
64U-71444	f	hom	54	R	0.82								0.82	yes	moderate
64U-71444	f	hom	54	L	0.72								0.72	yes	moderate
64U-71444	f	hom	60	R	1.13								1.13	yes	moderate
64U-71444	f	hom	60	L	1.28								1.28	yes	moderate
64U-68904	m	hom	36	R	0.2	0.27							0.47	no	mild
64U-68904	m	hom	36	L	0.08	0.2	0.38	0.25					0.91	no	mild
64U-68904	m	hom	42	R	0.14	0.2							0.34	no	mild
64U-68904	m	hom	42	L	0.12	0.21	0.47	0.26					1.06	no	mild
64U-68904	m	hom	48	R	0.1	0.28							0.38	no	mild
64U-68904	m	hom	48	L	0.17	0.93	0.2						1.3	yes	moderate
64U-68904	m	hom	54	R	0.18	0.33							0.51	no	mild
64U-68904	m	hom	54	L	1.81	0.08							1.89	yes	severe
64U-68904	m	hom	60	R	0.11	0.32							0.43	no	mild
64U-68904	m	hom	60	L	3.12								3.12	yes	severe
64U-68906	m	hom	36	R	2.83	0.08	0.13						3.04	yes	severe
64U-68906	m	hom	36	L	0.22	0.2	0.14						0.56	no	mild
64U-68906	m	hom	42	R	4.46	0.11							4.57	yes	severe
64U-68906	m	hom	42	L	0.26	0.21	0.19						0.66	no	mild
64U-68906	m	hom	48	R	5.25	0.18							5.43	yes	severe
64U-68906	m	hom	48	L	0.23	0.19	0.12						0.54	no	mild
64U-68906	m	hom	54	R	5.05	0.19	0.06	0.06					5.36	yes	severe
64U-68906	m	hom	54	L	0.28	0.2	0.15						0.63	no	mild
64U-68906	m	hom	60	R	6.03	0.31	0.06	0.02					6.42	yes	severe
64U-68906	m	hom	60	L	0.23	0.26	0.14						0.63	no	mild
64U-H5026	m	hom	48	R	0.19	0.13							0.32	no	mild
64U-H5026	m	hom	48	L	0.21	0.24							0.45	no	mild
64U-H5026	m	hom	54	R	0.2	0.17							0.37	no	mild
64U-H5026	m	hom	54	L	0.21	0.22							0.43	no	mild
64U-H5026	m	hom	60	R	0.31	0.2	0.14						0.65	no	mild
64U-H5026	m	hom	60	L	0.18	0.32	0.21						0.71	no	mild
83S-E5724	m	hom	30	R	0.04	0.06	0.15						0.25	no	mild
83S-E5724	m	hom	30	L	0.03	0.11	0.13	0.84					1.11	yes	moderate
83S-E5724	m	hom	36	R	0.09	0.18							0.27	no	mild

83S-E5724	m	hom	36	L	0.04	0.12	1.25						1.41	yes	moderate
83S-E5724	m	hom	42	R	0.07	0.08	0.14						0.29	no	mild
83S-E5724	m	hom	42	L	0.06	0.15	1.56						1.77	yes	severe
83S-E5724	m	hom	48	R	0.07	0.15	0.15						0.37	no	mild
83S-E5724	m	hom	48	L	0.07	0.25	1.78						2.1	yes	severe
83S-E5729	f	hom	30	R	1.55	0.11							1.66	yes	severe
83S-E5729	f	hom	30	L	0.19								0.19	no	mild
83S-E5729	f	hom	36	R	2.37								2.37	yes	severe
83S-E5729	f	hom	36	L	0.31	0.05							0.36	no	mild
83S-E5729	f	hom	42	R	2.46								2.46	yes	severe
83S-E5729	f	hom	42	L	0.37	0.02	0.12						0.51	no	mild
83S-E5729	f	hom	48	R	2.74								2.74	yes	severe
83S-E5729	f	hom	48	L	0.34	0.05	0.14						0.53	no	mild
83S-E6551	f	hom	18	L	0.03	0.09							0.12	no	mild
83S-E6551	f	hom	24	L	0.03	0.08							0.11	no	mild
83S-E6551	f	hom	30	L	0.03	0.09	0.05						0.17	no	mild
83S-E6551	f	hom	36	L	0.11	0.09							0.2	no	mild
83S-E6551	f	hom	42	L	0.05	0.04	0.12						0.21	no	mild
83S-E6551	f	hom	48	L	0.21								0.21	no	mild
83S-G9053	m	hom	6	R	0.55	0.13							0.68	no	mild
83S-G9053	m	hom	6	L	0.04	0.09	0.31						0.44	no	mild
83S-G9053	m	hom	12	R	0.7								0.7	yes	moderate
83S-G9053	m	hom	12	L	0.04	0.04	0.07	0.8					0.95	yes	moderate
83S-G9053	m	hom	18	R	1.33	0.69	0.05						2.07	yes	severe
83S-G9053	m	hom	18	L	0.06	0.12	0.08	0.95					1.21	yes	moderate
83S-G9053	m	hom	24	R	3.02								3.02	yes	severe
83S-G9053	m	hom	24	L	0.03	0.12	0.09	1.2					1.44	yes	moderate
83S-G9053	m	hom	30	R	3.77								3.77	yes	severe
83S-G9053	m	hom	30	L	0.15	0.17	2.35						2.67	yes	severe
83S-H5847	m	hom	6	R	0.16	0.09							0.25	no	mild
83S-H5847	m	hom	6	L	0.19	0.14	0.2						0.53	no	mild
83S-H5847	m	hom	12	R	0.23	0.07							0.3	no	mild
83S-H5847	m	hom	12	L	0.18	0.13	0.22						0.53	no	mild
83S-H6122	m	hom	6	R	0.3								0.3	no	mild
83S-H6122	m	hom	6	L	0.1	0.07	0.09						0.26	no	mild
83S-H6986	f	hom	6	R	0.04	0.11	0.07	0.09	0.04	0.16	0.11		0.62	no	mild
83S-H6986	f	hom	6	L	0.09	0.13	0.16	0.13	0.1				0.61	no	mild
83S-H6986	f	hom	12	R	0.14	0.16	0.05	0.13	0.05	0.19	0.06		0.78	no	mild
83S-H6986	f	hom	12	L	0.08	0.15	0.09	0.16	0.18	0.13			0.79	no	mild
83S-H6986	f	hom	18	R	0.18	0.16	0.07	0.16	0.12	0.04	0.09	0.24	1.06	no	mild
83S-H6986	f	hom	18	L	0.08	0.13	0.14	0.19	0.19	0.18			0.91	no	mild
83S-H6986	f	hom	24	R	0.15	0.06	0.16	0.21	0.04	0.3	0.11	0.04	1.07	no	mild
83S-H6986	f	hom	24	L	0.17	0.23	0.17	0.28	0.27	0.29			1.41	no	mild
83S-H6986	f	hom	30	R	0.27	0.22	0.05	0.23	0.25	0.07	0.35	0.18	1.62	no	moderate
83S-H6986	f	hom	30	L	0.07	0.22	0.24	0.29	0.13	0.14			1.09	no	mild
83S-H6985	m	hom	18	R	0.39	0.16							0.55	no	mild
83S-H6985	m	hom	18	L	0.34	0.3	0.24						0.88	no	mild
83S-H6985	m	hom	24	R	0.53	0.15							0.68	no	mild
83S-H6985	m	hom	24	L	0.79	0.24							1.03	no	mild
83S-H6985	m	hom	30	R	0.12	0.35	0.15						0.62	no	mild
83S-H6985	m	hom	30	L	0.87	0.31	0.31						1.49	no	mild
83S-I6264	m	hom	30	R	0.49	0.31	0.17						0.97	no	mild
83S-I6264	m	hom	30	L	1.71	0.16	0.21						2.08	yes	severe
83S-H7579	f	hom	30	R	0.21	0.33	4.42	0.68					5.64	yes	severe
83S-H7579	f	hom	30	L	0.2	1.36							1.56	yes	severe
83S-I7900	m	hom	18	R	0.09	0.22	0.15	0.1					0.56	no	mild
83S-I7900	m	hom	18	L	0.29	0.15	1.24						1.68	yes	severe
83S-I9538	m	hom	18	R	0.36	0.21	0.65	0.06	0.04				1.32	yes	moderate
83S-I9538	m	hom	18	L	0.16	0.32	0.05						0.53	no	mild
83S-I9539	f	hom	18	R	1.74	0.08	0.18						2	yes	severe
83S-I9539	f	hom	18	L	0.09	1.41							1.5	yes	moderate
83S-I9292	f	hom	18	L	0.22	0.21							0.43	no	mild

Supplementary Table 1: Retinal damage categorisation of FAM84B KO mice. All animals measured were assessed for retinal damage by viewing cross-sectional b-scans of wild type and FAM84B KO C57Bl/6NCRl mouse retinas. Borders of the areas of retinal damage were defined and marked on the corresponding fundus image, as seen in Figure 16. The area of retinal damage was calculated automatically by the built-in software. If multiple areas of damage were present, surface areas were added together. This was achieved by utilising HRA+OCT Spectralis® PLUS (Heidelberg Engineering, Germany). No wild type mice showed any sign of the retinal dysmorphology described in this thesis work and consequently, do not feature in this table. Each retina was given a severity grade (Mild, Moderate or Severe), which is defined in Table 4.

Age (weeks)	FAM84B Genotype	Retinal Thickness (μm)		
		Total	Outer	Inner
12	wt	257.96 $\pm$ 5.62	77.35 $\pm$ 1.77	180.61 $\pm$ 4.53
	ko	222.30 $\pm$ 10.36	69.95 $\pm$ 4.48	152.35 $\pm$ 9.76
24	wt	260.57 $\pm$ 4.25	78.15 $\pm$ 1.44	182.42 $\pm$ 3.78
	ko	210.49 $\pm$ 16.87	61.98 $\pm$ 6.28	148.51 $\pm$ 10.95
36	wt	261.66 $\pm$ 2.28	79.61 $\pm$ 1.10	182.04 $\pm$ 2.33
	ko	222.44 $\pm$ 9.27	68.40 $\pm$ 5.13	154.04 $\pm$ 10.36
48	wt	258.68 $\pm$ 2.17	78.32 $\pm$ 1.74	180.36 $\pm$ 1.25
	ko	223.05 $\pm$ 4.69	67.12 $\pm$ 4.49	155.93 $\pm$ 5.37
60	wt	256.31 $\pm$ 3.44	79.30 $\pm$ 1.26	177.00 $\pm$ 3.87
	ko	224.69 $\pm$ 6.86	72.91 $\pm$ 2.68	151.78 $\pm$ 5.94

Supplementary Table 2: Mean retinal thicknesses for each age group. The total, outer and inner retinal thickness was determined for each mouse retina using methods described previously in section 4.3.2. This table shows mean $\pm$ standard deviation layer thickness for each measured age group of wild type and FAM84B KO mice. The total retinal thickness (Total) was defined as the distance from the inner limiting membrane to the outer boundary of the retinal pigmented epithelium. The inner retinal thickness (Inner) was defined as the distance from the inner limiting membrane to the outer boundary of the outer nuclear layer (ONL). The outer retinal thickness (Outer) was defined as the distance from the outer boundary of the outer nuclear layer to outer boundary of the retinal pigmented epithelium (RPE).

Age (weeks)	FAM84B Genotype	Retinal Layer Thickness (μm)									
		RNFL	GCL	IPL	INL	OPL	ONL	Inner PR	Outer PR	Subret. Space	RPE
12	wt	23.17 $\pm$ 1.16	20.99 $\pm$ 3.10	27.06 $\pm$ 2.89	21.24 $\pm$ 2.40	20.01 $\pm$ 2.27	68.13 $\pm$ 2.97	13.41 $\pm$ 0.45	16.16 $\pm$ 2.28	26.70 $\pm$ 2.68	21.09 $\pm$ 1.19
	ko	22.57 $\pm$ 2.81	19.65 $\pm$ 0.82	23.64 $\pm$ 1.69	20.32 $\pm$ 0.96	18.95 $\pm$ 2.54	47.21 $\pm$ 8.78	13.05 $\pm$ 2.01	14.53 $\pm$ 1.12	22.83 $\pm$ 5.30	19.55 $\pm$ 1.71
24	wt	23.74 $\pm$ 1.94	24.59 $\pm$ 2.36	25.92 $\pm$ 1.55	21.44 $\pm$ 1.50	18.86 $\pm$ 0.96	67.86 $\pm$ 2.45	13.47 $\pm$ 0.83	17.86 $\pm$ 1.04	24.96 $\pm$ 2.35	21.84 $\pm$ 0.85
	ko	23.27 $\pm$ 2.08	19.17 $\pm$ 1.86	24.39 $\pm$ 1.69	19.44 $\pm$ 0.97	18.62 $\pm$ 4.09	43.61 $\pm$ 8.65	15.98 $\pm$ 3.24	12.10 $\pm$ 1.79	16.38 $\pm$ 3.30	17.52 $\pm$ 1.61
36	wt	25.71 $\pm$ 2.77	20.95 $\pm$ 2.46	29.20 $\pm$ 2.97	19.88 $\pm$ 1.64	19.40 $\pm$ 1.48	66.90 $\pm$ 0.97	13.81 $\pm$ 2.79	18.38 $\pm$ 1.65	29.53 $\pm$ 3.74	17.90 $\pm$ 2.00
	ko	25.01 $\pm$ 2.27	18.44 $\pm$ 2.60	22.95 $\pm$ 1.12	18.31 $\pm$ 1.36	17.64 $\pm$ 1.44	51.70 $\pm$ 6.39	16.70 $\pm$ 3.56	14.55 $\pm$ 3.24	18.51 $\pm$ 2.68	18.64 $\pm$ 1.90
48	wt	25.74 $\pm$ 2.39	22.53 $\pm$ 3.20	27.63 $\pm$ 2.15	20.74 $\pm$ 1.79	18.84 $\pm$ 1.43	64.88 $\pm$ 1.20	12.63 $\pm$ 1.55	18.12 $\pm$ 1.83	27.09 $\pm$ 3.51	20.47 $\pm$ 2.61
	ko	23.31 $\pm$ 2.67	19.44 $\pm$ 1.66	23.52 $\pm$ 0.55	18.22 $\pm$ 1.08	17.24 $\pm$ 0.91	54.21 $\pm$ 3.35	17.27 $\pm$ 2.70	13.13 $\pm$ 3.60	19.44 $\pm$ 3.49	17.28 $\pm$ 1.35
60	wt	25.65 $\pm$ 1.70	24.66 $\pm$ 1.36	24.96 $\pm$ 1.00	21.02 $\pm$ 1.20	17.68 $\pm$ 0.52	63.04 $\pm$ 1.76	15.70 $\pm$ 3.42	17.35 $\pm$ 2.29	26.08 $\pm$ 4.51	20.17 $\pm$ 1.66
	ko	24.94 $\pm$ 2.51	18.93 $\pm$ 1.83	23.27 $\pm$ 1.05	18.50 $\pm$ 0.83	17.54 $\pm$ 0.99	48.61 $\pm$ 2.93	19.34 $\pm$ 1.60	16.39 $\pm$ 1.41	20.40 $\pm$ 2.62	16.78 $\pm$ 1.16

Supplementary Table 3: Mean retinal layer thicknesses for each age group. The thickness of each retinal layer was determined for each retina using methods described previously in section 4.3.2. This table shows mean $\pm$ standard deviation layer thickness for each measured age group of wild type and FAM84B KO mice. RNFL, retinal nerve fibre layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; Inner PR, photoreceptor inner segments, Outer PR, photoreceptor outer segments.