

1 **Title: The zebrafish eye – a paradigm for investigating human ocular genetics**

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29 **Abstract**

30 Although human epidemiological and genetic studies are essential to elucidate the aetiology  
31 of normal and aberrant ocular development, animal models have provided us with an  
32 understanding of the pathogenesis of multiple developmental ocular malformations.  
33 Zebrafish eye development displays in depth molecular complexity and stringent  
34 spatiotemporal regulation that incorporates developmental contributions of the surface  
35 ectoderm, neuroectoderm and head mesenchyme, similar to that seen in humans. For this  
36 reason, and due to its genetic tractability, external fertilisation and early optical clarity, the  
37 zebrafish has become an invaluable vertebrate system to investigate human ocular  
38 development and disease.

39

40 Recently, zebrafish have been at the leading edge of preclinical therapy development, with  
41 their amenability to genetic manipulation facilitating the generation of robust ocular disease  
42 models required for large-scale genetic and drug screening programmes. This review  
43 presents an overview of human and zebrafish ocular development, genetic methodologies  
44 employed for zebrafish mutagenesis, relevant models of ocular disease, and finally  
45 therapeutic approaches, which may have translational leads in the future.

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## 57 **Zebrafish as a model organism**

58 The zebrafish (*Danio rerio*) has become an increasingly popular model organism for the  
59 study of genetic mechanisms of vertebrate development and disease. Zebrafish are easy to  
60 maintain and breed in large numbers at a low cost. They have a short generation time of 2-4  
61 months, with a single mating pair producing large clutches of fertilized eggs (~100-200) at  
62 weekly intervals. Fertilization is *ex utero* and the developing embryo is transparent facilitating  
63 easy visualisation of early organogenesis and amenability to embryological manipulation.

64

65 Seventy percent of human genes have at least one zebrafish orthologue, with 84% of known  
66 human disease-causing genes having a zebrafish counterpart.<sup>1</sup> In fact, zebrafish frequently  
67 have two orthologues of mammalian genes which map in duplicated chromosomal segments  
68 as a consequence of an additional round of whole genome duplication. The most likely fate  
69 of a duplicate gene is loss-of-function, although both copies can be retained and  
70 subfunctionalisation or neofunctionalisation can occur. Despite genome duplication,  
71 zebrafish have a similar number of chromosomes to humans (25 and 23, respectively), many  
72 of which are mosaically orthologous. These factors, in addition to the genetic versatility of  
73 the zebrafish, make it a prominent model organism for systematic mutational approaches in  
74 the study of human disease.

75

## 76 **Advantages of the zebrafish model pertaining to the eye**

77 The eyes of the zebrafish are large relative to the overall size of the fish, making eye bud  
78 manipulation feasible during early embryogenesis. Zebrafish are visually responsive by 72  
79 hours post fertilisation (hpf) by which time the retina resembles adult retinal morphology that  
80 is anatomically and functionally similar to humans (Fig1). The zebrafish retinal architecture  
81 possesses photoreceptor subtypes spatially arranged in a highly organised heterotypical  
82 photoreceptor mosaic, and, due to the diurnal nature of zebrafish, it is cone-rich akin to the  
83 human macula resulting in colour vision with a cone density close to humans.

84

85 Zebrafish behaviour is an invaluable tool for assaying visual function. Zebrafish alter their  
86 skin pigmentation when exposed to different light-intensities by expanding or contracting  
87 melanosomes; if a fish has impaired vision, it perceives itself to be in an environment with  
88 low light intensity, therefore appearing hyper-pigmented. More specific visual assays take  
89 advantage of visual reflexes such as the optokinetic or startle response, and an ability to  
90 monitor visual response when varying examination conditions.

91

## 92 **Zebrafish eye morphogenesis**

93 Ocular development in zebrafish closely resembles that of humans and other vertebrates  
94 (Fig2).<sup>2,3</sup> Both develop from three distinct embryological tissues, neuroectoderm which gives  
95 rise to the neural retina, retinal pigment epithelium, optic stalk, iris dilator and sphincter  
96 muscles, and ciliary body; surface ectoderm, which forms the lens and subsequently the  
97 conjunctival and corneal epithelia; and mesenchyme which originates from the neural crest  
98 cells forming the corneal endothelium and stroma, iris stroma, ciliary muscles and  
99 vasculature and sclera.

100

101 The formation of optic sulci as small grooves on either side of the developing forebrain by  
102 day 22 of gestation marks the establishment of rudimentary ocular development in human  
103 embryogenesis.<sup>4</sup> During the following week of gestation, evagination of the optic sulci leads  
104 to formation of optic pits, which deepen to form optic vesicles.<sup>4</sup> A key difference in zebrafish  
105 development is that the neural tube develops as a solid mass of cells referred to as the  
106 neural keel.<sup>2</sup> Consequently, optic primordia evaginate from the neural keel as a dense  
107 neuroepithelial cellular mass. The optic lumina form from cavitation within the optic  
108 primordia, and these spaces expand to become continuous with the ventricles of the neural  
109 keel by 14 hpf (12 somite stage). Similar to human ocular development, the zebrafish optic  
110 vesicle then undergoes a series of morphogenetic movements between 16-20 hpf giving rise  
111 to a two-layered optic cup composed of retinal neuroepithelium and pigmented epithelium  
112 (Fig2a,e).<sup>2,3</sup>

113 Although during both human and zebrafish development the lens placode is induced to form  
114 from the surface ectoderm cells overlying the optic cups, the morphogenetic processes  
115 resulting in placode formation differ. Cell fate tracking during zebrafish lens development  
116 suggested that delamination of the lens placode from the surface ectoderm results in  
117 formation of a solid lens mass which detaches by apoptosis of the intervening cells by 28 hpf  
118 (Fig2f-h).<sup>5</sup> In contrast, after thickening of the cells of the surface ectoderm overlying the optic  
119 vesicle at 27 days gestation, the human lens placode invaginates as a result of cessation of  
120 cell division at its centre, forming lens pits. The lens pit pinches off from the surface  
121 ectoderm as the cells of the pit delaminate from the head ectoderm and develop cell-cell  
122 interactions with the opposite pit edge (Fig2b-d).

123

124 During the fifth week of human gestation, the surface ectoderm, once separated from the  
125 lens vesicle, differentiates into the corneal epithelium.<sup>4</sup> This process occurs by 30 hpf during  
126 zebrafish embryogenesis.<sup>6</sup> Simultaneously, the corneal endothelial monolayer forms as  
127 migratory periorbital mesenchymal cells migrate into the cornea from peripheral regions of  
128 the optic cup between 30-36 hpf.<sup>6</sup> Subsequently, the neuroectodermal layers of the optic  
129 vesicle invaginate ventrally, meeting across the optic fissure along the proximo-distal axis,  
130 encircling the optic stalk by the end of the 4<sup>th</sup> week of human gestation and by 24 hpf in the  
131 zebrafish. The retina and retinal pigment epithelium are confined within the optic cup.  
132 Closure of the optic fissure is achieved by the seventh week of gestation during human  
133 embryogenesis and by 48 hpf in the zebrafish. By these time points morphogenesis of the  
134 eye is largely complete with only retinal neurogenesis proceeding.

135

### 136 **Vasculature**

137 Similar to transient hyaloid vascular anatomy in mammalian embryos, the primitive zebrafish  
138 retinal vasculature branches from the central retinal artery by angiogenesis between 24-29  
139 hpf. The optic artery enters the eye ventrally through the optic fissure and forms the single  
140 loop of the hyaloid artery within the eye, which exits the optic fissure as the hyaloid vein.

141 Endothelial cells between the lens and retina give rise to the first hyaloid vessel which is  
142 distinguishable as rudimentary vasculature by 60 hpf. The hyaloid vessels branch and lose  
143 contact with the lens, adhering to the inner limiting membrane of the juvenile retina by 30  
144 dpf. In mammals, remodelling of the hyaloid vessels involves a coordinated process of  
145 hyaloid regression and retinal angiogenesis. In zebrafish, the vessels gradually move away  
146 from the lens and deflect onto the retina as the vitreous forms; re-modelling of the vessels is  
147 sufficient to establish retinal vasculature in the zebrafish retina without re-growth, perhaps  
148 due to the close proximity of the lens to the retina.

149

### 150 **Retinal neurogenesis**

151 Human retinal neurogenesis is initiated during gestational week six. The ganglion cells exit  
152 the cell cycle and differentiate to establish the innermost layer of the retina by 20 weeks  
153 gestation.<sup>4</sup> Similarly, the ganglion cells are the first identifiable cells in the developing  
154 zebrafish retina. Differentiation of ganglion cells is initiated in the ventronasal retina and  
155 spreads dorsally to the ventrotemporal retina.<sup>2,7</sup> The first axons from the ganglion cells exit  
156 the retina and by 40 hpf reach the optic tectum. Ganglion cell differentiation is closely  
157 followed by the appearance of amacrine and horizontal cells. Lamination of the retina  
158 progresses rapidly, spreading across most of the retina by 48 hpf.<sup>3</sup> During the 10<sup>th</sup> week of  
159 gestation, photoreceptor cell bodies become apparent in the outer nuclear layer of the  
160 human retina, developing radially from the optic stalk. In the zebrafish retina, opsin  
161 expression can be detected in the ventral patch by 50 hpf, closely followed by the  
162 appearance of rod and cone outer segments and synaptic terminals by 55 hpf.<sup>3</sup> Rod cells  
163 and Müller glial cells are the last to differentiate in both human and zebrafish retinal  
164 development. During human embryogenesis, visual evoked potentials can be recorded by  
165 30 weeks gestation suggestive of a level of functional integrity. The zebrafish exhibits visual  
166 function by 72 hpf, with most major classes of cell being identifiable in the central retina.<sup>3,7,8</sup>  
167 Interestingly, by 72-74 hpf a specialised area in the temporal retina develops which is  
168 characterised by a high density of cones and corresponding reduction in rods. This “area

169 temporalis” is similar to the fovea centralis in humans, but has been suggested to provide  
170 zebrafish with an area of better visual acuity in their anterior visual field.<sup>3</sup> Additionally, a  
171 visual evoked startle response (body twitch induced by an abrupt decrease in light intensity)  
172 and electroretinogram (ERG) can be generated by 72 hpf.<sup>8</sup>

173

#### 174 **Mature retinal anatomy**

175 Comparable to the human eye, the mature zebrafish retina is composed of three nuclear  
176 layers separated by two plexiform layers. The photoreceptor rod and cone cell bodies reside  
177 in the outer nuclear layer; the amacrine, horizontal and Müller glial cell bodies occupy the  
178 inner nuclear layer and the ganglion cell bodies are contained in the ganglion cell layer.  
179 Synapsis between these nuclear layers occurs at the plexiform layers. Larval zebrafish  
180 vision is mediated almost entirely by cone photoreceptors; zebrafish possess blue, UV-  
181 sensitive, and red-green double cones and one rod cell type anatomically arranged in a  
182 mosaic pattern.<sup>9</sup> Short cones become identifiable by 4 dpf and by 12 dpf, all photoreceptor  
183 types are discernible based on morphological criteria. In contrast, the human retina lacks  
184 UV-sensitive cones.

185

#### 186 **Gene discovery**

187 The usefulness of the zebrafish model in ocular research originates not only from the striking  
188 anatomical similarities in ocular development between human and zebrafish embryos, but  
189 also from its amenability to experimental and genetic manipulation. Initial large-scale forward  
190 genetic screens in the 1990’s involved mutagenesis with ethylnitrosourea (ENU) and  
191 facilitated the isolation of over 2000 mutations affecting the first 5 days of zebrafish  
192 development. Included in the spectrum of mutant phenotypes obtained were multiple ocular  
193 defects<sup>10</sup>. More recently, targeted genetic screens have been employed to isolate mutations  
194 specifically affecting eye development. Such screening methods have involved behaviour  
195 assays, examination of eye morphology or the use of transgenic reporters.

196

197 **Zebrafish mutagenesis**

198 The development of sophisticated mutagenesis techniques, for example morpholino  
199 antisense oligonucleotide knockdown, transcription activator-like effector nucleases  
200 (TALENs) or the clustered regularly interspaced short palindromic repeats (CRISPR)  
201 system, has facilitated the identification of multiple zebrafish mutants that model human  
202 genetic eye disease.

203

204 Zebrafish transgenesis can be achieved simply by the injection of purified plasmid DNA into  
205 newly fertilised eggs, however, this approach does have drawbacks. The potential to induce  
206 mosaic distribution of injected DNA into injected embryos, and late transgene integration at  
207 high copy numbers or inefficient incorporation of the transgene into genomic DNA can make  
208 generation of transgenic zebrafish lines a laborious task. The development of autonomous  
209 and non-autonomous transposable elements, has improved the efficiency of germline  
210 transformation.<sup>11</sup>

211

212 Injection of an antisense oligonucleotide morpholino which is complementary to a specific  
213 mRNA of interest into fertilised zebrafish eggs, can inhibit its expression by sterically  
214 blocking the translation initiation complex to hinder ribosome assembly or by binding and  
215 blocking sites involved in pre-mRNA processing via inhibition of the spliceosome components.  
216 Despite highly efficient gene knockdown in early development, the transient activity of an  
217 injected morpholino oligonucleotide rarely persists beyond 5 dpf. This is however, sufficient  
218 for the timeframe of zebrafish ocular development. Morpholino antisense oligonucleotides  
219 have been an indispensable tool in studying gene function during both anterior and posterior  
220 segment development, although the reliability and reproducibility of the results are subject to  
221 increasing scrutiny with concerns raised with regard to poor correlation between morpholino-  
222 induced and mutant phenotypes. Additionally, the constitutive activity of these  
223 oligonucleotides limits their use for applications where embryonic development depends on  
224 specific temporal and spatial control of gene function; deciphering the molecular



225 mechanisms that underpin complex developmental processes requires methods for  
226 perturbing gene expression with similar precision. Photo-cleavable morpholino  
227 oligonucleotides allow the activation or deactivation of morpholino function by UV  
228 exposure.<sup>12</sup> Recently, RNA-interference mediated chromatin silencing has been used to  
229 induce sequence-specific gene knockdown by convergent transcription (in which a DNA  
230 sequence is simultaneously transcribed in sense and antisense orientations directed by two  
231 inducible promoters) in zebrafish.<sup>13</sup>

232

233 The targeted introduction of mutations using sequence-specific TALENs or the CRISPR  
234 system have recently been successfully applied to generate loss-of-function alleles by  
235 specifically targeting open reading frames or deletion or inversion of whole chromosomal  
236 regions *in vivo* with efficiencies in zebrafish similar to those obtained using zinc-finger  
237 nucleases and transcription-like nucleases.<sup>14</sup>

238

239 TALENs comprise a non-specific DNA-cleaving nuclease fused to an engineered DNA-  
240 binding domain, which contains a series of tandem repeats. Binding of two TALENs to their  
241 respective target sites, reconstitutes the active nuclease domain resulting in cleavage of the  
242 targeted genomic locus by inducing a double strand break. Subsequent DNA repair by  
243 homologous recombination or non-homologous end joining mediates DNA insertion, deletion  
244 and replacement, or introduces frame-shift mutations respectively. In the zebrafish, TALENs  
245 has been used to induce somatic tissue mutations in the *golden (gol)* gene, which encodes a  
246 putative cation exchanger (*slc24a5*) required for pigmentation in the embryo. Intense  
247 melanosome production occurs in the zebrafish RPE at 2 dpf, however homozygous null *gol*  
248 mutants appear pigmentless at this stage providing a robust method to assess somatic loss  
249 of gene function. Almost all TALEN RNA-injected wild-type embryos displayed mutant non-  
250 pigmented cells in the RPE, highlighting the efficiency with which TALENs can induce  
251 directed mutations in the zebrafish.<sup>15</sup>

252

253 CRISPR-Cas is a microbial adaptive immune system that uses targeted nucleases to initiate  
254 double strand breaks in foreign genetic elements. CRISPR RNAs that guide the Cas9-  
255 ribonucleoprotein complex to the target sequence can be injected into the zebrafish embryo  
256 and designed to target any 20 nucleotide genomic sequence to achieve phage or plasmid  
257 DNA cleavage with high specificity. The potential of CRISPR-mediated gene targeting has  
258 recently been demonstrated in a large scale reverse genetic screening strategy to identify  
259 zebrafish genes involved in electrical synaptogenesis.<sup>16</sup> Multiplexed pool guide RNA  
260 injections were designed to simultaneously examine multiple loci, allowing the identification  
261 of phenotypes induced by several gene deletions. With the breadth of accessible ocular  
262 phenotypes in zebrafish, such strategies could prove invaluable in the identification of  
263 candidate eye disease genes. This is particularly important in gene-knockdown  
264 investigations where the anticipated phenotype arises after the 5 dpf efficacy window of  
265 morpholinos.

266

## 267 **Zebrafish models of human ocular disorders**

268

### 269 **Ocular coloboma**

270 Failure of optic fissure closure underpins the aetiology of ocular coloboma, which is  
271 characterised by the persistence of a cleft potentially spanning the iris, ciliary body, zonules,  
272 retina, choroid and optic nerve. Ocular coloboma has been reported in up to 11.2% of blind  
273 children worldwide, with an estimated incidence of between 0.5-7.5 per 10,000 births and is  
274 often associated with other congenital anomalies of the eye.<sup>17</sup> Mutations in *PAX2*, *CHD7*,  
275 *SOX2*, *PAX6*, *GDF6*, *YAP1*, *OTX2*, *SHH*, *SIX3*, *FADD*, *MAF*, *ZFHX1B*, *RX*, *GDF3*,  
276 *MAB21L2*, *SALL2* and *ABCB6*, as well as multiple chromosomal aberrations have been  
277 associated with coloboma, although the molecular mechanisms that underpin this condition  
278 remain to be elucidated.

279

280 Zebrafish models of ocular coloboma have provided a vital paradigm for understanding optic  
281 fissure morphogenesis, with mutations in zebrafish orthologues of human ocular coloboma-  
282 causative genes resulting in an array of observed retinal and lens defects (Table 1). *PAX2*  
283 mutations are one of the most commonly identified genetic causes of renal-coloboma  
284 syndrome.<sup>18</sup> Zebrafish mutants which are homozygous for *pax2a* mutations exhibit optic  
285 fissure closure defects and lack the midbrain, midbrain-hindbrain boundary and cerebellum,  
286 fail to feed and die within two weeks.<sup>19</sup> This optic fissure closure defect has been attributed  
287 in part, to the inhibition of downstream effector caspases and deficiencies in the control of  
288 cellular proliferation, implicating a role for *pax2a* in the fine tuning of apoptotic cell death.<sup>20, 21</sup>

289

290 Recessive mutations in several other genes have been associated with ocular coloboma  
291 including *laminin-β1 (lamb1a)*, *laminin-γ1 (lamc1)*, *n-cadherin (ncad)*, *adenomatous*  
292 *polyposis coli (apc)*, *growth and differentiation factor 6 (gdf6a)* and *patched1 (ptc1)*.<sup>22-24</sup>  
293 Morphological defects exhibited by *lamb1* and *lamc1* mutants can likely be attributed to  
294 pathological changes in extracellular matrix deposition or cell-ECM interactions, where  
295 laminin proteins are a major component of the ocular basal lamina, playing a critical role in  
296 determining and maintaining tissue function.<sup>25</sup> Additionally, defective regulation of retinal  
297 progenitor cell number and proliferative activity and abnormal activation of apoptotic cell  
298 death pathways may contribute to persistence of the optic fissure in these mutants.<sup>20, 23</sup>

299

### 300 **Microphthalmia/anophthalmia**

301 Microphthalmia is characterised by an eye with total axial length that is at least two standard  
302 deviations below the mean for age, and anophthalmia refers to complete absence of ocular  
303 tissue in the orbit. Although the aetiology of microphthalmia/anophthalmia is complex,  
304 heterozygous loss-of-function mutations in *SOX2* or *OTX2* have been described as the most  
305 prevalent monogenic cause to date.<sup>26</sup> Microphthalmia-causing mutations have also been  
306 described in *CHX10*, *OTX2*, *PAX6*, *RAX*, *BMP4*, *GDF6* and *GDF3*.

307

308 During zebrafish ocular development *gdf6* expression is limited to the dorso-temporal retina,  
309 and morpholino-induced knockdown results in variable retinal defects including decrease in  
310 lenticular and ocular size, loss of retinal lamination and vacuolation of the lens.<sup>27</sup> The small  
311 eye phenotype in the *dark half*<sup>27</sup> mutant has been attributed to a nonsense mutation in the  
312 *gdf6a* gene which causes retinotectal mapping defects.<sup>28</sup> Research suggests that a gradient  
313 of *gdf6a* signalling works in conjunction with Sonic Hedgehog (Shh) to establish dorsal-  
314 ventral positional information in the retina. Similarly, a mutation in the zebrafish *out of sight*  
315 (*out*)<sup>m233</sup> locus, which eliminates the initiation codon in *gdf6a*, results in a severe small eye  
316 phenotype, likely caused by a transient wave of apoptosis at the onset of neurogenesis.<sup>29</sup>

317

318 Morpholino-induced knockdown of *rx1* and *rx2* in zebrafish results in optic vesical formation,  
319 but microphthalmia ensues.<sup>30</sup> Conversely, knockdown of the zebrafish paralogue *rx3* results  
320 in an eyeless phenotype and associated expanded forebrain suggesting a role for Rx3 in the  
321 regulation of spatiotemporal expression of eye field transcription factors during optic vesicle  
322 morphogenesis.<sup>31</sup>

323

324 *PAX6* haploinsufficiency in humans is the main cause of aniridia, but in rare cases results in  
325 microphthalmia. In zebrafish, two *pax6* paralogues, *pax6a* and *pax6b*, encode functionally  
326 redundant proteins involved in formation and differentiation of the retina and lens. Zebrafish  
327 homozygous for the *pax6b* missense mutation *sunrise (sri)*, display a mild microphthalmia  
328 phenotype which manifests in anterior segment dysgenesis.<sup>32</sup> Interestingly, morpholino-  
329 induced knockdown of *pax6a* or simultaneous knockdown of *pax6a* and *pax6b* results in a  
330 similar but more severe phenotype; morphants present with reduced body size and neural  
331 tube girth, morphologically abnormal brain development and microphthalmia.<sup>33</sup>

332

333 TALENs-mediated knockdown of *pax6a* and *pax6b* simultaneously, by targeting conserved  
334 sequences between these paralogs, resulted in eye phenotypes including microphthalmia,  
335 closely recapitulating the ocular morphant phenotype described.<sup>34</sup> The strategy employed

336 not only confirms the efficiency of TALENs-mediated gene disruption but also emphasizes  
337 the ability to induce bi-allelic and paralogous genome editing, which is important when  
338 investigating genes that are duplicated in the zebrafish genome.

339

### 340 **Cyclopia**

341 Cyclopia is characterized by the presence of a single eye with variable degrees of doubling  
342 of the intrinsic ocular structures. During embryogenesis, the splitting of the eye field occurs  
343 in parallel with establishment of the midline, a process mediated by SHH. Mutations in *SHH*  
344 cause midline patterning defects, which manifest as holoprosencephaly and cyclopia.

345

346 Zebrafish express three mammalian *SHH* orthologues in the embryonic midline, *sonic you*  
347 (*syu*), *tiggy-winkle hedgehog* (*twhh*) and *echidna hedgehog* (*ehh*). Zebrafish with  
348 simultaneous morpholino-induced knockdown of *syu* and *twhh* function, exhibit partial  
349 cyclopia suggesting that zebrafish contain two functionally redundant orthologues of  
350 mammalian *SHH*, with similar roles in anterior midline patterning.<sup>35</sup>

351

352 Additional zebrafish cyclopic mutations have been described. The zebrafish loci *squint* (*sqt*)  
353 and *cyclops* (*cyc*) encode Nodal-related proteins, Ndr1 and Ndr2, respectively. Both *squint*  
354 and *cyclops* mutations lead to severe defects in the development of the medial floor plate  
355 and ventral brain; mutants lack head and trunk mesoderm and endoderm derivatives which  
356 leads to cyclopia as a consequence of incomplete splitting of the eye field.<sup>36</sup> Similarly, the  
357 zebrafish *one-eyed pinhead* (*oep*) mutation also results in cyclopia and defects in the  
358 formation of endoderm, prechordal plate and ventral neuroectoderm.<sup>37</sup> Interestingly, the *oep*  
359 gene encodes an Epidermal growth factor (EGF)-related protein which acts as a co-receptor  
360 in the Nodal pathway,<sup>37</sup> hence linking the three zebrafish cyclopic mutations *cyc*, *sqt* and *oep*  
361 in a common signalling pathway central to mesendoderm formation and development.  
362 Importantly, research suggests that Nodal signalling acts upstream of the Shh pathway

363 during establishment of zebrafish ventral forebrain patterning indicating that known causative  
364 zebrafish cyclopic genes converge in a final common pathway.

365

366 Importantly, a novel strategy for heritable chromosomal editing in the zebrafish has recently  
367 been described using nodal-related genes; TALENs was used simultaneously with ZFNs to  
368 induce whole locus and transcription start site element-specific deletions in the zebrafish *sqt*  
369 and *cyc* genes.<sup>38</sup> Cyclopia and midline defects were observed in both *sqt* and *cyc* nuclease-  
370 injected embryos, consistent with previously reported mutant embryos, demonstrating the  
371 ability to induce specific deletions at desired locations with higher efficacy using the  
372 combined action of differing nuclease pairs.

373

#### 374 **Glaucoma**

375 Glaucoma is an optic neuropathy that can result in progressive and irreversible vision loss  
376 secondary to retinal ganglion cell death and subsequent optic nerve head damage. Many  
377 human genes have been implicated in glaucoma, although the incomplete penetrance  
378 reported within families is indicative of a multifactorial aetiology. Despite dissimilarity  
379 between the mammalian trabecular meshwork and the zebrafish annular ligament<sup>39</sup> and the  
380 vectorial (flowing from dorsal to ventral) flow of zebrafish aqueous humour which contrasts  
381 the circumferential flow of aqueous humour around and through the iridocorneal angle in  
382 mammals, the anatomy and overall ultrastructure of the tissues and cells facilitating aqueous  
383 humour dynamics in zebrafish, show conservation with that of mammals.<sup>40</sup>

384

385 Adult zebrafish and mammals share similar intraocular pressure (IOP) distributions.<sup>41</sup> With  
386 this, the disease-risk phenotype of glaucoma has been well studied in zebrafish; the reduced  
387 melanin zebrafish mutant *brass* (genetic basis unknown) exhibits mildly elevated IOP and iris  
388 hypoplasia whilst the *bugeye* mutant, which has a recessive mutation in the gene encoding  
389 Low density lipoprotein receptor related protein 2 (Lrp2), shows chronic elevated IOP

390 resulting in enlarged eyes, retinal stretch and RGC loss with progressive optic nerve  
391 damage.<sup>41, 42</sup>

392

393 In humans, mutation of the forkhead C1 (*FOXC1*) transcription factor gene results in a  
394 spectrum of anterior segment dysgenesis phenotypes, including glaucoma in some  
395 individuals.<sup>43</sup> Zebrafish *foxC1* expression is limited to the anterior segment and periocular  
396 mesenchyme<sup>44</sup> and has been implicated in the regulation of factors that mediate responses  
397 to oxidative stress and suppression of apoptosis in cells involved in aqueous humour  
398 dynamics.<sup>45</sup> Additionally, loss of *foxC1* results in defects of the vascular basement  
399 membrane integrity indicating that genetic interactions between *foxC1* and basement  
400 membrane components influence vascular stability which may impact glaucoma  
401 pathogenesis.<sup>46</sup>

402

403 Zebrafish were employed as a model to determine the normal nucleolar function of *wdr36*, a  
404 gene previously identified as causative for human primary open-angle glaucoma (POAG).<sup>47</sup>  
405 From this work, a role for *wdr36* in ribosomal RNA processing was established and  
406 subsequently confirmed in other species, highlighting the importance of zebrafish in the rapid  
407 functional elucidation of glaucoma-associated proteins. Similarly, mutations in the human  
408 LIM-homeodomain gene *LMX1B* are associated with Nail-Patella syndrome and an  
409 increased susceptibility to POAG.<sup>48</sup> Zebrafish *lmx1b.1* and *lmx1b.2*, orthologues of the  
410 mammalian *LMX1B* gene, are expressed in cells of the periocular mesenchyme and anti-  
411 sense morpholinos against *lmx1b.1* and *lmx1b.2* not only result in defective migration of  
412 periocular mesenchymal cells and subsequent apoptosis, but also alter the expression of  
413 *foxc1*.<sup>49</sup>

414

#### 415 **Corneal dystrophies**

416 Human genetic corneal dystrophies, although uncommon, can cause significant visual  
417 impairment when corneal transparency is lost. At 6 mpf, the zebrafish cornea contains all

418 five major layers found in the human cornea.<sup>6</sup> A number of genes associated with human  
419 corneal dystrophies encode proteins which are expressed in the zebrafish cornea including  
420 *tgfb1*, keratin 3 and corneal keratan sulfate proteoglycan.<sup>50</sup> Importantly, some human  
421 corneal dystrophy-linked genes show conserved expression in the zebrafish cornea,  
422 including the François-Neetens fleck corneal dystrophy-gene *PIP5K3*.<sup>51</sup>

423

424 The human gene Keratocan (*KERA*) encodes a corneal small leucine-rich proteoglycan  
425 which is essential in maintaining corneal transparency. Mutations in *KERA* are directly  
426 associated with inherited cornea plana, which manifests clinically as reduced visual acuity  
427 due to a flattened convex corneal curvature and corneal parenchymal opacity.<sup>52</sup> The  
428 zebrafish homologue *zKera* is widely expressed in the brain and ocular tissue at larval  
429 stages and limited to the cornea in the adult suggestive of an important role in the  
430 maintenance of corneal transparency and structure.<sup>53</sup>

431

432 The surface of the human eye is covered by corneal epithelial cells, which regenerate from a  
433 small population of limbal epithelial stem cells (LESCs) found in limbal epithelial crypts at the  
434 peripheral cornea.<sup>54</sup> The human *LAMA1* gene encodes an important basal membrane  
435 protein which is highly expressed in LESCs.<sup>55</sup> Interestingly, loss of the functional zebrafish  
436 homologue *lama1* in ocular tissues leads to focal corneal dysplasia in adult zebrafish.<sup>56</sup>

437

### 438 **Cataract**

439 In conjunction with the cornea, the transparent lens functions as a key refractive element  
440 required to focus light on the retina. Cataract is an opacity in the lens and represents the  
441 leading cause of preventable sight loss worldwide. Although most cases of cataracts are  
442 age-related and likely have both genetic and environmental causes, congenital cataracts  
443 occur in approximately 2-3 per 10,000 live births, often presenting during the first year of  
444 life.<sup>57</sup> The causative genes for many cases of human cataract remain unknown.

445



446 Several mutations affecting the zebrafish lens have been described. The *lens opaque (lop)*  
447 mutation which lies within the *cdipt* (phosphatidylinositol synthase) gene, causes cataract as  
448 a consequence of disrupted lens fibre differentiation and unregulated cell proliferation  
449 leading to retinal photoreceptor degeneration in the mutant.<sup>58</sup> Large-scale zebrafish  
450 mutagenesis screens have identified many more zebrafish which present with lens defects  
451 as a consequence of mutations in genes which encode crystallins, connexions, aquaporin,  
452 beaded filament proteins and heat shock factors. These proteins are important in  
453 maintaining the transparency and refractive power of the lens and the cornea. The *cloche*  
454 zebrafish mutant (genetic basis unknown) presents with cataract as a consequence of  
455 defects in haematopoiesis and vascular development due to  $\gamma$ -Crystallin insolubility and  
456 defective lens fibre cell differentiation.<sup>59</sup> Interestingly, the cataract phenotype was rescued by  
457 overexpression of  $\alpha$ -Crystallin, a protein that shows reduced expression in the *cloche* mutant  
458 lens. Importantly, mutations in lens crystallins have also been associated with cases of  
459 human cataract and current research focuses on therapeutic strategies that may revive lens  
460 transparency by pharmacological targeting to safeguard crystalline chaperone activity,  
461 thereby evading the need for cataract surgery.

462

463 The lens capsule contains a heterotrimer of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of the extracellular matrix  
464 component laminin. Mutations in human *LAMB2* result in severe renal disease coupled to  
465 multiple ocular defects including lens malformation and cataract.<sup>60</sup> Similarly, mutations in  
466 zebrafish *lama1*, *lamb1* and *lamc1* genes result in retinal lamination and lens defects  
467 including ectopic position of the lens within the retina, loss of lens capsule integrity and lens  
468 fragmentation, highlighting the importance of laminin in lens development and structural  
469 integrity.<sup>25, 61</sup> Extracellular matrix-dependent myosin dynamics also contribute to the  
470 cytoskeletal organisation of the lens. Heterozygous mutations in the myosin chaperone  
471 *UNC45B* have recently been associated with congenital cataract.<sup>62</sup> Zebrafish *steif* mutants  
472 carrying an *unc45b* nonsense mutation present with a small lens and lens fibre cell nuclear  
473 retention, where degradation of all cytoplasmic organelles within the lens fibre cells is

474 necessary for the establishment and maintenance of normal lens transparency. Injection of  
475 RNA encoding the human UNC45B protein into homozygous mutant zebrafish embryos  
476 rescued the ectopic nuclei phenotype, highlighting the potential of novel therapeutic targets  
477 to treat ocular pathologies.

478

### 479 **Aniridia**

480 Aniridia is a pan-ocular disease that manifests by alterations in the structure and function of  
481 the eye, including variable degrees of iris hypoplasia, glaucoma, foveal hypoplasia,  
482 nystagmus, glaucoma, cataract and corneal abnormalities. This congenital eye malformation  
483 has been linked to haploinsufficiency of human *PAX6* and aberrations in the genomic region  
484 downstream of *PAX6*,<sup>63</sup> with different clinicopathological phenotypes determined by *PAX6*-  
485 mutation dosage. A point mutation in the *PAX6* regulatory cis-element (SIMO), that resides  
486 in intron 9 of the gene *ELP4*, positioned downstream of the *PAX6* promoter, has been  
487 described in a familial case of classical aniridia.<sup>64</sup> Interestingly, SIMO cis-element-driven  
488 reporter expression was apparent in the lens, and more variably in the diencephalon and  
489 hindbrain of a transgenic zebrafish reporter line by 48 hpf, suggestive of the importance of  
490 this conserved cis-regulatory element in zebrafish ocular development.<sup>64</sup> Deletion of the  
491 SIMO element from the *pax6* zebrafish locus resulted in complete abolition of Pax6 reporter  
492 expression in the lens, despite the persistence of other controlled lens-specific enhancers,  
493 indicating that the SIMO enhancer element is specifically important for maintained tissue-  
494 specific expression from *pax6* promoters during ocular development. This work supports the  
495 notion of the existence of conserved cis-regulatory mechanisms that govern gene  
496 expression in both human and zebrafish developing ocular tissues.

497

### 498 **Ocular albinism and associated syndromes**

499 Oculocutaneous albinism (OCA; affects the eyes, skin and hair) and ocular albinism (OA;  
500 affects only the eye) are recessive pigmentation disorders caused by defective melanin  
501 synthesis or trafficking which manifest in a broad phenotypic range. OCA type 1 and 3 are

502 associated with mutations in tyrosinase (*TYR*) and tyrosinase-related protein 1 (*TYRP1*),  
503 both of which are directly involved in melanin synthesis. OCA type 2 is associated with  
504 mutations in *OCA2* which encodes melanosomal transmembrane protein. OCA type 4 and 6  
505 are caused by mutations in *SLC45A2* and *SLC24A5* respectively, which encode  
506 melanosomal membrane-associated transporter proteins. OCA type 7 is due to mutations in  
507 the *C10ORF11* gene. Knockdown of the zebrafish orthologues *tyr* (*TYR*), *oca21p* (*OCA2*),  
508 *tyrp1a* and *tyrp1b* simultaneously (*TYRP1*), *slc45a2* (*SLC45A2*), *slc24a5* (*SLC24A5*) or  
509 *c10orf11* (*C10ORF11*) lead to a reduction or complete absence of melanin in the eye, with  
510 varying responses to visual testing immediately after light exposure, phenocopying the  
511 varying degrees of hypopigmentation of the eyes of OCA patients and exemplifying the use  
512 of zebrafish to model human pathologies of the eye.<sup>65-69</sup>

513

514 Multiple zebrafish mutants have been identified with visual defects secondary to functional  
515 deficits in the RPE. The *vps39* mutant carries a mutation in the zebrafish orthologue of  
516 *VPS39*, a gene encoding a component of the vacuole protein sorting (HOPS) membrane-  
517 tethering complex, which coordinates vesicle fusion and transport.<sup>70</sup> The *vps39* mutant  
518 presents with hypopigmentation of skin melanocytes and RPE, internal organ defects and  
519 innate immunologic function, therefore phenocopying patients with Arthrogryposis-Renal  
520 dysfunction-Cholestasis syndrome, Chediak-Higashi syndrome, Hermansky-Pudlak  
521 syndrome and Griscelli syndrome.

522

523 In the RPE, intracellular trafficking is required for receptor-mediated phagocytosis and  
524 degradation of disc membranes shed from the apical tips of photoreceptor outer segments in  
525 a diurnal rhythm, and is essential for long-term viability and functionality of photoreceptors.  
526 Molecular analysis of the *vps39* mutant confirmed defects in vision as a consequence of  
527 increased RPE cell size, fewer immature melanosomes and an accumulation of vesicles  
528 containing phagocytosed photoreceptor cell outer segments, consistent with a defect in the

529 fusion of endocytic vesicles with lysosomes, supporting a role for *VPS39* in the aetiology of  
530 human pathologies associated with defective intracellular trafficking.<sup>70</sup>

531

### 532 **Choroideremia (CHM)**

533 The small GTP-binding protein Rab Escort Protein 1 (REP1) is involved in the control of  
534 intracellular transport. REP1 binds newly synthesised Rab proteins and facilitates the  
535 addition of geranyl-geranyl groups, a post-translational modification which is essential for  
536 Rab function in the regulation of intracellular trafficking in the RPE and photoreceptors. CHM  
537 is a progressive chorioretinal dystrophy caused by mutations in the *CHM* gene, which  
538 encodes REP1. Loss-of-function of REP1 causes defective prenylation of a subset of Rab  
539 proteins with subsequent disruption of intracellular trafficking leading to a progressive  
540 degeneration of the choroid, RPE and photoreceptors. Zebrafish carrying a recessive  
541 nonsense mutation in *chm* initially exhibit areas of RPE hypertrophy and atrophy in the  
542 periphery followed by progressive cell death in the RPE and peripheral retina which leads to  
543 severe loss of retinal lamination and degeneration throughout, consistent with CHM  
544 patients(Fig3).<sup>21</sup>

545

546 The absence of REP1 in CHM, is compensated for by the homologous protein REP2, which  
547 is coded for by the intronless gene *CHML* (*choroideremia-like*), thought to arise from a  
548 retrogene insertion of the *REP1* mRNA transcript during vertebrate evolution. The *chm*  
549 mutant zebrafish only has one rep isoform, therefore lacking any compensatory function  
550 resulting in early lethality at 5 dpf as a consequence of multisystemic organ failure.<sup>71</sup> It has  
551 been hypothesised that the maternal supply of rep1 stored in the embryonic yolk sac allows  
552 for sufficient Rab function to ensure proper intracellular cycling up to 4 dpf, thereafter the  
553 disease phenotype manifests.<sup>71</sup> The *chm* mutant model provides a useful tool to characterise  
554 therapies aimed at boosting REP1 activity, for example novel drug classes that facilitate  
555 read-through of nonsense mutations.<sup>21</sup>

556

557 **Lebers congenital amaurosis (LCA)**

558 LCA is a group of inherited severe early onset retinal dystrophies with clinical and genetic  
559 heterogeneity. Currently at least 15 genes have been linked to LCA including *CEP290*,  
560 *RPE65*, *CRB1*, *KCNJ13*, *GUCY2D*, *AIP1*, *CRX*, *IMPDH1*, *LCA5*, *LRAT*, *RD3*, *RDH12*,  
561 *RPGRIP1*, *SPATA7* and *TULP1*. Knockdown of the zebrafish orthologue *gucy2f* results in  
562 early visual dysfunction with visible outer segment and photoreceptor layer loss.<sup>72</sup>  
563 Morpholino-induced knockdown of *centrosomal protein 290kDa (cep290)* resulted in delayed  
564 intracellular transport and reduced visual acuity despite a fully laminated retina, consistent  
565 with human LCA patients.<sup>73</sup> Importantly, injection of *cep290* mutants with an N-terminal  
566 CEP290 construct rescued visual function, supporting a potential treatment for LCA patients.  
567 Mutations in the zebrafish orthologue *crb1* result in severe corneal defects<sup>74</sup> which, in  
568 comparison, appear more pronounced than changes in the human LCA cornea. Conversely,  
569 the *oko meduzy (ome)* zebrafish mutant which harbours mutations in *crb2a*, result in  
570 neuronal patterning defects of the retina, preceded by reduced neuroepithelial cell integrity  
571 more consistent with human CRB1-related LCA (Fig3).<sup>75</sup> The *rpe65a*-deficient zebrafish also  
572 exhibits changes in retinal physiology, presenting with shortened, deteriorating rod outer  
573 segments which interfere with photoreceptor functionality.<sup>76</sup>

574

575 **Retinitis pigmentosa (RP)**

576 RP is a class of diseases that leads to progressive retinal degeneration characterised by  
577 dysfunction of the photoreceptors with retinal vessel attenuation, and progressive cell death.

578

579 X-linked RP is one of the most severe forms of RP, characterised by early onset and rapid  
580 progression of vision loss before the fourth decade. Multiple X-linked RP causative genetic  
581 loci have been mapped on the X-chromosome, although mutations in *retinitis pigmentosa 2*  
582 (*RP2*) and *retinitis pigmentosa GTPase regulator (RPGR)* genes account for the vast  
583 majority of all X-linked RP cases.

584

585 Morpholino-induced knockdown of the *RP2* zebrafish orthologue *rp2* results in  
586 microphthalmia, defective retinal lamination and abnormal photoreceptor morphology  
587 including lack of outer segments with extensive retinal cell death and retinal degeneration,  
588 consistent with features of human X-linked RP.<sup>77</sup> The *rpgr* zebrafish morphant presents with  
589 a similar retinal phenotype as a consequence of cell death in the dysplastic retina.<sup>78</sup> These  
590 zebrafish morphant phenotypes can be rescued by injection of human *RP2* or *RPGR* mRNA,  
591 respectively, indicative of a functional role for *RP2* and *RPGR* in the pathogenesis of human  
592 X-linked RP.<sup>78</sup>

593

594 Mutations in the (*RHO*) gene are the most common cause of human autosomal dominant  
595 RP. Rhodopsin is a member of the G protein-coupled receptor family and plays a role in  
596 phototransduction in rod photoreceptors. Transgenic fish with the human rhodopsin *Q344X*  
597 mutation under the control of the zebrafish rhodopsin promoter show photoreceptor  
598 degeneration as a consequence of increased apoptotic cell death. Importantly, cone  
599 photoreceptor number remained unchanged in the transgenic zebrafish retina, therefore  
600 mimicking human RP with the *RHO* mutation.<sup>79</sup>

601

602 Mutations in ceramide kinase-like gene (*CERKL*) have been associated with the severe  
603 retinal degeneration described in patients with RP26. The zebrafish *cerkl* protein protects  
604 retinal cells from oxidative stress-induced apoptosis. Morpholino-induced knockdown of the  
605 zebrafish *cerkl* resulted in increased retinal cell death with rod and cone photoreceptor  
606 degeneration.<sup>80</sup> Additionally, mutations in *c2orf71*, the zebrafish orthologue of *C2ORF71*  
607 (responsible for human RP54), resulted in shortened photoreceptor outer segments and  
608 attenuated visual response to light exposure.<sup>81</sup> The ability to simulate discrete phenotypes  
609 associated with human RP in zebrafish models such as these, has proved invaluable in  
610 confirming human genes as causative for RP.

611

612 RP can also occur in association with systemic disease like in the genetically heterogeneous  
613 ciliopathy Bardet Biedl syndrome, which is characterised by RP, obesity, kidney dysfunction,  
614 polydactyly, behavioural dysfunction and hypogonadism. The zebrafish photoreceptor  
615 mutant *oval* (*ovl*) which encodes the cilium gene, *IFT88* displays loss of outer segments due  
616 to mislocalised visual pigment and cilia dysfunction, similar to the mislocalisation observed in  
617 human photoreceptor cell death caused by RP, suggesting that ectopic phototransduction  
618 may play an important role in photoreceptor cell death.<sup>82</sup>

619

620 Usher syndrome (USH) is an autosomal recessive genetic disease characterised by  
621 combined hearing and vision loss and occasionally balance problems. USH is classified into  
622 three subtypes according to clinical severity and symptoms; Usher type 1 (USH1) presents  
623 as severe to profound congenital sensorineural deafness and vestibular areflexia with onset  
624 of RP within the first decade of life; type 2 (USH2) patients show moderate to severe hearing  
625 loss, normal vestibular function and pre-/post-pubertal onset of RP; and type 3 (USH3)  
626 patients have progressive hearing loss, sporadic vestibular dysfunction and variable onset of  
627 RP. The exact function of USH proteins remains contentious, however the identification of  
628 multiple USH-causative genes and their analysis in zebrafish models suggests that  
629 compromised photoreceptors, RPE cells and Müller cells may all underlie the cellular  
630 pathogenesis of USH-associated RP.

631

632 The gene *MYO7A* encodes human myosin VIIA, a protein responsible for USH1B, is  
633 expressed in RPE and photoreceptor cells of the human retina. Homozygous mutations in  
634 zebrafish *myo7aa* resulted in mild retinal degeneration by 10 dpf; mutants presented with  
635 elevated cell death in the outer nuclear layer of the retina and photoreceptor degeneration.<sup>83</sup>

636 A number of large acellular holes were observed in the RPE of *myo7aa* mutants following  
637 light damage by exposure to constant light conditions, consistent with an inability to clear  
638 outer segment debris and subsequent non-autonomous RPE degeneration.

639

640 *USH1C* encodes the PDZ-domain-containing protein harmonin. The *harmonin (ush1c)*  
641 mutant presented with defective photoreceptor function attributed to a primary defect in  
642 Müller glial cells and subsequent ribbon synapse stability and function.<sup>84</sup> Interestingly, *PDZ*  
643 *domain-containing 7 (PDZD7)* encodes a ciliary protein with homology to the *USH1C* and  
644 *USH2D* proteins. Although morpholino-induced knockdown of *ush2a* or *pdzd7a* alone  
645 resulted in moderate levels of photoreceptor cell death in the retina, combined morpholino-  
646 induced partial *ush2a;pdzd7a* or *pdzd7a:gpr98* knockdown exacerbated photoreceptor  
647 death, consistent with the possibility of human digenic inheritance of *USH*-associated  
648 mutations and retinal disease modifiers in patients with *USH2A*.<sup>85</sup>

649

650 Point mutations and large deletions in *PCDH15* are the cause of human *USH1*.<sup>86, 87</sup>  
651 Reduction in the zebrafish orthologue *pcdh15b* resulted in short and disorganised outer  
652 segments that lack interdigitation with the RPE.<sup>88</sup> This early-onset photoreceptor  
653 malformation is likely attributed to progressive photoreceptor death as a consequence of  
654 impaired contact between the RPE and outer segments, suggesting a role for *PCDH15* in  
655 maintaining the structural integrity of the photoreceptor outer segment.

656

### 657 **Vascular disease**

658 Mutations in *plexin D1 (plxnd1)* in the zebrafish mutant *out of bounds (obd)* result in  
659 patterning defects of intersegmental vessels and increased primary branching of the hyaloid  
660 vessels, providing a model to explore the mechanisms that govern normal and aberrant  
661 human retinal angiogenesis.<sup>89</sup> Importantly, zebrafish *obd* mutants survive to adulthood unlike  
662 knockout mice, enabling characterisation of the hyaloid vasculature.

663

664 Additionally, zebrafish with mutations in the *von Hippel-Lindau (VHL)* orthologue, *vhl* present  
665 with increased choroidal and hyaloid vascular networks and display a systemic hypoxic  
666 response, including vascular leakage in the retina, lesions throughout the retinal layers and  
667 retinal detachment of the retinal nerve layers from the RPE, consistent with *VHL* patients



668 that develop retinal neovascularisation.<sup>90</sup> Importantly, overproduction of hypoxia-induced  
669 mRNAs is a hallmark of highly vascularised neoplasms associated with inactivation of the  
670 *VHL* tumour suppressor gene in human retinal haemangioblastomas, highlighting the clinical  
671 relevance of the zebrafish model for the study of hypoxia-induced pathological angiogenesis.

672

673 Familial exudative vitreoretinopathy (FEVR) is characterised by abnormal retinal  
674 angiogenesis, resulting in retinal detachment and sight impairment. Mutations in *zinc finger*  
675 *protein 408* (*ZNF408*) have been associated with autosomal dominant FEVR. Morpholino-  
676 induced knockdown of zebrafish *znf408* resulted in defects in retinal radial vessel sprouting,  
677 highlighting a functional link between *znf408* and retinal blood vessel formation.<sup>91</sup>

678

#### 679 **Therapies for ocular disorders**

680 Gene therapy approaches represent the most promising therapeutic option for the treatment  
681 of genetic eye disease; the routes of administration are local and systemic for ocular  
682 diseases. Vectors derived from adeno-associated virus (AAV) are most frequently used for  
683 ocular gene delivery due their small size and ability to efficiently transduce retinal cell types  
684 *in vivo*.

685

686 Although viral gene transfer in zebrafish has been achieved using multiple retroviral  
687 integrases, significant drawbacks have included toxicity, complexity of virus production and  
688 modification, and difficulty in achieving high titres. As an alternative, effective gene delivery  
689 and expression using the *Sleeping Beauty* transposon system has been described.<sup>92</sup>  
690 Injection of a transposon construct containing GFP under control of a lens-specific  
691 expression cassette from the *γ-crystallin* gene produced embryos with eye-specific GFP  
692 expression. Similarly, the Tol2 transposon-based vector system can drive overexpression of  
693 *crb3a* in the zebrafish otic vesicle under the control of the heat-shock promoter.<sup>93</sup> Induction  
694 of *crb3a* expression by heat exposure resulted in profound epithelial polarity defects limited  
695 to the retinal neuroepithelium, and disorganised retinal architecture similar to *crb2a* mutants

696 providing proof-of-concept for vector-based and tissue-specific gene delivery. Established  
697 vector based gene delivery also provides an opportunity to carry out conditional phenotypic  
698 rescue experiments applicable against zebrafish mutants and morphants, representing a  
699 powerful tool for high-throughput drug screening.

700

### 701 **Small molecule drugs**

702 Pharmacological screens in zebrafish can be used to identify small molecule drugs that  
703 affect biological processes, by perturbing protein function. Although little precedent exists for  
704 small molecule screens focusing on zebrafish retinal development, this approach has been  
705 successfully employed to investigate other zebrafish organs and behaviours. Recently, a  
706 quantitative whole-organism screening method combining high-throughput screening  
707 instrumentation with reporter-based assays was developed to identify compounds that  
708 elevated *insulin* reporter activity.<sup>94</sup> Over 500,000 zebrafish embryos were screened and 177  
709 drugs implicated as candidates to treat  $\beta$ -cell paucity in diabetic patients. Importantly, a  
710 proportion of these candidates were already approved for use in humans highlighting the  
711 plausibility of rapid clinical translation of such screening approaches.

712

713 Current therapies for delaying defective ocular angiogenesis include laser surgery or  
714 molecular inhibition of pro-angiogenic factors. The small molecule drug LY294002, a PI3  
715 kinase inhibitor, was identified as a selective inhibitor of both developmental and ectopic  
716 hyaloid angiogenesis in the eye in a targeted screen of known regulators of angiogenesis  
717 using zebrafish. Intraocular injection inhibited ocular angiogenesis without secondary  
718 systemic effects or threatening visual function, demonstrating the potential to effectively and  
719 safely treat unwanted neovascularisation in eye disease with isoform-specific inhibitors or  
720 vascular-targeted prodrugs as monotherapies or part of combination angiostatic  
721 approaches.<sup>95</sup> Similarly, a random pharmacological screen involved treatment of zebrafish  
722 with 2000 small molecules from The Spectrum library (MicroSource Discovery Systems Inc.)  
723 to identify compounds affecting retinal vasculature that could be of therapeutic importance.<sup>96</sup>

724 Compounds which caused collapse and loss of retinal vessels, or increased vessel diameter  
725 were identified, with the potential to alleviate symptoms of persistent hyperplastic foetal  
726 vasculature or retinal blood vessel occlusion, respectively.

727

728 Nonsense mutations that introduce premature stop codons account for approximately 30%  
729 of genetic eye disease. Molecular therapies that target premature stop codons offer a  
730 practical treatment option. *PAX2* nonsense mutations have been identified in ocular  
731 coloboma. The *no isthmus* (*noi*<sup>tu29a</sup>) zebrafish mutant has a recessive nonsense mutation in  
732 *pax2.1* and the *grumpy* (*gup*<sup>m189</sup>) zebrafish mutant has a recessive nonsense mutation in  
733 *lamb1*, both resulting in optic fissure closure defects. Mutant *noi* and *gup* larvae dosed with  
734 aminoglycosides, gentamicin and paromomycin, demonstrated complete fusion of the optic  
735 fissure and regular retinal lamination by 9 dpf.<sup>21</sup> The choroideremia mutant zebrafish  
736 (*chm*<sup>ru848</sup>) has a recessive nonsense mutation in the second exon of the *chm* gene, which  
737 manifests with severe retinal degeneration, microphthalmia and cataract.<sup>71</sup> Treatment with  
738 gentamicin or paromomycin resulted in normalization of retinal lamination with no signs of  
739 chorioretinal degeneration at 9 dpf.<sup>21</sup> highlighting the applicability of pharmacological agents  
740 to readthrough premature stop codons in the treatment of inherited eye disorders caused by  
741 nonsense mutations.

742

### 743 **Molecular therapies; targeting molecular pathways for therapeutic intervention**

744 Reverse genetics has proved to be a powerful way to delineate roles of canonical signalling  
745 pathways involved in zebrafish ocular development and disease. Ocular coloboma is  
746 associated with increased apoptotic activity at the site of defective optic fissure fusion.<sup>21</sup>  
747 Curcumin (chemical structure diferuloylmethane) has a wide range of pharmacological  
748 effects, including anti-apoptotic by inhibition of caspase-3 expression. zVAD-FMK  
749 (fluoromethyl ketone molecule) is a specific cell-permeable pan-caspase inhibitor. Treatment  
750 of the *gup* coloboma mutant with curcumin or zVAD-FMK resulted in a reduction in apoptotic  
751 activity and a milder coloboma phenotype.<sup>97</sup> Additionally, inhibition of RIP1, a regulatory

752 molecule of necroptosis and apoptosis, with the small molecule drug necrostatin-1, rescued  
753 the colobomatous defect in *pax2.1*-deficient zebrafish mutants. Future therapeutic strategies  
754 may be based on small molecule drugs that bypass the gene defects causing common  
755 congenital tissue fusion defects.<sup>20</sup>

756

### 757 **Photoreceptor regeneration**

758 The adult fish retina possesses a robust capacity to replace lost neurons following injury.  
759 Several lesion paradigms have been employed to investigate zebrafish retinal regeneration  
760 including light lesions which specifically destroy photoreceptors, intravitreal injection of  
761 ouabain neurotoxin which destroys ganglion cells and the inner nuclear layer and physical  
762 lesions causing localised retinal damage. In all cases, lost retinal neurons were regenerated,  
763 retinal lamination normalised and visual function restored.

764

765 Although a number of genetic, molecular, and cell biological techniques have proven  
766 invaluable in understanding mechanisms that underpin zebrafish retinal degeneration and  
767 regeneration, most modalities are unable to generate a dynamic retinal picture during this  
768 neuronal cell death and regeneration. Optical coherence tomography (OCT) is a non-  
769 invasive imaging technique essential in diagnosing and monitoring human retinal disease.  
770 Research has demonstrated a good correlation between representative OCT retinal layers  
771 and retinal histopathology in humans, facilitating the ability to discern important features of  
772 anterior and posterior segments of the eye. Importantly, OCT has also been employed to  
773 assay dynamic morphological changes during zebrafish retinal lamination and photoreceptor  
774 loss and regeneration with light-induced or ouabain-induced damage *in vivo*.<sup>98</sup> It is possible  
775 to not only resolve individual retinal layers, from the retinal nerve fiber layer to the RPE, but  
776 also the photoreceptor mosaic, emphasizing the high resolution imaging capacity of OCT in  
777 small animal imaging (Fig4). This work potentiates the ability to study dynamic processes of  
778 zebrafish retinal development, damage and regeneration whilst providing a means to  
779 characterise retinal degenerative disorders and assess the efficacy of potential treatments.

780

781 The efficacy of pathway-specific compounds has been investigated in zebrafish retinal  
782 regeneration models.  $\beta$ -catenin/Wnt signalling has been implicated in Müller-glia  
783 proliferation in the regenerating adult zebrafish retina.<sup>99</sup> Following retinal injury,  $\beta$ -catenin  
784 accumulates in the nucleus of the Müller glial-derived progenitors. The tankyrase inhibitor,  
785 XAV939, selectively inhibits  $\beta$ -catenin-mediated transcription and pyrvinium, a casein kinase  
786 1- $\alpha$  activator, promotes  $\beta$ -catenin degradation. Blocking  $\beta$ -catenin accumulation by injection  
787 of XAV939 or pyrvinium at the time of retinal injury resulted in reduced proliferation of Müller  
788 glial-derived progenitors indicating that  $\beta$ -catenin is required for retinal progenitor cell  
789 proliferation.<sup>100</sup> Importantly,  $\beta$ -catenin signalling can be enhanced by injection of the GSK-3 $\beta$ -  
790 inhibitor, lithium chloride in injured and uninjured zebrafish retinas, stimulating Müller glial  
791 dedifferentiation and proliferation into multiple retinal neuronal subtypes.<sup>100</sup>

792

793 Reprogramming of endogenous Müller glial cells to a stem cell function, or transplantation of  
794 stem cells or progenitor cells into diseased retinas may provide therapeutic potential for  
795 degenerative retinal disorders. Inspiration for Müller-cell therapies comes from findings that  
796 suggest Müller glia function as multipotent retinal stem cells, which can generate retinal  
797 neurons in response to loss of photoreceptors in the differentiated zebrafish retina.  
798 Mammalian Müller glia also exhibit some neurogenic properties indicative of an ability to  
799 regenerate retinal neurons. Elucidating the specific properties of zebrafish Müller glia that  
800 facilitate their innate capacity to regenerate retinal neurons and the identification of chemical  
801 modulators of this process will provide invaluable information to harness alternative  
802 therapies to treat human retinal degeneration.

803

## 804 **Summary**

805 The zebrafish has emerged as a robust model system for studying cellular and molecular  
806 mechanisms that underpin normal ocular development and human eye disease. Large-scale  
807 genetic screens have allowed the identification of candidate genes, which provide a deeper

808 understanding of human pathogenicity and support the development of potential therapies to  
809 combat untreatable genetic disorders. The development of reliable zebrafish models of eye  
810 disease through technologies such as CRISPR, coupled with more sophisticated transgenic  
811 approaches to visualise cellular processes *in vivo*, support the expanded use of the  
812 zebrafish as a tool to functionally annotate human ocular disease alleles. Ongoing work to  
813 dissect the signalling pathways that converge to regulate ocular development will not only  
814 infer the pathophysiological processes that underlie complex ocular diseases, but also  
815 facilitate the development of gene- and cell-mediated therapeutic strategies for their  
816 treatment and prevention.

817

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824

### 825 **Conflict of Interests**

826 The authors declare that they have no conflict of interest.

827

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## 1081 **Titles and legends to figures**

1082 **Figure 1.** Cross sectional histology of the human and zebrafish retina demonstrating  
1083 similarities in the arrangement of cells and structural features that define the distinct retinal



1084 layers. RPE, pigmented epithelium; IS, inner segment; OS, outer segment; PR,  
1085 photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer;  
1086 GCL, ganglion cell layer; NFL, nerve fiber layer.

1087

## 1088 **Figure 2. Schematic of human and zebrafish ocular development**

1089 The lens placode and optic vesicle are formed as the central eye field splits at 27 days (d) of  
1090 gestation in the human and 16 hours post fertilisation (hpf) in the zebrafish (**a, e**). The distal  
1091 portion of the optic vesicle invaginates so that the presumptive neural retina is apposed to  
1092 the presumptive RPE in a double-walled cup structure (**b, f**). The optic cup grows  
1093 circumferentially. The inner layer differentiates into the neural retina from 28-35 days of  
1094 gestation in the human and from 16 hpf in the zebrafish. The outer layer of the optic cup  
1095 gives rise to the RPE. The lens develops concomitantly with the retina in both human and  
1096 zebrafish development. The human lens placode invaginates to become the lens pit, which  
1097 deepens and closes before pinching off from the surrounding surface ectoderm (yellow) as  
1098 the lens vesicle by 35 days of gestation (**c**). Cells of the central lens placode migrate to the  
1099 posterior lens vesicle and elongate to form primary lens fibre cells (blue), filling the lens  
1100 vesicle lumen by 50 days of gestation (**d**). Cells of the peripheral lens placode migrate to the  
1101 anterior lens vesicle forming the anterior epithelium (orange). Similar to humans, the  
1102 zebrafish lens begins as a lens placode (**e**). Progressive delamination of cells of the lens  
1103 placode results in the formation of a solid lens mass by 22 hpf (**f, g**). Cells of the central lens  
1104 placode migrate to the posterior lens mass, elongate and differentiate to form primary lens  
1105 fibre cells (blue). Cells of the peripheral lens placode migrate to the anterior lens mass to  
1106 form the anterior epithelium (orange). In both human and zebrafish ocular development, the  
1107 cornea (yellow) develops when the surface ectoderm closes after detachment of the lens  
1108 vesicle or lens mass, respectively, from the surface ectoderm (**d, h**). Human ocular  
1109 development is represented here based on the widely accepted mammalian lens cell fate  
1110 map.

1111

1112 **Figure 3.** Gross morphology and retinal histology of wild-type (wt), *ome* and *chm* mutants at  
1113 6 dpf. Left panel: Coronal retinal sections. The wild-type retina shows characteristic  
1114 stratification in three nuclear and two plexiform layers. The *ome* (*crb2a*<sup>289/289</sup>) mutant shows  
1115 widespread retinal degeneration with loss of lamination and irregular patchy islands of  
1116 plexiform tissue, with absence of large areas of RPE. In *chm* (*chm*<sup>ru848/ru848</sup>) mutant embryos,  
1117 there is a hard compacted cataractous lens, extensive retinal degeneration with pyknotic  
1118 nuclei, areas of photoreceptor cell loss and RPE hypertrophy and atrophy. Middle and left  
1119 panel: Bright-field images showing whole eye and fish morphology. The *ome* mutant shows  
1120 irregular curvature of the spine, cardiac oedema, persistent yolk sac, absent swimbladder.  
1121 The *chm* mutant displays a smaller eye size with a cataract lens, gross morphological  
1122 abnormalities include cardiac and abdominal oedema, persistent yolk sac, absent  
1123 swimbladder, reduced body length and curvature of the spine. e, eye; ea, ear; h, heart; y,  
1124 yolk sac; sb, swimbladder, df, dorsal fin; tf, tail fin. Scale bars 100  $\mu$ m.

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1126 **Figure 4.** *In vivo* OCT horizontal scans of the human and zebrafish retina. In the human  
1127 OCT image, the site of the IS/OS border aligns with the ellipsoids of the IS, therefore  
1128 referred to as the IS ellipsoid (ISE) band. The interdigitation zone corresponds to the  
1129 configuration of the RPE apices with the photoreceptor outer segments. Standard resolution  
1130 OCT imaging allows the identification of individual photoreceptor inner and outer segments  
1131 in the living zebrafish retina. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner  
1132 plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear  
1133 layer; ELM, external limiting membrane; ISE, inner segment ellipsoid; OS, outer segment; IZ,  
1134 interdigitation zone; RPE, retinal pigment epithelium. Scale bars 200  $\mu$ m.