1 **Zebrafish models for human ALA-dehydratase-deficient porphyria (ADP) and**

2 **hereditary coproporphyria (HCP) generated with TALEN and CRISPR-Cas9**

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21 **Running title**: Zebrafish models for human porphyrias ADP and HCP
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25   **ABSTRACT**: Defects in the enzymes involved in heme biosynthesis result in a group

26   of human metabolic genetic disorders known as porphyrias. Using a zebrafish model

27   for human hepatoerythropoietic porphyria (HEP), caused by defective

28   uroporphyrinogen decarboxylase (Urod), the fifth enzyme in the heme biosynthesis

29   pathway, we recently have found a novel aspect of porphyria pathogenesis. However,

30   no hereditable zebrafish models with genetic mutations of *alad* and *cpox*, encoding the

31   second enzyme delta-aminolevulinate dehydratase (Alad) and the sixth enzyme

32   coproporphyrinogen oxidase (Cpox), have been established to date. Here we employed

33   site-specific genome-editing tools transcription activator-like effector nuclease

34   (TALEN) and clustered regularly interspaced short palindromic repeats

35   (CRISPR)/CRISPR-associated protein 9 (Cas9) to generate zebrafish mutants for *alad*

36   and *cpox*. These zebrafish mutants display phenotypes of heme deficiency,

37   hypochromia, abnormal erythrocytic maturation and accumulation of heme precursor

38   intermediates, reminiscent of human ALA-dehydratase-deficient porphyria (ADP) and

39   hereditary coproporphyrian (HCP), respectively. Further, we observed altered

40   expression of genes involved in heme biosynthesis and degradation and particularly

41   down-regulation of exocrine pancreatic zymogens in ADP (*alad-/-*) and HCP (*cpox-/-*)

42   fishes. These two zebrafish porphyria models can survive at least 7 days and thus

43   provide invaluable resources for elucidating novel pathological aspects of porphyrias,

44   evaluating mutated forms of human *ALAD* and *CPOX*, discovering new therapeutic

45   targets and developing effective drugs for these complex genetic diseases. Our studies

46   also highlight generation of zebrafish models for human diseases with two versatile

47   genome-editing tools.

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49   **Keywords:** Zebrafish, heme, porphyria, *alad*, *cpox*, TALEN, CRISPR-Cas9

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52   **Introduction**

53   The porphyrias are a group of rare and clinically complex metabolic disorders, each

54   caused by genetic mutations that result in defective enzymes involved in eight highly

55   conserved reactions of heme biosynthesis (Balwani and Desnick, 2012; Puy et al.,

56   2010). Deficiencies of the second enzyme delta-aminolevulinate dehydratase (ALAD;

57   EC 4.2.1.24) in the heme biosynthesis pathway cause autosomal recessive

58   ALA-dehydratase-deficient porphyria (ADP; Online Mendelian Inheritance in Man,

59   OMIM 125270, John Hopkins University, Baltimore, MD) (Jaffe and Stith, 2007; Yano

60   and Kondo, 1998); whereas deficiencies of the sixth enzyme coproporphyrinogen

61   oxidase (CPOX, EC 1.3.3.3) lead to autosomal dominant hereditary coproporphyria

62   (HCP; OMIM 121300) (Whatley et al., 2009). Both porphyrias are acute hepatic and

63   clinically manifest neurologic attacks, abdominal pain, nausea, vomiting, tachycardia,

64   and hypertension (Siegesmund et al., 2010). The diagnosis of the two porphyrias is

65   primarily through detection of elevated levels of delta-aminolevulinic acid (ALA),

66   coproporphyrin III and porphobilinogen (PBG) in urine, feces and blood, combined

67   with enzymatic assays and mutation analysis (Balwani and Desnick, 2012; Cappellini

68   et al., 2010; Maruno et al., 2001; Puy et al., 2010).

69   The zebrafish (*Danio rerio*) has been a model for investigating molecular genetic

70   mechanisms underlying numerous human disorders including hematopoietic diseases

71   (Dooley and Zon, 2000; Lieschke and Currie, 2007), and also has been particularly

72   effective for high-throughput drug screens in whole organism (MacRae and Peterson,

73   2015; North et al., 2007; Poureetezadi et al., 2014; Rennekamp and Peterson, 2015;

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74   Zon and Peterson, 2005). Using a zebrafish model for human hepatoerythropoietic

75   porphyria (HEP; OMIM 176100) (Wang et al., 1998), we recently have uncovered a

76   novel aspect of porphyric pathogenesis, i.e., heme regulates exocrine pancreatic

77   zymogens through the Bach1b/Nrf2a-MafK pathway (Wang et al., 2007; Zhang et al.,

78   2014), demonstrating that the utilities and power of zebrafish disease models for

79   delineating novel pathogenesis mechanisms (Santoriello and Zon, 2012). However, no

80   hereditable zebrafish *alad* and *cpox* models with genetic mutations have been generated

81   to date. A medaka (*Oryzias latipes*) *whiteout* (*who*) mutant was previously

82   characterized as a model for human ADP based upon its phenotype of hypochromic

83   anemia (Sakamoto et al., 2004), but medaka spawns relative fewer eggs (20~40) each

84   time (Furutani-Seiki and Wittbrodt, 2004) and has not yet been developed for

85   large-scale drug screens. Even though *cpox*-Morpholino-injected zebrafish embryos

86   were reported as an *in vivo* assay system for assessing biological activities of human

87   *CPOX* mutations (Hanaoka et al., 2006), these *cpox* morphants are not genetically

88   stable and particularly difficult to scale up for drug discoveries. A Nakano mouse

89   displays reduced CPOX enzymatic activities resulted from a *Cpox* mutation (Mori et

90   al., 2013), however, the Nakano mouse also harbors another mutation in the *Crybg3*

91   gene tightly linked with *Cpox* on mouse chromosome 16. Thus the Nakano mouse also

92   manifests cataracts that human HCP patients do not develop, whereby compounding its

93   phenotype and limiting its use in investigating novel aspects of porphyria pathogenesis.

94   Hence, zebrafish *alad* and *cpox* genetic models are needed.

95   Transcription activator-like effector nuclease (TALEN) and clustered regularly

96   interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)

97   are two site-specific genome-editing systems, each composed of a DNA-recognizing

98   component and a DNA-cleavage component (Hwang et al., 2013; Irion et al., 2014;

99   Wei et al., 2013). In the TALEN system, the two TALE domains are the

100   DNA-recognizing component and the two FokI domains are the DNA-cleavage

101   component (Huang et al., 2014), while in the CRISPR-Cas9 system, the single

102   guide-RNA (gRNA) is in charge of DNA-recognizing and the Cas9 endonuclease in

103   charge of DNA-cleavage (Hsu et al., 2014). A pair of TALENs or one gRNA along

104   with the Cas9 protein suffice to generate site-specific DNA double-strand breaks

105   (DSBs) that trigger the endogenous nonhomologous end joining (NHEJ) DNA repair

106   pathway to induce indel mutations in targeted genes in numerous species including

107   zebrafish (Cong and Zhang, 2015; Wei et al., 2013).

108   Here we used TALEN and CRISPR-Cas9 to generate zebrafish mutants for *alad* and

109   *cpox*. Characterization of these two zebrafish mutants found that they both manifest

110   phenotypes of heme deficiency, hypochromia, abnormal erythrocytic maturation and

111   accumulation of heme precursor intermediates, and in particular that *cpox* mutants

112   display reddish autofluorescence, resembling human ALA-dehydratase-deficient

113   porphyria (ADP) and hereditary coproporphyrian (HCP), respectively. These zebrafish

114   new models of human porphyrias are invaluable for elucidating novel pathological

115   aspects of porphyrias, in vivo assessments of mutations of these human disease genes,

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116   discovering new therapeutic targets and developing effective drugs for these complex

117   genetic diseases.

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119   **Results**

120   **Generation of zebrafish *alad* mutants with TALEN**

121   A pair of TALENs was designed to target exon 3 of zebrafish *alad*

122   (ENSDARG00000052815) with a BsmA I restriction endonuclease site in the targeted

123   fragment for subsequent mutant identification (Fig. 1A). Following microinjection of

124   this pair of *alad* TALEN mRNAs into single-cell zebrafish embryos, we extracted

125   DNAs from injected and control embryos. By PCR amplification and restriction

126   endonuclease digestion analysis, we estimated a mutagenesis rate of approximately

127   44% in F_0 embryos (Fig. S1A). To identify the induced mutation types, the uncleaved

128   DNA fragments were then cloned and sequenced. Sequencing results showed that there

129   are several types of deletions in the targeted fragment (Fig. 1B) (Xiao et al., 2013). The

130   remaining F_0 fish were raised to adulthood and then crossed with wild-type fish to

131   produce F_1. To screen for inheritable mutations, DNAs extracted from approximately

132   10 F_1 embryos of each cross were used for PCR amplification and enzymatic digestion

133   analysis. The germline transmission efficiency of *alad* TALEN-induced mutations was

134   estimated to be approximately 20% (Supplementary Table S1). Siblings of the F_1 fish

135   carrying inheritable mutations were raised to adulthood and re-identified by PCR,

136   restriction endonuclease digestion and sequencing of fin-clipped DNAs. An *alad*

137   mutant line carrying a 14-bp deletion resulting in a frame shift starting the 51th amino

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138   acid (ZFIN allele nomenclature designation, $alad^{sus003}$) (Fig. 1C) was used for the

139   further experiments.

Generation of zebrafish *cpox* mutants with CRISPR-Cas9

141   To generate zebrafish mutants for *cpox* (ENSDARG00000062025), we selected a 20-nt

142   gRNA immediate to a PAM (protospacer adjacent motif) sequence (NGG) located at

143   exon 2 of *cpox*, and the targeted fragment also contains a Hinf I restriction

144   endonuclease site for subsequent genotyping (Fig. 1D). Single-cell zebrafish embryos

145   were microinjected with the synthetic Cas9 mRNA and *cpox* gRNA. DNAs extracted

146   from injected and control embryos were PCR amplified. Restriction endonuclease

147   digestion of the PCR products detected approximately 44% to 49% of mutagenesis

148   frequency induced by gRNA-Cas9 (Fig. S1B). Cloning and sequencing of the

149   uncleaved DNA fragments showed several types of small deletions adjacent to PAM in

150   F_0 embryos (Fig. 1E). The remaining F_0 embryos were raised to adulthood. DNAs

151   extracted from the F_1 embryos produced by crossing F_0 fish with wild-type fish were

152   PCR amplified. Restriction endonuclease digestion of the PCR products identified the

153   corresponding F_0 fish carrying inheritable mutations. The germline transmission

154   efficiency of the *cpox* CRISPR/Cas9-induced mutations was approximately 17%

155   (Supplementary Table S1). Siblings of the F_1 fish identified as carrying inheritable

156   mutations were raised to adulthood and re-identified by PCR, restriction endonuclease

157   digestion and sequencing of fin-clipped DNAs. Two *cpox* mutant lines, one harboring a

158   6-bp deletion resulting in deletion of 2 amino acids (ZFIN allele nomenclature

159   designation, $cpox^{sus004}$) in a highly conserved region (Hanaoka et al., 2006) and the

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160   other possessing a 11-bp deletion resulting in a truncated peptide with only 208 amino

161   acids (ZFIN allele nomenclature designation, $cpox^{sus005}$), were identified (Fig. 1F).

162   These two *cpox* mutant fishes display similar phenotypes and were used in the

163   following experiments.

164   **Zebrafish *alad-/-* and *cpox-/-* mutants display severe hypochromic anemia**

165   The *alad-/-* and *cpox-/-* mutant embryos exhibit hypochromic phenotype as early as 48

166   hours post fertilization (hpf) (Fig. 2A). The circulating blood cells of the wild-type

167   pericardia are reddish, while those of the *alad-/-* and *cpox-/-* mutant pericardia are pale

168   (Fig. 2A). In addition, the *cpox-/-* mutant displays reddish autofluorescence (see the

169   result below), presumably due to the excessive accumulation of photosensitive

170   porphyrin. Although heterozygous *alad+/-* and *cpox+/-* fish appear unaffected

171   outwardly and can grow to adulthood, homozygous embryos die due to severe

172   embryonic anemia. The mass death of *alad-/-* homozygotes starts at 7 dpf, and their

173   survival rate drops to 0% at 14 dpf (Fig. 2D), suggesting that *alad-/-* homozygous

174   larvae cannot survive longer than 13 days. The mass death of *cpox-/-* starts at 6 dpf, and

175   *cpox-/-* homozygous larvae cannot survive longer than 11 days (Fig. 2D). Homozygous

176   *alad-/-* and *cpox-/-* larvae that survive past day 10 (Fig. 2B) are pale, and have much

177   shorter body length (Fig. 2C) but do not have observable ingested food in the intestine

178   compared to wild-type control larvae (Fig. 2B).

179   **Abnormal erythrocytic maturation in *alad-/-* and *cpox-/-* mutant larvae**

180   Whole-mount *in situ* hybridization and qRT-PCR show the expression of *slc4a1a*, a

181   terminal erythroid marker gene, significantly down regulated in *alad-/-* and *cpox-/-*

182   mutants (Fig. 2E, F and G), suggesting insufficient erythroid cells in these two

183   porphyric mutant zebrafish. Whole-embryo *o*-dianisidine staining also revealed that the

184   hemoglobin contents of blood cells are severely reduced in *alad-/-* and *cpox-/-* mutants

185   compared with wild types. Hemoglobin of wild-type embryos is distributed widely in

186   the ventral part of the yolk from 32hpf to 72hpf (Fig. 3A, D and G), while that of *alad-/-*

187   (Fig. 3B, E and H) and *cpox-/-* (Fig. 3C, F and I) mutant embryos can be observed only

188   in a small and narrow area and is markedly reduced compared with wild types (Fig. 3J).

189   We also performed the hematoxylin-eosin (HE) staining after *o*-dianisidine staining.

190   Results show that blood cells in *alad-/-* and *cpox-/-* lack the red color of *o*-dianisidine

191   staining signal compared with wild-type blood cells, suggesting loss of hemoglobin in

192   these two porphyric mutant zebrafish (Fig. S2A). The blood cell density in section

193   slides is also significantly reduced in *alad-/-* and *cpox-/-* (Fig. S2B), suggesting the

194   number of blood cells is significantly decreased in *alad-/-* and *cpox-/-*.

195   Wright staining of circulating embryonic red blood cells showed absence of

196   acidophilic materials (Fig. 3L-T), especially the cytoplasm in *alad-/-* does not show any

197   red color at 108 hpf (Fig. 3S). Blood smears assay showed that blood cells of *alad-/-*

198   and *cpox-/-* mutants have larger nuclei compared with wild-type cells at 72 hpf and 108

199   hpf (Fig. 3U), suggesting that the erythrocytes of the two mutants differentiate

200   abnormally.

201   Furthermore, heme contents of the circulating embryonic red blood cells are

202   significantly reduced in *alad-/-* and *cpox-/-* mutants than wild types (Fig. 3V), and the

203   mRNA levels of *hbae1* and *hbbe2*, encoding α_{e1}-globin and β_{e2}-globin, are significantly

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204   down-regulated in *alad*-/- and *cpox*-/- mutant fishes (Fig. 3K), indicating that heme

205   biosynthesis is disrupted in *alad*-/- and *cpox*-/- mutants, and subsequently leading to the

206   reduced hemoglobin (Tahara et al., 2004a; Tahara et al., 2004b) and abnormal

207   erythrocyte differentiation.

208   **Homozygous *alad*-/- and *cpox*-/- fishes resemble ADP and HCP**

209   The substrate of aminolevulinic acid dehydrase (Alad) is porphyrin precursor

210   δ-aminolevulinic acid (ALA), which normally does not accumulate in significant

211   amounts. The hepatic porphyrias are characterized by overproduction and

212   accumulation of ALA (Cappellini et al., 2010; Costa et al., 1997; Puy et al., 2010; Yano

213   and Kondo, 1998). HPLC (high-performance liquid chromatography) assays showed

214   excessive amounts of ALA in *alad*-/- embryos compared with wild-type embryos (Fig.

215   4A), indicative of defective Alad in the *alad*-/- mutant. In *cpox*-/-, the reddish

216   autofluorescence of the blood cells can be observed in the yolk as early as 30hpf (Fig.

217   4C, D), and HPLC assays also showed excessive amounts coproporphyrinogen

218   III (CP), the substrate of coproporphyrinogen oxidase (Cpox), in *copx*-/- embryos

219   compared with wild types, indicating the excessive accumulation of photosensitive

220   coproporphyrinogen III of the heme biosynthesis pathway.

221   To determine whether *alad* mRNA or *cpox* mRNA can rescue the phenotypes of

222   *alad*-/- or *cpox*-/- mutant fishes, we microinjected theses mRNAs into the mutant and

223   wild-type control embryos (Fig. 5). *o*-Dianisidine staining showed that the hemoglobin

224   content was increased to 60% of the normal level in *alald*-/- and 80% in *cpox*-/-

225   following mRNA microinjection, indicating that the hypochromic anemia phenotype of

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226 *alald-/-* and *cpox-/-* is resulted from the mutated *alad* or *cpox* genes. . Taken together,

227 homozygous *alad-/-* and *cpox-/-* fishes display phenotypes of hypochromia, heme

228 deficiency, abnormal erythrocytic maturation and accumulation of heme precursor

229 intermediates, mimicking human ALA-dehydratase-deficient porphyria (ADP) and

230 hereditary coproporphyrian (HCP).

231 **Evaluating the mutated forms of human ALAD and CPOX in homozygous *alad-/-***

232 **and *cpox-/-* fishes**

233 The zebrafish Alad and Cpox proteins are highly similar in sequence to human and

234 mouse counterparts (Fig. S3) (Hanaoka et al., 2006). In order to examine whether the

235 biological activities of these two enzymes are conserved between zebrafish and human,

236 *alad-/-* and *cpox-/-* embryos were microinjected with human *ALAD* or *CPOX* mRNAs

237 at one-cell stage, respectively. *o*-Dianisidine staining showed that hemoglobin was

238 significantly increased after injection compared with the mutant embryos without

239 injection at 48hpf (Fig. 5), indicating that the hypochromic anemia phenotype of *alad-/-*

240 and *cpox-/-* also can be rescued by human *ALAD* and *CPOX*. In contrast, microinjected

241 with mutated forms of human *ALAD* or *CPOX* mRNAs from the ADP and HCP patients

242 (Ishida et al., 1992; Rosipal et al., 1999) did not show any rescue effect (Fig. 5). These

243 results suggest that the functions of these two enzymes are conserved between

244 zebrafish and human, and we are able to effectively use homozygous *alad-/-* and

245 *cpox-/-* fishes to evaluate mutations of these two human disease genes.

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247 **Expression patterns of genes involved in heme biosynthesis and degradation, and**

248 **exocrine pancreatic zymogens are disrupted in ADP (*alad-/-*) and HCP (*cpox-/-*)**

249 **fishes**

250   The heme biosynthesis pathway is tightly regulated, and in particular heme

251 negatively regulates the first and rate-limiting enzyme Alas1 in the pathway in

252 non-erythroid cells (Furuyama et al., 2007). qRT-PCR analysis showed that *alas1* is

253 up-regulated in both ADP and HCP fishes (Fig. 6A, B), likely resulted from heme

254 deficiencies caused by *alad* and *cpox* mutations. In contrast, heme can enhance its own

255 synthesis in erythroid cells by up-regulating *alas2* (Chiabrando et al., 2014), and we

256 observed two *hmbs* (encoding the third enzyme Hmbs in the heme biosynthesis

257 pathway) genes, *hmbsa* and *hmbsb* in zebrafish (data not shown). While *alas2* and

258 *hmbsa* are up-regulated but *alad* itself and *hmbsb* are down-regulated in ADP larvae

259 (Fig. 6A), all genes including upstream and downstream of *cpox* except *ppox* are

260 up-regulated in HCP larvae (Fig. 6B).

261   Heme oxygenase (Hmox) is the rate-limiting enzyme of the heme degradation

262 pathway, and can be induced by heme and the oxidative stress of organisms (Applegate

263 et al., 1991; Igarashi and Sun, 2006). Zebrafish contain three *hmox* genes, *hmox1*,

264 *hmox2a* and *hmox2b*. While *hmox1*, *hmox2a* and *hmox2b* are up-regulated in HCP

265 larvae, only *hmox2a* is up-regulated in ADP larvae (Fig. 6C), indicating high levels of

266 the oxidative stress in these two mutants especially in HCP larvae, likely due to

267 accumulation of intermediate products of the heme biosynthesis pathway.

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268   Our previous studies found that heme deficiencies result in down-regulation of six

269   peptidase precursor genes in the zebrafish exocrine pancreas (Wang et al., 2007; Zhang

270   et al., 2014). Here qRT-PCR analysis showed that all the six zymogens are also

271   down-regulated in both ADP and HCP larvae (Fig. 6D), providing strong support for

272   our previous finding (Wang et al., 2007; Zhang et al., 2014).

273   **Complex syndromes in different zebrafish porphyria models**

274   Porphyrias show a group of complex syndromes, characteristic of diverse clinical

275   manifestations (Balwani and Desnick, 2012; Cappellini et al., 2010; Puy et al., 2010).

276   We also observed diverse phenotypes in the four zebrafish porphyria models with

277   defective Urod (Wang et al., 1998), Cpox (this study), Ppox (Dooley et al., 2008) and

278   Fech (Childs et al., 2000). First, different autofluorescence patterns can be observed in

279   these four zebrafish heme-deficient models (Fig. 4E-4N). Reddish fluorescence

280   displays primarily in blood cells and internal organs of HEP (*urod-/-*) larvae (Fig. 4H),

281   primarily in the internal organs and yolk of HCP (*cpox-/-*) (Fig. 4J) and VP (*ppox-/-*)

282   larvae (Fig. 4L), and in the whole body, especially in the lens, brain and liver of EPP

283   (*fech-/-*) larvae (Fig. 4N). Second, light-induced pericardial edemata have been

284   observed in HEP (*urod-/-*) larvae but not other heme-deficient zebrafish models. As

285   shown in Fig. S4, the appearance of HEP (*urod-/-*) larvae protected under dark

286   condition are normal, while HEP (*urod-/-*) larvae under light exposure display an

287   exacerbating pericardial edema phenotype from 72 hpf to 144 hpf, which cannot be

288   observed in HCP (*cpox-/-*) larvae. The distinct patterns of reddish fluorescence and

289   light-induced pericardial edemata may be resulted from accumulation of different

290   porphyrins or porphyrin precursors, and these mutants provide tools for reveal the

291   complex pathological mechanisms of human porphyrias.

292

293   **Discussion**

294   Zebrafish have become a bona fide vertebrate model for studying numerous

295   human diseases (Dooley and Zon, 2000; Lieschke and Currie, 2007). Heme

296   biosynthesis is conserved throughout vertebrate evolution (Detrich et al., 1995), all 8 of

297   human genes involved in heme biosynthesis pathway have zebrafish orthologues (Tzou

298   et al., 2014). Four zebrafish mutants *sauternes/alas2* (Brownlie et al., 1998),

299   *yquem/urod* (Wang et al., 1998), *montalcino/ppox* (Dooley et al., 2008) and

300   *dracula/fech* (Childs et al., 2000) were identified in large-scale forward genetic

301   mutagenesis screens (Ransom et al., 1996; Weinstein et al., 1996). A knock-in mouse

302   carrying human *UROS* P248Q missense mutation ($Uros^{mut248}$) represents a model of the

303   congenital erythropoietic porphyria (CEP) (Ged et al., 2006), *Pbgd*-deficient mice

304   generated by gene targeting was reported to exhibit the typical biochemical

305   characteristics of human acute intermittent porphyria (AIP) (Ged et al., 2006; Lindberg

306   et al., 1996), a *URO-D*$^{+/-}$ mouse was generated by homologous recombination as a

307   model of familial porphyria cutanea tarda (PCT) (Phillips et al., 2001), a South African

308   variegate porphyria (VP) mouse model was generated by knocking in the human R57W

309   mutation (Medlock et al., 2002; Meissner et al., 1996), and a BALB/c *Fech*^{m1Pas} mouse

310   was generated by a mutagenesis screen using ethylnitrosourea (ENU) as a model of

311   erythropoietic protoporphyria (EPP) (Tutois et al., 1991)(Table 1). Nevertheless,

312   zebrafish models for ALA-dehydratase deficient porphyria (ADP), acute intermittent

313   porphyria (AIP), congenital erythropoietic porphyria (CEP) and hereditary

314   coproporphyria (HCP) have not been reported to date. Here, we generated two

315   zebrafish models for ADP and HCP with TALEN and CRISPR-Cas9, respectively. Our

316   work emphasizes that the engineered endonucleases (EENs) TALEN and

317   CRISPR-Cas9 are efficient tools to generate mutations in zebrafish as models for

318   heritable human disease.

319   Our studies of *alad*-/- and *cpox*-/- mutants demonstrate that defects in Alad or

320   Cpox cause hypochromic anemia and abnormal erythrocytic maturation in zebrafish.

321   The accumulation of ALA and CP, the metabolic intermediates, in the heme synthetic

322   pathway in *alad*-/- and *cpox*-/- mutants (Fig. 4A, B) indicates that Alad and Cpox

323   enzymatic activities are impaired by these mutations. Expression of the genes involved

324   in the heme biosynthetic pathway is altered in *alad*-/- and *cpox*-/-, which may be caused

325   by the accumulation of metabolic intermediates and/or heme deficiency. The heme

326   oxygenases participate in the cellular defense from the oxidative damage of reactive

327   oxygen species (ROS) generated by heme precursors (Ryter and Tyrrell, 2000).

328   Although heme levels were reduced in *alad*-/- and *cpox*-/-, the heme-induced heme

329   oxygenase genes were up-regulated likely due to the increased level of oxidative stress.

330   Interestingly, consistent with our previous finding of heme deficiency-induced

331   down-regulation of exocrine pancreatic zymogens (Wang et al., 2007; Zhang et al.,

332   2014), all these six exocrine pancreatic zymogens are significantly down-regulated in

333   ADP (*alad*-/-) and HCP (*cpox*-/-) fishes (Fig. 6D).

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334   Most of the reported human ADP patients are compound heterozygous or

335   heterozygous, with point mutations of *ALAD* gene resulting in amino acid changes and

336   reduced enzyme activity (Ishida et al., 1992; Maruno et al., 2001). For the HCP

337   patients, most are heterozygotes with reduced enzymatic activities and homozygotes

338   are rare (Kuhnel et al., 2000; Lamoril et al., 1995; Nordmann et al., 1983). The

339   enzymatic activities of human ALAD or CPOX were investigated through *in vitro*

340   expression of mutated genes from patients (Martasek et al., 1997; Maruno et al., 2001).

341   Our rescue experiments showed that the biological activities of these two enzymes are

342   conserved between zebrafish and human (Fig. 5), these homozygous zebrafish provided

343   *in vivo* tools for evaluating these human mutated enzymes.

344   Reddish fluorescence was observed in *cpox-/-* embryos under an epifluorescent

345   stereomicroscope using a rhodamine filter (Fig. 4D). We found that four

346   heme-deficient zebrafish mutants exhibit distinct patterns of autofluorescence in

347   different organs (Fig. 4E-N), indicating the complex syndromes and distinct underlying

348   pathological mechanisms in different types of porphyrias. All eight types of porphyrias

349   have animal models, providing invaluable resources to study the pathogenesis of

350   human porphyrias, and six of them are zebrafish models thus far (Table 1). Circulating

351   blood cells of ADP (*alad-/-*) and HCP (*cpox-/-*) fish lack red color, characteristic of

352   hypochromic anemia in zebrafish, which can be observed as early as 48 hpf. Moreover,

353   both of these two homozygous mutants can live at least 7 days, providing a time

354   window for using these zebrafish models to investigate novel aspects of porphyria

355   pathogenesis.

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356   Zebrafish have been employed as an ideal vertebrate model organism for

357   high-throughout drug screens and development in whole organism (MacRae and

358   Peterson, 2015; North et al., 2007; Rennekamp and Peterson, 2015; Zon and Peterson,

359   2005). The adult heterozygous zebrafish with deficiencies of *alad* or *cpox* can spawn a

360   large number of embryos each week, homozygous mutant embryos can be easily

361   identified according to conspicuously pale blood cells (Fig. 2, Fig. S4), and the *cpox*-/-

362   homozygous embryos even can be identified as early as 32 hpf according to

363   autofluorescent traits under an epifluorescent stereomicroscope (Fig. 4), which all can

364   facilitate high-throughput chemical screening for discovering of potential drugs for

365   preventing or ameliorating the similar symptoms of ADP and HCP in humans. Potential

366   drugs can be dissolved in the embryo medium and the extent of recovery phenotypes

367   can be easily detected also through *o*-dianisidine staining (Fig. 5), which provide an

368   effective assay for drug screening for human porphyrias using these two zebrafish

369   models.

370   **Materials and Methods**

371   **Fish husbandry and embryo production**

372   The Soochow University Animal Use and Care Committee approved all animal

373   protocols. Zebrafish wild-type AB strain, and mutant line *alad*-/-, *cpox*-/-, *urod*-/-

374   (Wang et al., 1998), *ppox*-/- (Dooley et al., 2008) and *fech*-/- (Childs et al., 2000) are

375   raised at the Soochow University Zebrafish Facility. Wild-type (WT) and mutant

376   embryos were produced by pair mating, collected for RNA isolation at specified stages.

377   Homozygous mutants were obtained by mating heterozygous fish and then identified

378   under an epifluorescent stereomicroscope (Leica M165 FC). The zebrafish mutant lines

379   *alad* and *cpox* generated in this study (See below) have been deposited in both the

380   China Zebrafish Resource Center (CZRC, http://en.zfish.cn/) and the Zebrafish

381   International Resource Center (ZIRC, http://zebrafish.org/home/guide.php), and the

382   *alad* mutant ZFIN allele nomenclature ID is *alad*^{sus003} (14-bp deletion), and the *cpox*

383   ZFIN allele nomenclature mutant IDs are *cpox*^{sus004} (6-bp deletion) and *cpox*^{sus005}

384   (11-bp deletion), respectively.

385   **alad-TALEN construction and microinjection**

386   The TALEN sites targeting the third exon of zebrafish *alad* were designed using a

387   web-tool TALEN-NT (https://boglab.plp.iastate.edu/) (Doyle et al., 2012). TALEN

388   expression vectors were constructed using the 'Unit Assembly' method with

389   Sharkey-AS and Sharkey-R forms of FokI cleavage domains as described previously

390   (Huang et al., 2014), were linearized by NotI and used as templates for TALEN mRNA

391   synthesis with SP6 mMESSAGE mMACHINE Kit (Ambion). TALEN mRNAs

392   encoding each monomer were injected in pair into one-cell zebrafish embryos at

393   concentrations of 300 pg. Primers for amplifying the TALEN-targeted fragment are

394   listed in Supplementary Table S2.

395   **cpox-gRNA construction and microinjection**

396   A 20-nt gRNA was selected to target exon 2 of zebrafish *cpox*. The PCR primers for

397   the generation of the DNA template for *cpox*-gRNA were designed, and a T7 promoter

398   sequence was added to 5'upstream of the gRNA sequence. Cas9 mRNA and gRNA

399   were synthesized as described previously (Hwang et al., 2013). Briefly, the Cas9

19

400   mRNA was synthesized by *in vitro* transcription using T7 mMESSAGE mMACHINE

401   Kit (Ambion). The gRNA was *in vitro* transcribed and purified using T7 Riboprobe

402   Systems (Promega). Approximately 300 pg of Cas9 mRNA and 75 pg of gRNA were

403   co-injected into one-cell zebrafish embryos. Primers for amplifying the CRISPR-Cas9

404   targeted fragment are listed in Supplementary Table S2.

405   **RNA isolation and quantitative RT (Real Time)-PCR**

406   Total RNAs were extracted from approximately 20-30 larvae with the TRIzol®

407   Reagent according to the manufacturer's instructions (Invitrogen). The cDNAs

408   synthesized by reverse transcription with the M-MLV reverse transcription kit

409   (Invitrogen) were used as template for quantitative RT-PCR (qRT-PCR) analysis.

410   qRT-PCRs were carried out with the ABI StepOnePlus^{TM} systems, using SYBR$^{®}$

411   Premix Ex Taq™(TaKaRa) and the following thermal profile: 95$^{°}$C for 3 min, and 40

412   cycles of 95$^{°}$C , 10 sec; and 58$^{°}$C, 30 sec. Primers are listed in Supplementary Table S2.

413   *actb1* was used as an internal control. Relative mRNA expression levels were

414   quantified using the comparative Ct (ΔCt) method and expressed as $2^{-(\Delta\Delta Ct)}$. Each PCR

415   assay was done with three biological samples.

416   **Construction of the expression vectors**

417   The *alad* and *cpox* ORF (open reading frame) cDNAs were PCR amplified using

418   zebrafish larvae (120hpf) cDNAs as template, then cloned to pcDNA3.1+, and named

419   *alad*-pcDNA3.1 and *cpox*-pcDNA3.1, respectively. Primers for *alad* and *cpox* ORF

420   cloning are listed in Supplementary Table S2. The human *ALAD* and *CPOX* ORF

421   cDNAs were PCR amplified using the NIH 293T cells' cDNAs as template, and then

20

422   cloned to pcDNA3.1+, and named *ALAD*-pcDNA3.1 and *CPOX*-pcDNA3.1,

423   respectively. The mutated ORF cDNAs of human *ALAD* (C^{718}-T,)(Ishida et al., 1992)

424   and *CPOX* (G^{589}-T)(Rosipal et al., 1999) were generated with Q5$^{®}$ Site-Directed

425   Mutagenesis Kit (NEB) using *ALAD*-pcDNA3.1 and *CPOX*-pcDNA3.1, respectively.

Rescue experiments and *o*-dianisidine staining

427   The plasmids of expression vectors *alad*-pcDNA3.1, *cpox*-pcDNA3.1,

428   *ALAD*-pcDNA3.1 and *CPOX*-pcDNA3.1 were linearized, and used as templates for

429   generating capped mRNAs with mMESSAGE mMACHINE$^{®}$ Kit according to the

430   manufacturer's instructions (Ambion). A mixture of capped mRNA in Tris-HCl

431   (0.01M, pH7.0) were microinjected into one-cell zebrafish embryos. Microinjection

432   controls with the vehicle solution free of mRNAs also were performed. Embryos were

433   collected at 48hpf for the *o*-dianisidine staining. Detection of hemoglobin by

434   *o*-dianisidine was performed as described previously (Ransom et al., 1996). The images

435   were acquired with a stereomicroscope microscope (Leica M165 FC) and a digital

436   camera.

Blood smears assay, Wright staining and heme level determination

438   We obtained embryonic blood cells and performed Wright staining as described

439   previously (Brownlie et al., 1998; Hanaoka et al., 2006). Embryos were bled by tail

440   amputation and immediately spread on glass slides, allowed to air dry and stained with

441   wright's dye for 10 minutes, then washed with 1 X PBS. The images were acquired with

442   a stereomicroscope microscope (Olympus U-HGLGPS) and a digital camera.

443   Circulating blood cells maintained in 1 X PBS were collected from 20 embryos and

444   were counted. The heme level assay was performed with the QuantiChrom^{TM} Heme

445   Assay Kit (BioAssay Systems).

446   **Whole-mount *in situ* hybridization and HE (hematoxylin-eosin) staining**

447   Whole-mount in situ hybridization experiments were conducted as described

448   previously (Wang et al., 2007). Following the whole-embryo *o*-dianisidine staining,

449   selected embryos were rehydrated in 30% sucrose at 4°C overnight for cryostat

450   sectioning. Frozen embryos were sectioned to 8 μm thickness, and hematoxylin-eosin

451   staining was performed as standard protocol. Sections were photographed using a

452   stereomicroscope (Leica M165 FC) with a digital camera, and the images were

453   analyzed by using ImageJ (National Institutes of Health).

454   **HPLC (High-performance liquid chromatography) assay**

455   For the ALA assay, approximately 300 larvae each for *alad-/-* and WT control were

456   collected at 7 days postfertilization (dpf) for HPLC assay. These larvae were then

457   promptly rinsed and homogenized in 10 mM HEPES buffer for ALA assay as

458   previously described (Costa et al., 1997). HPLC assay was conducted using

459   5-Aminolevulinic acid hydrochloride (Sigma) as ALA standard.

460   For the CP assay, approximately 300 zebrafish larvae at 7 dpf were pulverized into

461   fine powder and homogenized in 1 mL 0.2 M HCl aqueous solution by a tissue grinder.

462   The porphyrins were extracted twice into ether as described (Dailey et al., 2015). The

463   extracts were combined and dried under a stream of nitrogen. Standards and samples

464   were resuspended in 100 μl of ethyl acetate/acetic acid (4/1, v/v). The compounds are

465   separated by liquid chromatography on the Accela LC Systems (Thermo Fisher

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466   Scientific, San Jose, CA) using a Hypersil GOLD column (3 μm, 150 × 2.1 mm, Part

467   Number: 25003-152130, Thermo Fisher Scientific). Chromatographic separation was

468   conducted in binary gradient using 0.1% formic acid in water as mobile phase A and 0.1%

469   formic acid in methanol as mobile phase B. The gradient elution program started at 40%

470   B for 5 min, increased to 100% B over 7 min, where it was then held for 6 min before

471   returning to 40% B. The flow rate was 0.2 mL/min. MS analysis of Coproporphyrin III

472   was performed on a TSQ Vantage mass spectrometer (Thermo Fisher Scientific) in

473   positive mode using SRM as described(Fyrestam et al., 2015). Coproprophyrinogen III

474   (Frontier Scientific) was used as CP standard (Tomokuni et al., 1987).

475   **Statistical analysis**

476   The data were analyzed with unpaired, two-tailed Student's *t*-test with Microsoft

477   Excel and the results were shown as mean ± SD. The level of significance was accepted

478   at $P<0.05$. *, ** and *** represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

479

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483   suggestions on this study.

484

485   **Competing interests**

486   The authors declare no competing financial interests.

487

488   **Author contributions**

489   H.W. and S.Z. conceived and designed the experiments. S.Z., J.M., Z.N., and J.W.

490   performed the experiments. Y.H. and X.S. helped measure CP contents. Y.Z. provided

491   reagents. S.Z. and H.W. analyzed the data and wrote the paper.

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506   **Supplementary materials**

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508   Supplementary materials available online at

509

510   **Reference**

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Table 1. Animal models for human porphyrias

Human disease	Gene	Animal model	Species
ALA dehydratase deficient porphyria	ALAD	*whiteout* *alad*	Medaka Zebrafish
Acute intermittent porphyria	HMBS	T1/T2(-/-)	Mouse
Congenital erythropoietic porphyria	UROS	$Uros^{mut248}$	Mouse
Porphyria cutanea tarda /hepatoerythropoietic porphyria	UROD	*yquem* *URO-D*+/-	Zebrafish Mouse
Hereditary coproporphyria	CPOX	Nakano *cpox*	Mouse Zebrafish
Variegate porphyria	PPOX	*Montalcino* Human R59W mutation Knock-in	Zebrafish Mouse
Erythropoietic protoporphyria	FECH	*dracula* BALB/c $Fech^{m1Pas}$	Zebrafish Mouse

679

680

681

682   **Figure legends**

683   **Fig. 1. TALEN and CRISPR-Cas9 targeted sites and mutated sequences.** (A)

684   TALENs were designed to target the third exon of the zebrafish *alad* gene. The

685   wild-type (WT) sequence with the two TALEN arms is highlighted in blue, and the

686   BsmA I restriction endonuclease site for genotyping is underlined. (B) WT sequence

687   and F_0 mutated sequence with indels at the *alad* TALEN-targeted fragment. (C) A

688   *alad* mutant line contains a 14-bp deletion resulting in a frame shift starting at the 51st

689   amino acid. (D) A 20-nt gRNA was designed to target the second exon of the zebrafish

690   *cpox* gene. The WT sequence with the PAM is highlighted in blue, and the Hinf I

691   restriction endonuclease site for genotyping is underlined. (E) WT sequence and F_0

692   mutated sequences with indels at the *cpox* CRISPR-Cas9-targeted fragment. (F) Two

693   *cpox* mutant lines, one containing a 6-bp deletion resulting in deletion of the 2 amino

694   acids (210 Leu and 211 Thr) and the other having a 11-bp delection resulting a

695   truncated peptide with only 208 amino acids.

696   **Fig. 2. Phenotypes of homozygous mutants with mutated *alad* and *cpox* genes.** (A)

697   Lateral views of WT, *alad-/-* and *cpox-/-* (6-bp deletion) zebrafish embryos at 48 hpf.

698   Arrows indicate the pericardium. (B) Lateral views of WT, *alad-/-* and *cpox-/-* (6-bp

699   deletion) zebrafish larvae at 10 dpf. (C) Body lengths of *alad-/-* and *cpox-/-* (6-bp

700   deletion) larvae at 10 dpf compared with wild types. 25 larvae per group were

701   measured for statistical analysis. Student's *t*-tests were conducted. ***P<0.001. (D)

702   Survival rate of *alad-/-* and *cpox-/-* (6-bp deletion) from 1 dpf to 13d pf compared with

703   wild types. 40 larvae per group were examined for statistical analysis . (E) *In situ*

30

704   hybridization staining shows the down-regulation of *slc4a1a* in *alad-/-* and *cpox-/-*

705   (11-bp deletion) embryos at 48 hpf. (F) Mean optic densities of *in situ* hybridization

706   staining of a group of larvae (10-12 each) corresponding to Fig. 2E were quantified by

707   using ImageJ. Student's *t*-tests were conducted. *P<0.05. (G) qRT-PCR analysis

708   shows down-regulation of *slc4a1a* in *alad-/-* and *cpox-/-* (11-bp deletion). Student's

709   *t*-tests were conducted. *P<0.05.

710   **Fig. 3. Abnormality of blood cells in *alad-/-* and *cpxo-/-* mutants.** Whole-mount

711   *o*-dianisidine staining of WT (A, D and G), *alad-/-* (B, E and H) and *cpxo-/-* (6-bp

712   deletion) (C, F and I) embryos at 32 hpf (A, B and C), 48 hpf (D, E and F), and 72 hpf

713   (G, H and I). Shown are ventral views. (J) Statistical analysis of optic densities of

714   whole-mount *o*-dianisidine staining, shown in A, B, C, D, E, F, G, H and I, measured

715   by ImageJ. 20 embryos per group were measured for statistical analysis. Student's

716   *t*-tests were conducted. *P<0.05, **P<0.01, ***P<0.001. (K) Relative mRNA levels

717   of *hbae1* and *hbbe2* in WT, *alad-/-* and *cpxo-/-* (6-bp deletion) embryos at 48hpf.

718   Circulating red blood cells of WT (L, O and R), *alad-/-* (M, P and S) and *cpxo-/-* (6-bp

719   deletion) (N, Q and T) embryos/larvae at 36 hpf (L, M and N), 72 hpf (O, P and Q),

720   and 108 hpf (R, S and T) with Wright staining. (U) Statistical analysis of optic nuclear

721   areas shown in L, M, N, O, P, Q, R, S, and T. The nuclei of *alad-/-* and *cpox-/-* (6-bp

722   deletion) are larger than those of WT at 72 hpf and 108 hpf. Optic nuclear areas were

723   measured by ImageJ. 30 blood cells per group were measured for statistical analysis.

724   Student's *t*-tests were conducted. ***P<0.001. (V) Relative heme levels of circulating

31

725 red blood cells in WT, *alad*-/- and *cpxo*-/- (6-bp deletion) embryos at 7 dpf,

726 determined by the QuantiChrom^{TM} Heme Assay Kit.

727 **Fig. 4. Accumulation of metabolic intermediates in *alad*-/- and *cpxo*-/- mutants.**

728 (A) ALA measurements with HPLC (High-performance liquid chromatography) in

729 WT and *alad*-/- larvae at 7 dpf. Relative fluorescence of ALA peak in WT and *alad*-/-.

730 (B) CP measurements with HPLC in WT and *cpox*-/- (11-bp deletion) larvae at 7 dpf.

731 Relative fluorescence of CP peak in WT and *cpox*-/- (11-bp deletion). Reddish

732 autofluorescence of *cpox*-/- embryo at 32 hpf (C, D), the top embryo is *cpox*-/-

733 mutants, the bottom one is the wild-type siblings. (C) Bright light image. (D) Image

734 under a fluorescent microscope with a rhodamine filter. The arrow indicates

735 autofluorescence of the yolk sac of the *cpox*-/- (6-bp deletion) embryo.

736 Autofluorescence analysis of larvae of wild-type (E, F), *urod*-/- (G, H), *cpox*-/- (6-bp

737 deletion) (I, J), *ppox*-/- (L, L) and *fech*-/- (M, N) at 72hpf. (E, G, I, K and M), Bright

738 light image. (F, H, J, L and N) Image under a fluorescent microscope with a

739 rhodamine filter.

740 **Fig. 5. Rescuing the phenotypes of zebrafish *alad*-/- and *cpxo*-/- mutants by**

741 **zebrafish and human genes.** (A) Whole-mount *o*-dianisidine staining of wild-type

742 embryos, *alad*-/- embryos, *alad*-/- embryos injected with zebrafish *alad* mRNA and

743 *alad*-/- embryos injected with human *ALAD* mRNA at 48 hpf. (B) Statistical analysis

744 of optic densities measured by ImageJ. 25 embryos per group were measured for

745 statistical analysis. Student's *t*-tests were conducted. *P<0.05, ***P<0.001. (C)

746 Whole-mount *o*-dianisidine staining of wild-type embryos, *cpox*-/- (6-bp deletion)

32

747   embryos, *cpox*-/- (6-bp deletion) embryos injected with zebrafish *cpox* mRNA and

748   *cpox*-/- embryos injected with human *CPOX* mRNA at 48 hpf. (D) Statistical analysis

749   of optic blood cells about C. Optic densities were measured by ImageJ, 20 embryos

750   per group were measured for statistical analysis. Student's *t*-tests were conducted.

751   *P<0.05, ***P<0.001.

752   **Fig. 6. Altered expression of genes involved in heme biosynthesis and degradation,**

753   **and exocrine pancreatic zymogens in *alad*-/- and *cpox*-/- mutants detected by**

754   **qRT-PCR analysis.** (A) Altered expression of genes involved in heme biosynthesis

755   including *alas1*, *alas2*, *alad*, *hmbsa* and *hmsb* in *alald*-/- at 48 hpf. (B) Altered

756   expression of genes involved in heme biosynthesis including *alas1*, *alas2*, *urod*, *cpox*

757   and *ppox* in *cpox*-/- (6-bp deletion) at 48 hpf. (C) Altered expression of genes involved

758   in heme degradation including *hmox1*, *hmox2a* and *hmox2b* in *alald*-/- and *cpox*-/-

759   (6-bp deletion) at 48 hpf. (D) Down-regulation of exocrine pancreatic zymogens in

760   *alald*-/- and *cpox*-/- (6-bp deletion) at 72 hpf. Student's *t*-tests were conducted.

761   *P<0.05, **P<0.01, ***P<0.001, n.s., not significant.

762

763

764   **Resource Impact**

765

766   **Clinical issue**

767   Heme is the prosthetic group for a number of important proteins and enzymes such as

768   hemoglobin, catalases, and cytochromes. Heme biosynthesis is catalyzed by a cascade

769   of highly conserved eight enzymatic reactions occurred in the mitochondria and the

770   cytoplasm. Defects in the heme biosynthetic enzymes result in a group of human

771   metabolic genetic disorders known as porphyrias. Using a zebrafish model for human

772   hepatoerythropoietic porphyria (HEP), caused by defective uroporphyrinogen

773   decarboxylase (UROD), the fifth enzyme in the heme biosynthesis pathway, we

774   recently have found a novel aspect of porphyria pathogenesis. However, no hereditable

775   zebrafish models with genetic mutations of *alad* and *cpox*, encoding the second

776   enzyme delta-aminolevulinate dehydratase (ALAD) and the sixth enzyme

777   coproporphyrinogen oxidase (CPOX), have been established to date.

778   **Results**

779   We employed site-specific genome-editing tools transcription activator-like effector

780   nuclease (TALEN) and clustered regularly interspaced short palindromic repeats

781   (CRISPR)/CRISPR-associated protein 9 (Cas9) to generate zebrafish mutants for *alad*

782   and *cpox*. Characterization of these two zebrafish mutants revealed that they display

783   phenotypes of heme deficiency, hypochromia, abnormal erythrocytic maturation and

784   accumulation of heme precursor intermediates, reminiscent of human

785   ALA-dehydratase-deficient porphyria (ADP) and hereditary coproporphyrian (HCP).
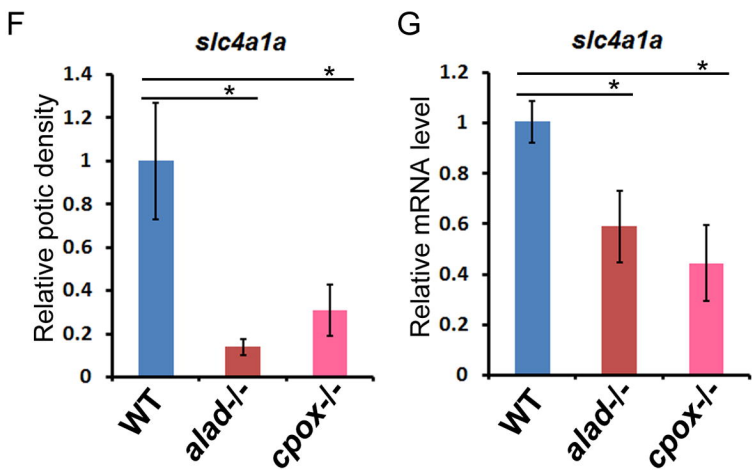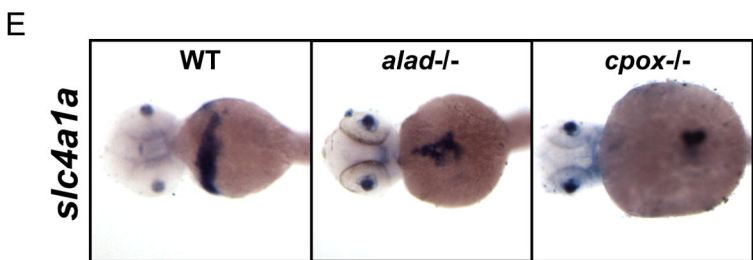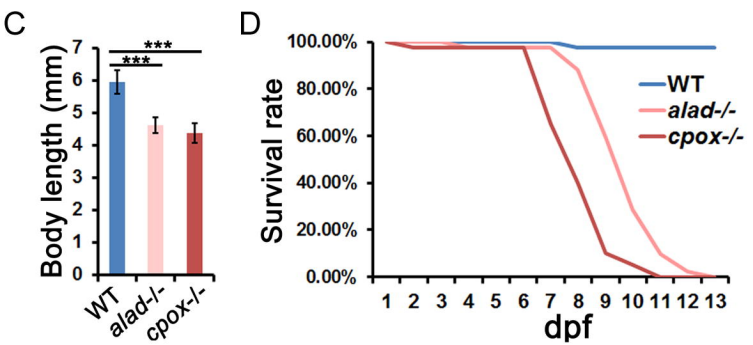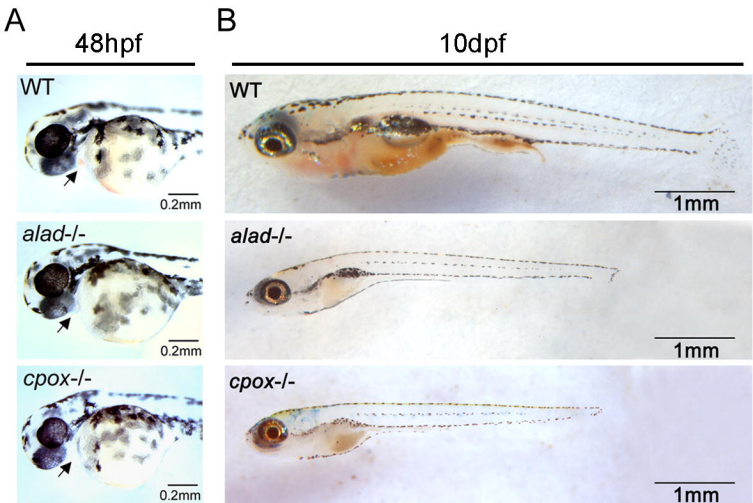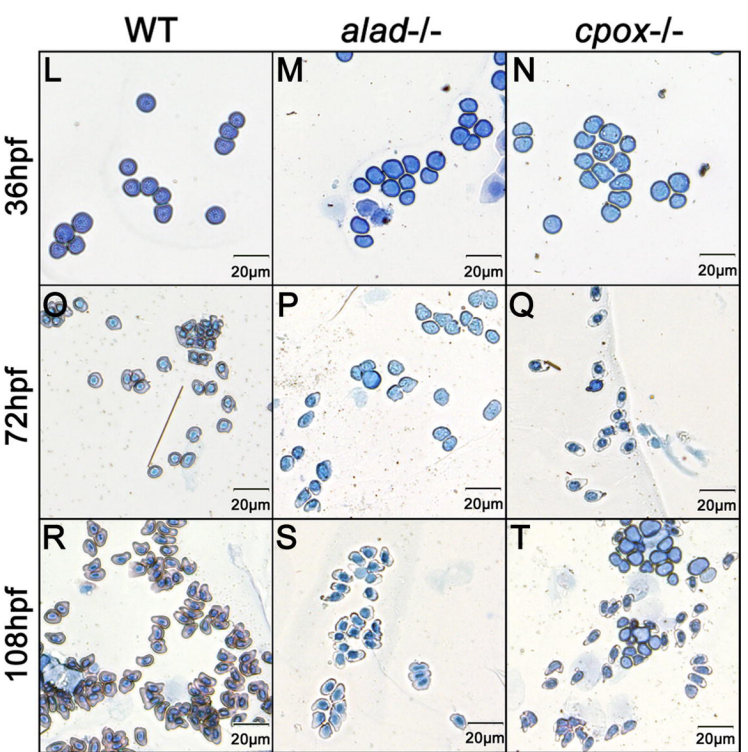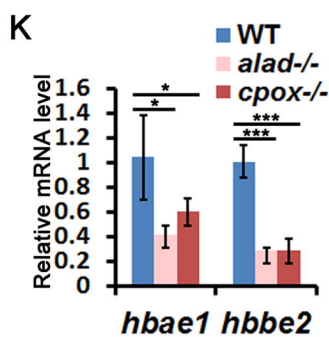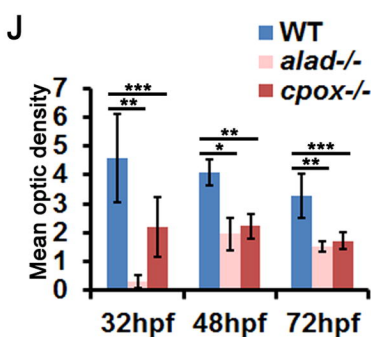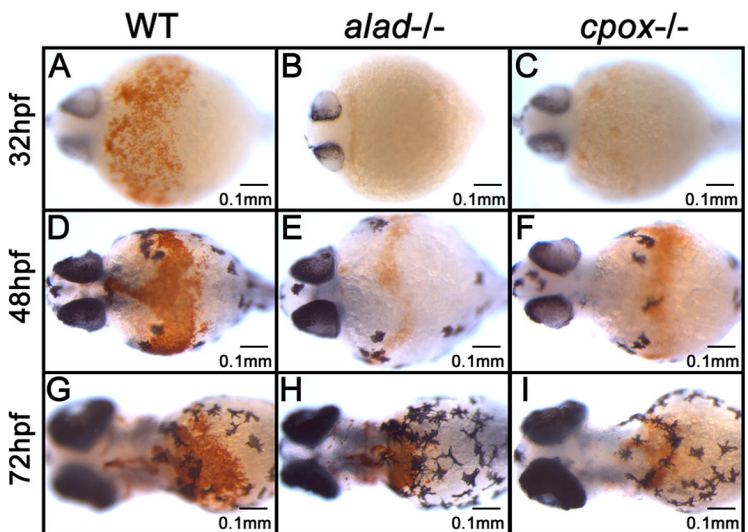
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Implications and future directions
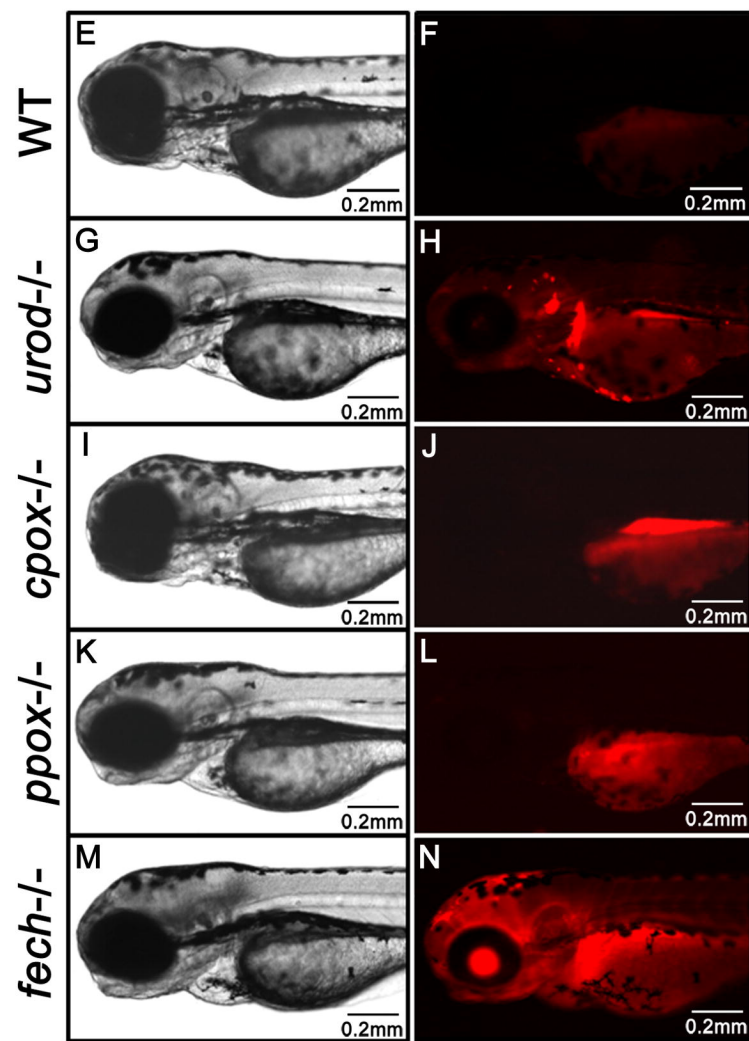
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788   These two zebrafish porphyria models provide invaluable resources for elucidating

789   novel pathological aspects of porphyrias, *in vivo* tools for evaluating human mutated

790   forms of these two enzymes, discovering new therapeutic targets and developing

791   effective drugs for these complex genetic diseases. Our studies also highlight

792   generation of zebrafish models for human diseases with two new versatile

793   genome-editing tools.

794

35

A

E1 E2 E3 E4 E5 E11 E12

alad locus

Left   BsmA I   Right

TGCAGTTGAACCAATTGCAAGTCTCCCTGGTCAAGCAAGGTATCAATTATA

TALEN target site in *alad*

B

WT sequence | TGCAGTTGAACCAATTGCAAGTCTCCCTGGTCAAGCAAGGTATCAATTATA

Mutated
sequence

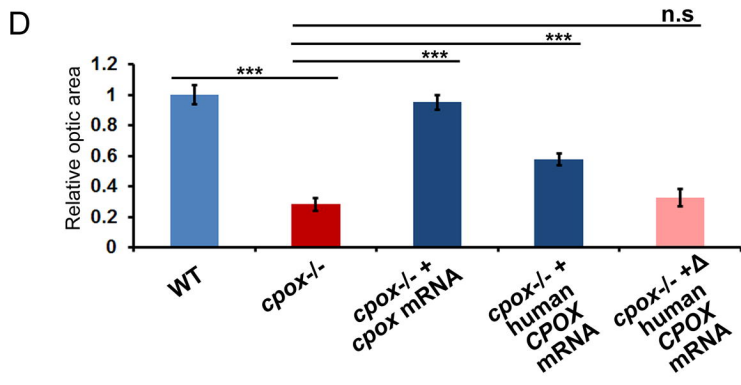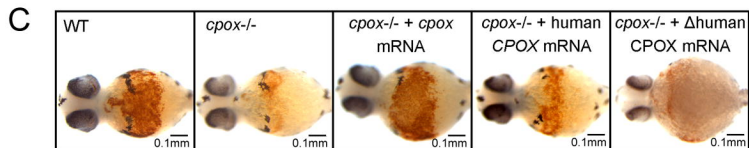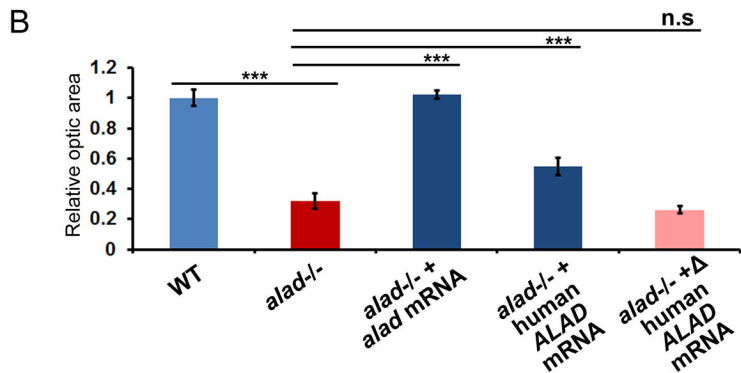TGCAGTTGAACC-----T--------------------TGGTCAAGCAAGGTATCAATTATA (-15bp)
TGCAGTTGAACCAATTGCAA-------------------GTCAAGCAAGGTATCAATTATA (-9bp)
TGCAG-----------------------CTGGTCAAGCAAGGTATCAATTATA (-21bp)
TGCAGTTGAACCAATTGCAAG---------------CAAGGTATCAATTATA (-14bp)

C

WT Alad   N [================================] C
           1   332

Mutated Alad N [====////////////////////////] C
              1 51   332

D

E1 E2 E3 E4 E5 E6 E7

cpox locus

Hinf I

GGTGTATGGGAATCTTACTGAGG

CRISPR/Cas9 target site in *cpox*

E

WT sequence | GGTGTATGGGAATCTTACTGAGG

Mutated
sequence

GGTGTAT--G----------------G--GG (-12bp)
GGTGTATGGGAAT---------GAGG (-6bp)
GGTG--------------------TACTGAGG (-11bp)

F

WT Cpox   N [================================] C
          1   209 212   450

Mutated Cpox N [////////////////////////////] C
              1   209   448

Mutated Cpox N [==============////////////////] C
              1   208   450

A 48hpf — WT, *alad-/-*, *cpox-/-* (scale bars 0.2mm)

B 10dpf — WT, *alad-/-*, *cpox-/-* (scale bars 1mm)

C

Body length (mm)

*** ***

WT *alad-/-* *cpox-/-*

D

Survival rate

100.00%
80.00%
60.00%
40.00%
20.00%
0.00%

1 2 3 4 5 6 7 8 9 10 11 12 13 dpf

WT
alad-/-
cpox-/-

E

slc4a1a

WT   *alad-/-*   *cpox-/-*

F

slc4a1a

Relative potic density

1.4
1.2
1
0.8
0.6
0.4
0.2
0

*   *

WT *alad-/-* *cpox-/-*

G

slc4a1a

Relative mRNA level

1.2
1
0.8
0.6
0.4
0.2
0

*   *

WT *alad-/-* *cpox-/-*

WT
alad-/-
cpox-/-

A 32hpf
B
C

D 48hpf
E
F

G 72hpf
H
I

J

K

L WT 36hpf
M *alad-/-*
N *cpox-/-*

O 72hpf
P
Q

R 108hpf
S
T

U

V

A

B

C

cpox-/-

WT

D

cpox-/-

WT

E   WT

F

G   urod-/-

H

I   cpox-/-

J

K   ppox-/-

L

M   fech-/-

N

A

B

C

D