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1 **Loss-of-function mutations in the melanocortin-1-receptor (Mc1r) cause**
2 **disruption of dorso-ventral countershading in teleost fish**

3

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6

7 Short title: Fish pigmentation and melanocortin system

8

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31 **ABSTRACT**

32 The Melanocortin 1 receptor (MC1R) is the central melanocortin receptor involved in
33 vertebrate pigmentation. Mutations in this gene cause variations in coat coloration in
34 amniotes. Additionally, in mammals MC1R is the main receptor for agouti signaling
35 protein (ASIP), making it the critical receptor for the establishment of dorsal-ventral
36 countershading. In fish, *Mc1r* is also involved in pigmentation but it has been almost
37 exclusively studied in relation to melanosome dispersion activity and as a putative
38 genetic factor involved in dark/light adaptation. However, its role as the crucial
39 component for the *Asip1*-dependent control of dorsal-ventral pigmentation remains
40 unexplored. Using CRISPR/Cas9 we created *mc1r* homozygous knockout zebrafish
41 and found that loss-of-function of *mc1r* causes a reduction of countershading and a
42 general paling of the animals. We find ectopic development of melanophores and
43 xanthophores, accompanied by a decrease in iridophore numbers in the ventral region
44 of *mc1r* mutants. We also reveal subtle differences in the role of *mc1r* in repressing
45 pigment cell development between the skin and scale niches in ventral regions

46

47 **Keywords:** *Mc1r*, *Asip1*, pigmentation, melanophores, xanthophores, iridophores,
48 chromatophore, zebrafish, countershading, CRISPR

49

50 **SIGNIFICANCE**

51 Countershading is a widespread pigmentary adaptation throughout vertebrates and
52 graded agouti-signaling peptide (ASIP) is the main regulatory effector. In mammals,
53 *Asip* expression signals through melanocortin receptor type-1 (MC1R) to inhibit
54 melanogenesis and melanocyte differentiation *in vitro*. Strikingly, in fish *Asip*-
55 dependent dorso-ventral patterning depends upon multiple types of chromatophore,

56 but the skin expresses multiple MCRs and the role for Mc1r remains untested. We
57 show here that Mc1r is required to block melanophore and xanthophore, and to
58 promote iridophore, development in ventral skin. In contrast, loss of Mc1r function
59 only partially reduced melanophores and xanthophores dorsally, suggesting
60 involvement of additional signaling systems.

61

62 **INTRODUCTION**

63 Pigment patterns have always intrigued scientists for both their evolutionary and
64 developmental aspects, and fish constitute excellent models because of their great
65 color pattern diversity. Zebrafish (*Danio rerio*) has become an important model to
66 study color pattern formation and cell fate decisions in vertebrates. Its striped
67 pigmentation is achieved by the patterned distribution of three types of pigment cells
68 (chromatophores), melanophores, xanthophores and iridophores. Both melanophores
69 and xanthophores absorb light due to the synthesis of melanin and pteridine pigments,
70 respectively; whereas iridophores reflect light because of guanine crystals (Hirata et
71 al., 2003, Hirata et al., 2005) . This striped pattern is superimposed on an ancient dorso-
72 ventral countershading pattern, with dark dorsum and light ventrum (Cal et al., 2017;
73 Ceinos et al., 2015). Dorso-ventral pigment polarity is a highly conserved evolutionary
74 trait (Linnen et al., 2013). It is regulated by the local presence/absence of agouti-
75 signaling protein (ASIP, simply called Agouti in mouse) (Vrieling et al., 1994). In
76 mammals ASIP binds to the melanocortin receptor 1 (MC1R), encoded by the
77 extension (e) locus, and which is the receptor for melanin-stimulating hormone (MSH)
78 (Robbins et al., 1993). Binding of agouti to MC1R lowers the ratio of eumelanin
79 (dark/brown pigment) to pheomelanin (red/yellow pigment) produced in the
80 melanocytes (mammalian melanophores) (Michaud et al., 1993; Miller et al., 1993),

81 inhibits melanoblast differentiation and proliferation (Aberdam et al., 1998;
82 Sviderskaya et al., 2001) and promotes dedifferentiation of melanocytes *in vitro* (Le
83 Pape et al., 2009). Agouti expression in different regions of the body is controlled by
84 distinct regulatory regions of the agouti gene. It results in either ventral specific or hair
85 cycle specific isoforms controlled by distinct regulatory enhancer regions of the *agouti*
86 gene. These result in either ventral specific or hair cycle specific isoforms. The ventral
87 isoform is constitutively expressed during development thus driving melanocytes to
88 synthesize pheomelanin and resulting in pale coloration. Dorsal-specific isoform
89 expression is temporally regulated during the hair cycle, thus producing the distinctive
90 yellow subapical band of the agouti fur coloration (Vrieling et al., 1994). Dorsoventral
91 pigmentation gradients in fish also depend on a dorso-ventral expression gradient of
92 *Asip1* (the fish ortholog of mammalian ASIP) (Cerdá-Reverter et al., 2005; Ceinos et
93 al., 2015; Guillot et al., 2012;), but the cellular and biochemical mechanisms seem to
94 be different from that of mammalian species. Fish melanophores synthesize only
95 eumelanin (Kottler et al., 2015), therefore changes in dorsoventral pigmentation can
96 be achieved either by inhibition of melanogenesis or by differential chromatophore
97 distribution. In zebrafish, *asip1* overexpression inhibits melanogenesis in the dorsal
98 region (Ceinos et al., 2015; Guillot et al., 2012) by antagonizing melanocortin
99 signaling (Cerdá-Reverter., 2005), but it also promotes the proliferation of dorsal
100 iridophores (Ceinos et al., 2015). Accordingly, *asip1* knockout has no effect on dorsal
101 pigmentation but the lack of *Asip1* in the belly promotes a dorsalization of the ventral
102 pigmentation by increasing melanophore differentiation and xanthophore numbers in
103 parallel with the disappearance of most ventral iridophores (Cal et al., 2019). Taken
104 together, the gradient of *asip1* expression is responsible for the dorso-ventral

105 pigmentation gradient in fish by using a different mechanism from tetrapods, i.e. by
106 inhibiting ventral melanogenesis and modulating chromatoblast fate.

107 This analysis leaves an important question concerning the signaling mechanism that
108 *Asip1* uses in zebrafish to regulate pigmentation. To date, five different melanocortin
109 receptors (Mc1r-Mc5r) have been characterized in vertebrates (Cortés et al., 2014).
110 Mc2r is unique because it is exclusively activated by the adrenocorticotrophic hormone
111 (ACTH) and it requires the interaction with the melanocortin receptor accessory
112 protein 1 (Mrap1) (Cerdá-Reverter et al., 2013). All other receptors bind the diverse
113 MSH-ligands with different affinities (Schiöth et al., 2005), and ACTH can also
114 activate Mc4r in combination with Mrap2 (Agulleiro et al., 2013). *Asip1* has been
115 shown to antagonize the effects of Msh on Mc1r and Mc4r in goldfish (Cerdá-Reverter
116 et al., 2005). In addition, *Asip1* can inhibit (Nle4, D-Phe7)- α -MSH-stimulated
117 melanosome dispersion (Cerdá-Reverter et al., 2005). Zebrafish has six melanocortin
118 receptors, since Mc5r is duplicated, as Mc5ra and Mc5rb (Cortés et al., 2014). They are
119 antagonized by *Asip1* but the protein seems to work also as an inverse agonist at Mc1r
120 (Guillot et al., 2012), with low Mc1R activity occurring in the absence of MSH or the
121 presence of *Asip1*. Although the involvement of Mc1r signaling in the agouti
122 phenotype of zebrafish has been suggested, we cannot rule out the participation of
123 other Mcr receptors since their expression in fish skin has been reported, e.g. Mc4r in
124 cyprinids (Cerdá-Reverter et al., 2003; Wei et al., 2013), Mc5r in flatfishes, goldfish,
125 and bass (Cerdá-Reverter et al., 2003; Kobayashi et al., 2012; Sánchez et al., 2009)
126 and Mc2r in bass (Agulleiro et al., 2013). Here, we investigate the role of Mc1r in
127 zebrafish pigmentation *in vivo* by generating knockout mutants using the
128 CRISPR/Cas9 genome-editing tool (Bassett et al., 2013). We demonstrate a disruption
129 of the dorso-ventral pigmentation polarity in *mc1r* knock-out fish, consistent with it

130 being the key receptor mediating Asip1 function in zebrafish dorsoventral
131 countershading.

132

133 **MATERIALS AND METHODS**

134 **Fish**

135 Zebrafish were cultured as previously described (Westerfield., 2007) and staged by
136 standard criteria (Kimmel et al., 1995). Fish of the following genotypes were used: Tü
137 strain (Tübingen, Nüsslein-Volhard Lab), Tg(Xla.Eef1a1:Cau.Asip1)iim05 (Ceinos et
138 al., 2015), Tg(TDL358:GFP)(Levesque et al., 2013) , Tg(kita:GalTA4:UAS:mCherry)
139 (Anelli et al., 2009). Ethical approval (Ref. Number: AGL2011-23581) for all studies
140 was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC
141 Institute in accordance with the National Advisory Committee for Laboratory Animal
142 Research Guidelines licensed by the Spanish Authority (RD53/2013) and conformed
143 to European animal directive (2010/63/UE) for the protection of experimental animals.

144

145 **Generation and analysis of *mc1r* knockout mutants**

146 The *mc1r* loss-of-function mutation was generated using the CRISPR-Cas9 gene
147 editing system. The CRISPR protocol, originally adapted from Bassett et al. (Bassett
148 et al., 2013), was kindly provided by Sam Peterson (U Oregon). The possible target
149 sequence was identified with the ChopChop web tool (Montague et al., 2014). Two
150 long oligonucleotides (Scaffold oligo: 5`-
151 GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT
152 TATTTTAACTTGCTATTTCTAGCTCTAAAAC-3`, and two different gene-
153 specific oligo GS1: 5`-
154 AATTAATACGACTCACTATAGCTAGTGAGCGTCAGTAATGGTTTTAGAGC

155 TAGAAATAGC-3' and GS2: 5'-
156 AATTAATACGACTCACTATAGGGCCAAGATGAACATGTGCAGGGTTTTAG
157 AGCTAGAAATAGC-3') were used to perform a template-free PCR to obtain a 125
158 bp DNA fragment that includes the two previously identified target site sequences
159 (TS1: 5'- GCTAGTGAGCGTCAGTAATGTGG-3' and TS2: 5'-
160 GGGCCAAGATGAACATGTGCAGG-3'). The PCR reaction was performed in 20
161 μ L containing 10 μ L of 2x Phusion High-Fidelity PCR Master Mix Buffer (New
162 England Biolabs, UK), 1 μ L of gene specific oligo (10 μ M), 1 μ L of gRNA scaffold
163 oligo (10 μ M) and H₂O nuclease free to 20 μ L. PCR conditions were 98°C for 30 sec,
164 40 cycles of 98°C for 10 sec, 60°C for 10 sec, 72°C for 15 sec, and a final step of 72°C
165 for 10 min. The PCR product was purified using DNA Clean&Concentration-5 Kit
166 (Zymo Research, USA) according to the manufacturer's instructions. Purified PCR
167 product was used as template for in vitro transcription with MEGAscript T7 High yield
168 transcription Kit (Ambion, USA) according to the manufacturer's instructions. The
169 gRNA was purified with RNA Clean&Concentrator 5 (Zymo Research, USA). The
170 gRNA was injected in a concentration of 25 ng/ μ L together with Cas9 mRNA (from
171 the pT3TS-nCas9n plasmid, Addgene, USA) at 50 ng/ μ L and Phenol red solution
172 (0,1%). Around 2 nL of this mix was microinjected into zebrafish eggs. Different
173 mutations were found and three different potential non-functional mutations were
174 raised as separate mutant allele lines (see results section). Primer sequence for
175 genotyping PCR were: TS1-F: CTTCAGCATGAAACACATGGA; TS1-R:
176 ATGGTGCACAGAAACGACAA; TS2-F: CTTCAGCATGAAACACATGGA;
177 TS2-R: AAGGGTTTGTGGGACAGGTG. For microscope imaging, zebrafish of
178 5dpf, 15 dpf, 30 dpf and 210 dpf were anesthetized with tricaine methasulfonate (MS-
179 222-, Sigma-Aldrich), skin sections and scales were isolated from the ventral and

180 dorsal areas and immersed in PBS on a glass slide and photographed.
181 Transgenic/mutant lines were obtained by setting up crosses between the *mc1r* mutant
182 lines and the reporter transgenic line Tg(TDL358:GFP), which labels iridophores
183 (Levesque et al., 2013), or Tg(kita:GalTA4:UAS:mCherry), which labels
184 melanophores (Anelli et al., 2009). The offspring of these crosses were incrossed to
185 obtain homozygous *mc1r* mutants. Confocal imaging was carried out on a Leica TCS
186 SP5 confocal microscope.

187

188 **Melanophore and xanthophore counts**

189 The melanophore pattern of *mc1r* knockout mutant fish (*mc1r*^{K.O.}) was compared with
190 control fish by quantification of melanized melanophores in both groups (Fig. 3, 4).
191 Selected regions for melanophore counting were different in each stage of
192 development. In early stage (5dpf), we counted melanophores in a 1 mm² area in a
193 dorsal view on the head and the dorsal area, on the horizontal myoseptum and in a
194 ventral view of the head. In early metamorphic stage (15dpf), we counted
195 melanophores in a 1 mm² area in a dorsal view on the head, on the dorsal area, on the
196 horizontal myoseptum and in a ventral view of the head and the belly. In late
197 metamorphic stage (30dpf), we counted melanophores in a 1 mm² area in a dorsal view
198 on the head, dorsal area, horizontal myoseptum and in a ventral view on the head and
199 belly. In adult fish (60 and 210 dpf) melanophores within 1 mm² area were counted in
200 several distinct positions: in a dorsal view on the head (head area) and on the dorsal
201 area (from the edge of the head to edge of the dorsal fin); in a lateral view, on the
202 dorsalmost dark stripe (2D), 1D, first ventral dark stripe under the myoseptum (1V)
203 and 2V anterior areas (pectoral to pelvic fin); and finally, in a ventral view of the head
204 and the belly (pectoral to pelvic fin) (see Fig. 4A; Fig 7Q-T). The dorsal-ventral

205 xanthophore pattern of *mc1r* knockout fish mutant was compared with control fish by
206 quantification of pigmented xanthophores in post-metamorphic fish (60 and 210 dpf).
207 For xanthophore counting, selected regions on the dorsal area (from the edge of the
208 head to edge of the dorsal fin), and in a ventral view of the belly (pectoral to pelvic
209 fin) were selected (see Fig. 4A). To analyze the number of melanophores and
210 xanthophores, seven fish per group were anesthetized with tricaine methasulfonate
211 (MS-222-, Sigma-Aldrich) and immersed in 10 mg/ml epinephrine (Sigma) solution
212 for 30 min to contract melanosomes. Fish were photographed on a Leica M165FC
213 stereomicroscope equipped with a Leica DFC310FX camera. Melanophores were
214 counted using ADOBE PHOTOSHOP CS2 software (Adobe Systems Software Adobe
215 Systems Ibérica SL, Barcelona, Spain) and the ImageJ software (National Institutes of
216 Health, NIH, Maryland, USA). Data values were statistically evaluated by Student's
217 t-test and data expressed as mean standard error of the mean (SEM).

218

219 **Functional *Asip1*/*Mc1r* interaction**

220 Transgenic/knockout line (*mc1r^{K.O.}/Asip1-Tg*) were obtained by setting up crosses
221 between the CRISPR1-*mc1r.iim02* mutant line and the transgenic reporter line
222 Tg(Xla.Eef1a1:Cau.*Asip1*)iim05 (Ceinos et al., 2015), which ectopically
223 overexpresses *asip1* and produces a dorsal-ventral disruption of pigment pattern
224 phenotype with dorsal skin as pale colored as ventral skin. The offspring was then
225 incrossed to obtain the F2 generation and the *mc1r* locus was sequenced to confirm
226 the homozygous knockout mutation (*mc1r^{K.O.}*) that carries the dominant *asip1*
227 transgene, localized by PCR using specific primers for the Tol2 vector (forward: 5'-
228 GCCCCTCTGCTAACCATGTTC-3', reverse: 5'-
229 TCATCAATGTATCTTATCATGTCTGG-3'). Adult double transgenic/mutant

230 zebrafish (100dpf) were anesthetized with MS-222 and photographed. Microscope
231 imaging was carried out in a Leica M165FC stereomicroscope equipped with a Leica
232 DFC310FX camera.

233

234 **RESULTS**

235 **Generation and selection of induced *mc1r* loss-of-function mutations**

236 Loss-of-function mutations in the zebrafish *mc1r* gene were generated using the
237 CRISPR/Cas9 system. We selected two different target sites in the single coding exon
238 (target T1: 286 bp after ATG and target T2: 636 bp after ATG) (Fig. 1A, B) and found
239 six different mutated alleles (Fig. 1B). Alleles M1 and M6 conserved the original open
240 reading frame; therefore, they could potentially generate a functional protein lacking
241 only one or two amino acids but keeping most of the complete amino acid sequence.
242 Alleles M2, M3, M4 and M5 show different open reading frames downstream of the
243 target site. The *mc1r* gene encodes a predicted protein of 323 amino acids. We selected
244 three mutations with predicted non-functional protein and established stable
245 homozygous mutant lines of each one to characterize the phenotype: M2 (CRISPR1-
246 *mc1r.iim02*), M3 (CRISPR1-*mc1r.iim03*), and M4 (CRISPR1-*mc1r.iim04*) (Fig. 1B).
247 The *mc1r^{iim02}* allele lacks 7 bp (Del 264-271) and carries 11 bp insertion at position
248 264 downstream of the predicted ATG. The *mc1r^{iim03}* allele has lost 2 bp (Del 642-
249 644), and *mc1r^{iim04}* lacks 2 bp (Del 642-644) and carries 16 bp insertion at position
250 642 downstream of the predicted ATG (Fig. 1B). In those three alleles, the mutations
251 result in premature stop codons. The *mc1r^{iim02}* encodes a 123 amino acids mutant
252 protein with 95 shared amino acids with WT Mc1r , the *mc1r^{iim03}* encodes a mutant
253 protein with 231 amino acids with 214 shared amino acids with WT Mc1r , and
254 *mc1r^{iim04}* encodes a mutant protein with 243 amino acids with 214 shared amino acids

255 with WT Mc1r (Fig. 1C). None of the predicted mutated proteins have the last three
256 α -helical transmembrane (TM) domains and an intracellular C-terminus with a
257 palmitoylation site which are required for ligand-receptor binding and the appropriate
258 tertiary structure of the receptor, therefore none of these mutant proteins would be
259 predicted to retain partial function. All *mc1r* knockout mutant lines examined resulted
260 in a similar dorso-ventral pigment phenotype as described below.

261

262 ***mc1r* functions in dorso-ventral pigment patterning**

263 All three homozygous *mc1r*-CRISPR knockout lines exhibited an indistinguishable
264 reduction of dorso-ventral countershading (Fig. 2), therefore we focused on the study
265 of one homozygous line, CRISPR1-*mc1r*.iim02 (referred to as *mc1r^{K.O.}*). *mc1r^{K.O.}* fish
266 displayed enhanced pigmentation over the entire ventral region (Fig. 2 B, D, F, H), as
267 well as a significant reduction over the dorsal and lateral regions (Fig. 2 D, J) compared
268 to WT siblings. In particular, in *mc1r^{K.O.}* fish, the number of melanophores and
269 xanthophores is increased in all ventral regions (Fig 2. G, H) together with a
270 concomitant reduction of these two pigment cell types in all dorsal regions (Fig.2 I, J).
271 This phenotype results in an apparent paling of the animal with a corresponding
272 reduction of countershading. Mutations of the *mc1r* gene had no major effect on the
273 characteristic striped pattern (Fig.2 A-D). However, we observe a significant decrease
274 of melanophore numbers in the dorsal 2D dark stripe, and the incipient 3V-stripe is
275 more pronounced in *mc1r^{K.O.}* mutants compared to wild type (Fig. 2C,D). In addition,
276 the abdominal ventral region exhibits a decrease in the number of iridophores that
277 results in an apparent breakup of the ventral iridophore layer into smaller fragments,
278 thus conferring a darker color to the ventral region of *mc1r^{K.O.}* fish (Fig. 2H).

279

280 **The development of the zebrafish *mc1r^{K.O.}* phenotype**

281 To establish the time point when the phenotype of the *mc1r^{K.O.}* mutants first becomes
282 apparent during development, melanophores were counted at larval (5dpf, SL 3 mm),
283 metamorphic (15 dpf, SL 6.3 mm and 30 dpf, SL 7 mm) and two adult stages (60 dpf,
284 SL 13 mm and 210 dpf, SL 25 mm) (Fig. 3 and 4). It has been shown that pigment
285 pattern changes during development can be quantified by an increase in melanophore
286 numbers and variations in their distribution (Kelsh., 2004; Parichy et al., 2009) . We
287 determined the melanophore distribution in *mc1r^{K.O.}* and WT siblings along the dorso-
288 ventral axis, by counting at defined positions in the dorsal and ventral head, the lateral
289 stripes and the belly (see Materials and Methods and Figs. 3 and 4 for details). We
290 found a significant general reduction of melanophore density in *mc1r^{K.O.}* at all
291 developmental stages evaluated. At pre-metamorphic (15dpf) and metamorphic stages,
292 the melanophore density were significantly lower in all areas examined. During pre-
293 metamorphic stages (15dpf), melanophore density was lower in the dorsal, lateral and
294 belly regions of *mc1r^{K.O.}* mutants compared to WT fish (Fig. 3A). During late
295 metamorphosis (30dpf), melanophore density was also lower in the dorsal, lateral
296 region and belly of *mc1r^{K.O.}* mutants compared to WT fish (Fig. 3B). At adult stages
297 (60 and 210 dpf), *mc1r^{K.O.}* mutants exhibited an obvious dorso-ventral pigmentation
298 defect characterized by an increase of melanophore and xanthophore density in the
299 ventral areas (Fig. 4B) together with a significant decrease of melanophore and
300 xanthophore density in the dorsal and lateral body sections, resulting in a general
301 homogenization of melanophores densities across the dorsoventral axis (Fig. 4C). At
302 60 dpf, the density of melanophores in *mc1r^{K.O.}* mutants compared to WT fish was
303 lower in the dorsal region, the dark stripe 2D, the dark stripe 1D and in the dark stripe
304 1V. However, in the ventral region of the head, the density of melanophores was higher

305 in *mc1r^{K.O.}* mutants compared to WT fish. No differences were found in the dark stripe
306 2V and the belly (Fig. 4C). Additionally, the density of xanthophores was also
307 affected. *mc1r^{K.O.}* mutants show a significant increase in the number of ventral
308 xanthophores (P<0.01) compared to WT fish (Fig. 4D). A similar phenotype was
309 found at 210 dpf; melanophore densities in 210 dpf *mc1r^{K.O.}* mutants were
310 considerably lower in the dorsal region of the head, in the dorsal region, in the dark
311 stripe 2D and in the dark stripe 1D compared to WT. In contrast, the density of
312 melanophores in the ventral region, in the dark stripe 3V and in the belly of *mc1r^{K.O.}*
313 fish was significantly higher than in their WT siblings (Fig. 4E). The density of
314 xanthophores in the dorsal region of *mc1r^{K.O.}* mutants was lower than in WT fish.
315 However, the density of xanthophores in the ventral regions of *mc1r^{K.O.}* fish was
316 considerably higher than in WT fish (Fig. 4F).

317 We also compared the distribution of transgenic markers for melanophores and
318 iridophores in *mc1r^{K.O.}* mutants and their WT siblings to assess densities of possible
319 unpigmented pigment cells or their precursors. Firstly, we imaged fish carrying the
320 Tg(Kita:GalTA4,UAS:mCherry) transgene, which labels melanophores with
321 membrane-bound mCherry (Anelli et al., 2009). WT fish showed no pigmented
322 melanophores in the ventral skin (Fig. 5A, B), but there were also no unpigmented
323 mCherry-expressing cells suggesting a complete absence of melanophores and
324 melanoblasts (Fig. 5B). In contrast, *mc1r^{K.O.}* mutants showed an increase in mCherry
325 labelled melanophores in the ventral skin region (Fig. 5C, D). This is in agreement
326 with the detected increase in the number of melanophores in ventral regions (Fig. 4).
327 By analyzing fish carrying Tg(TDL358:GFP), a transgene labeling iridophores and
328 glia with cytosolic GFP (Levesque et al., 2013), we confirmed the presence of a dense
329 and uniform sheet of iridophores in the ventral abdominal skin of WT fish (Figs. 5E,

330 F) and showed that this layer is broken up into smaller fragments in *mc1r^{K.O.}* mutants
331 (Fig. 5G, H). Although it is difficult to quantify the contribution of changed cell
332 numbers to this phenotype, *mc1r^{K.O.}* mutants display a significant reduction of the
333 extent of iridophores, together with a significantly increased number of melanophores
334 and xanthophores (Fig. 5G) within the ventral abdominal skin.
335 Finally, we characterized the contribution of pigment cells in the scales to the disrupted
336 countershading and pale phenotype in *mc1r^{K.O.}* mutants. Scales isolated from the
337 dorsum of *mc1r^{K.O.}* mutants displayed a substantial reduction of melanophores (Fig.
338 6B, black arrowheads) compared to WT fish (Fig. 6A). In contrast to ventral scales of
339 WT siblings which lack all pigmented cell-types (Fig. 6C), ventral scales from *mc1r^{K.O.}*
340 mutants display some ectopic melanophores (Fig. 6D, black arrowheads) and
341 numerous xanthophores (Fig. 6D, yellow arrowheads).

342

343 **Zebrafish *Asip1* is likely acting as an inverse agonist of Mc1r**

344 To determine if *Asip1* signaling is mediated by Mc1r, we analyzed the combination of
345 *mc1r^{K.O.}* with the *asip1*-Tg zebrafish line that overexpresses *asip1* in the entire body.
346 We predicted that, if *Asip1* signaling functions solely through Mc1r that the *mc1r^{K.O.}/*
347 *asip1*-Tg fish would show a pattern identical to the *mc1r^{K.O.}* line, whereas if another
348 receptor contributed to *Asip1* signaling, the pattern would be more similar to the *asip1*-
349 Tg line. Both *mc1r^{K.O.}* and *asip1*-Tg zebrafish lines differ significantly in their overall
350 pigmentation phenotype compared to WT fish (Fig. 7). WT fish (Fig. 7A) show a
351 specific striped pattern (Fig. 7 B), a light ventrum (Fig. 7C) and a darker dorsum (Fig.
352 7D). In *mc1r^{K.O.}* mutants (Fig. 7E) the striped pattern is barely affected (Fig. 7F), but
353 they show a darker belly (Fig. 7G) and a paler dorsum with fewer melanophores than
354 WT fish (Fig. 7 H,R). The *asip1*-Tg zebrafish phenotype presents a slightly affected

355 striped pattern (Fig 7J), a light belly similar to WT fish (Fig. 7K), but a drastic
356 reduction of dorsal melanophores (Fig. 7L,S) due to the ectopic overexpression of
357 *asip1* (Ceinos et al., 2015). In the combination of *mc1r^{K.O.}* with *asip1*-Tg, the *asip1*-
358 Tg phenotype is suppressed and the *mc1r^{K.O.}* phenotype prevails (Fig. 7M). The
359 *mc1r^{K.O.}* + *asip1*-Tg zebrafish line presented the same barely affected stripe pattern
360 like *mc1r^{K.O.}* alone (Fig. 7 N), the pigmented belly (Fig. 7O) and the dark dorsum with
361 similar numbers of melanophores as the *mc1r^{K.O.}* fish (Fig. 7P,T). Fish overexpressing
362 *asip1* are paler than fish lacking Mc1r. This suggests that Asip1 has additional effects
363 to those promoted by Mc1r, either by competitive antagonism or inverse agonism, as
364 the overexpression phenotype is stronger than absence of the receptor. Fish that
365 overexpress Asip1 but also lack Mc1r have a ventrally dorsalized pigmentation
366 phenotype, similar to fish only lacking Mc1r, indicating that Asip1 is probably acting
367 as an inverse agonist of Mc1r to elicit the opposite response from that produced by α -
368 Msh. Additionally, scales isolated from the dorsum of the *mc1r^{K.O.}/ asip1*-Tg fish
369 displayed a similar substantial reduction of melanophores than *mc1r^{K.O.}* mutants (Fig.
370 S1A,C) compared to WT fish (Fig. 6A) and an increase of melanophores compared to
371 *asip1*-Tg fish (Fig.S1B) . Ventral scales from *mc1r^{K.O.}/ asip1*-Tg also showed some
372 melanophores and numerous xanthophores (Fig. S1F).

373

374 **DISCUSSION**

375 Zebrafish, a major teleost fish model for pigmentation studies, utilizes two distinct
376 mechanisms to generate the adult pigmentation, the striped patterning and the dorso-
377 ventral countershading mechanism (Ceinos et al., 2015). Both mechanisms function
378 largely independently, with the resultant patterns superimposed to give the full pattern
379 (Ceinos et al., 2015). The dorso-ventral expression gradient of *asip1* is responsible for

380 the countershading, through inhibition of melanogenesis in the ventral region where
381 *asip1* is highly expressed. However, *Asip1* is also involved in the regulation of
382 chromatophore numbers since its overexpression modifies the melanophore/iridophore
383 ratio by promoting iridophore differentiation in absence of melanophores (Ceinos et
384 al., 2015). Our previous studies have demonstrated that *Asip1* works as a competitive
385 antagonist of both *Mc1r* and *Mc4r* (Cerdá-Reverter et al.,2005; Guillot et al., 2016)
386 but likely also as an inverse agonist of the constitutively activated *Mc1r* (Guillot et al.,
387 2016; Sánchez et al., 2010). *Asip1* overexpression in zebrafish results in a
388 ventralization of the dorsal skin pigmentation due to a substantial reduction in the
389 number of melanophores and the concomitant production of extra iridophores, without
390 effects on the ventral pigmentation (Ceinos et al., 2015). In blind Mexican cave tetra
391 (*Astyanax mexicanus*) *Mc1r* plays a key role in the establishment of the adult pigment
392 pattern; inactivating mutations are responsible for the reduction in the number of
393 melanophores, a phenotype that can be recapitulated in zebrafish by *mc1r* morpholino
394 knockdown (Gross et al., 2009). We now demonstrate that inactivating mutations of
395 *mc1r* in zebrafish lead to a reduction in the number of dorsal melanophores, and that
396 also extends to the dorsalmost stripe. In addition they lead to a significant reduction in
397 the number of dorsal xanthophores during all post-metamorphic stages studied. If
398 *Asip1* works as an inverse agonist of the constitutively activated *Mc1r*, inactivating
399 mutations of the cognate receptor would be predicted to recapitulate the *asip1-Tg*
400 dorsal effect. However, the number of dorsal melanophores in *asip1-Tg* fish was less
401 reduced in an *mc1r^{K.O.}* background when compared to a WT background. Also, *asip1-*
402 *Tg* zebrafish exhibit an extra dorsal band of iridophores (Ceinos et al., 2015), which is
403 absent in *mc1r^{K.O.}* mutants. Therefore, overexpression of *asip1* in *mc1r^{K.O.}* background
404 yields a similar phenotype to that of *mc1r* knockout mutant unmasking what is

405 essentially an epistatic relationship between *asip1* and *mc1r*. Our data demonstrate
406 that *Asip1* displays a more pronounced effect when *Mc1r* is present and suggests that
407 the effect is more than simply blocking of the constitutive activity of *Mc1r* (Ollmann
408 et al., 1998). Similar results have been reported in mouse showing that individuals
409 ubiquitously expressing high levels of *asip* exhibit paler yellow fur than that seem
410 when *mc1r* is absent (Ollmann et al., 1998; Jackson et al., 2007).

411 The dorsal root ganglia (DRG) host multipotent stem cells that can generate all three
412 pigment-cell types found in the post-metamorphic skin of zebrafish (Singh et al.,
413 2016). These stem cells remain multipotent until well into metamorphosis when
414 individual pigment cell fates become specified (Singh et al., 2016). Under our original
415 model, the constitutive or MSH-induced activation of *Mc1r* promotes melanocyte fate
416 decisions and differentiation in the dorsal skin, but in the more ventral regions, the
417 inverse agonism or competitive antagonism of *Asip1* would block *Mc1r* activity,
418 instead promoting the differentiation of iridophores. Accordingly, we would expect
419 the phenotype of the *asip1-Tg* line (where *Mc1r* inactivation is driven by dorsal
420 overexpression of *asip1*) to be similar to the phenotype of the *mc1r^{K.O.}* line (where all
421 *Mc1r* is eliminated by mutation). As discussed above both dorsal phenotypes (*asip1-*
422 *Tg* vs *mc1r^{K.O.}*) differed extensively thus challenging this original hypothesis. Our
423 results suggest that melanophore lineage specification and differentiation in the dorsal
424 skin are not fully dependent on *Mc1r* activity and thus, some other receptors,
425 presumably other *Mcrs*, interact with *Asip1*. The presence of different *Mcrs* has been
426 reported in several species (see introduction for references). However, we cannot
427 exclude that some other non-melanocortin receptors could be involved in *Asip1*
428 function, since *Agouti* is able to increase intracellular Ca^{2+} levels in skeletal myocytes
429 via mechanisms that may not involve *Mcr* antagonism (Zemel et al., 1995). In mice, it

430 has been suggested that the stronger pheomelaninic phenotype of *asip* overexpressing
431 mice carrying *mc1r* mutation might result from a β -arrestin-mediated mechanism
432 leading to increased cAMP degradation, reducing the signaling to greater level than
433 when *mc1r* is absent (Jackson et al., 2007). Such a mechanism would also be
434 conceivable here in zebrafish.

435 Our data suggest that most precursors in the dorsal region need an *Asip1*-free
436 environment to become melanophores but the *mc1r^{K.O.}* phenotype suggests that only
437 some of them seem to require a functional *Mc1r*. However, the *mc1r^{K.O.}+asip1-Tg*
438 phenotype is similar to the *mc1r^{K.O.}* phenotype, suggesting that the inhibitory effects
439 of *Asip1* on melanophores require a functional *Mc1r*.

440 We note that only the melanophores along the dorsal midline are apparently resistant
441 to the presence of high *Asip1* levels (see schematic representation on Fig. 8).
442 However, these melanophores are distinct in that they are localized immediately dorsal
443 to the CNS and not in the skin; these dorsal midline cells might be insensitive to *Asip1*,
444 for example, because they lack *mc1r* expression. Consistent with this explanation, we
445 note that these dorsal midline melanophores are unaffected in *mc1r* mutants and the
446 *mc1r^{K.O.};Asip1-Tg* combination. Furthermore, our observations indicate that these cells
447 are likely to derive from persisting embryonic melanocytes of the early larval dorsal
448 stripe, rather than from adult pigment stem cells. Alternatively, a trivial explanation
449 of the difference might be that the *Asip1-Tg* shows less robust expression of *Asip1* in
450 the adjacent tissues (CNS and/or dorsal myotome).

451 In contrast to the dorsal region, the effects of *Mc1r* dysfunction in the ventral region
452 depend on the developmental stage. During the early post-metamorphic stages, *Mc1r*
453 knockout induces similar effects to those observed in the dorsal region, i.e. a decrease
454 in the density of melanophores. However, in adult animals, the effects are opposite to

455 those observed in the dorsal area or during the early post-metamorphic stages in the
456 ventral skin. This we interpret as a dorsalisation of the ventral region. Therefore,
457 functional Mc1r is necessary to block melanophores and xanthophores in favor of
458 iridophores in the ventral skin of adult animals. Ventral *Asip1* levels would limit
459 melanophore/xanthophore specification, differentiation and/or proliferation and
460 conversely promote iridophores, but since this pathway requires the expression of
461 *mc1r*, its absence in the mutants will allow the production of ventral melanophores and
462 xanthophores and result in a reduction in the iridophore number. This dorsalisation
463 phenomenon of the ventral region extends also to the ventral-most stripes, with a
464 thickened 2V-stripe and a fully-developed 3V-stripe in the ventrum of *mc1r^{K.O.}* mutant
465 lines. Therefore, *Asip1*-induced inhibition of melanogenesis and melanophore
466 differentiation *via* Mc1r may limit the addition of new dark stripes in the ventral
467 region.

468 Finally, we also analyzed the effect of Mc1r dysfunction in the dorsal and ventral
469 scales. As expected, ventral scales in WT fish lack all chromatophores but the absence
470 of a functional Mc1r results in the development of ectopic melanophores and numerous
471 xanthophores, again consistent with our interpretation of this dorsalisation of the
472 ventral skin. However, the effect on dorsal scales was slightly different from that
473 observed in the dorsal skin. Dorsal scales exhibit a reduction in the number of
474 melanophore similar to that recorded in the dorsal skin but the number of xanthophores
475 is increased dramatically. Further work will be necessary to understand the different
476 responses to Mc1r absence of these progenitors in scales versus the skin.

477 In summary, we demonstrate that Mc1r is involved in the establishment of the dorso-
478 ventral pigment pattern in zebrafish. The ventral region requires a functional Mc1r to
479 block the production of melanophores and xanthophores and to promote iridophores.

480 In the dorsal region, the absence of Mc1r only results in a partial reduction in the
481 number of melanophores and xanthophores suggesting the potential involvement of
482 additional Mcrs in mediating the antagonistic signal of Asip1.

483

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499

500 **REFERENCES**

501 Aberdam, E., Bertolotto, C., Sviderskaya, E.V., de Thillot, V., Hemesath, T.J.,
502 Fisher, D.E., Bennett, D.C., Ortonne, J.P. and Ballotti, R. (1998) Involvement of
503 microphthalmia in the inhibition of melanocyte lineage differentiation and of
504 melanogenesis by agouti signal protein. *J Biol Chem* **273**, 19560–19565.

505 Agulleiro, M., Sánchez, E., Leal, E., Cortés, R., Fernández-Durán, B., Guillot, R.,
506 Davis, P., Dores, R.M., Gallo-Payet, N. and Cerdá-Reverte, J.M. (2013) Molecular
507 characterization and functional regulation of melanocortin 2 receptor (MC2R) in
508 the sea bass. A putative role in the adaptation to stress. *PLoS One* **8**, e65450.

509 Anelli, V., Santoriello, C., Distel, M., Köster, R.W., Ciccarelli, F.D. and Mione M.
510 (2009) Global repression of cancer gene expression in a zebrafish model of
511 melanoma is linked to epigenetic regulation. *Zebrafish* **6**, 417–424.

512 Bassett, A.R., Tibbit, C., Ponting, C.P. and Liu, J.L. (2013) Highly Efficient
513 Targeted Mutagenesis of Drosophila with the CRISPR/Cas9 System. *Cell Rep* **4**,
514 220–8.

515 Cal, L., Megías, M., Cerdá-reverter, J. , Postlethwait. J.H., Braasch, I. and Rotllant,
516 J. (2017) BAC Recombineering of the Agouti Loci from Spotted Gar and Zebrafish
517 Reveals the Evolutionary Ancestry of Dorsal–Ventral Pigment Asymmetry in Fish.
518 *J Exp Zool Part B Mol Dev Evol* **328**, 697–708.

519 Cal, L., Suarez-Bregua, P., Comesaña, P., Owen, J., Braasch, I., Kelsh, R., Cerdá-
520 Reverter, J.M., Rotllant J. (2019) Countershading in zebrafish results from an
521 *Asip1* controlled dorsoventral gradient of pigment cell differentiation. *Sci Rep*.
522 **9**(1):3449. doi: 10.1038/s41598-019-40251-z.

523 Ceinos, R.M., Guillot, R., Kelsh, R.N., Cerdá-Reverter, J.M. and Rotllant, J.
524 (2015) Pigment patterns in adult fish result from superimposition of two largely
525 independent pigmentation mechanisms. *Pigment Cell Melanoma Res* **28**, 196–209.

526 Cerdá-Reverter, J.M, Agulleiro, M.J., Cortés, R., Sánchez, E., Guillot, R., Leal,
527 E., Ferández-Durán, B., Puchol, S. and Eley, M. (2013) Involvement of
528 melanocortin receptor accessory proteins (MRAPs) in the function of
529 melanocortin receptors. *Gen Comp Endocrinol* **188**:133-6 doi:
530 10.1016/j.ygcen.2013.01.017.

531 Cerdá-Reverter, J.M., Haitina, T., Schiöth, H.B. and Peter, R.E. (2005) Gene
532 Structure of the Goldfish Agouti-Signaling Protein: A Putative Role in the Dorsal-
533 Ventral Pigment Pattern of Fish. *Endocrinology* **146**, 1597–1610.

534 Cerdá-Reverter, J.M., Ling, M.K., Schiöth, H.B. and Peter, R.E. (2003) Molecular
535 cloning, characterization and brain mapping of the melanocortin 5 receptor in the
536 goldfish. *J Neurochem* **87**, 1354–1367.

537 Cerdá-Reverter, J.M., Ringholm, A., Schiöth, H.B. and Peter, R.E. (2003)
538 Molecular cloning, pharmacological characterization, and brain mapping of the
539 melanocortin 4 receptor in the goldfish: involvement in the control of food intake.
540 *Endocrinology* **144**, 2336–2349.

541 Cortés, R., Navarro, S., Agulleiro, M.J., Guillot, R., García-Herranz, V., Sánchez,
542 E. and Cerdá-Reverter, J.M. (2014) Evolution of the melanocortin system. *Gen*
543 *Comp Endocrinol* **209**:3-10. doi: <http://dx.doi.org/10.1016/j.ygcen.2014.04.005>

544 Gross, J.B., Borowsky, R. and Tabin, C.J. (2009) A novel role for Mc1r in the
545 parallel evolution of depigmentation in independent populations of the cavefish
546 *Astyanax mexicanus*. *PLoS Genet* **5**, e1000326.

547 Guillot, R., Ceinos, R.M., Cal, R., Rotllant, J. and Cerdá-Reverter, J.M. (2012)
548 Transient ectopic overexpression of agouti-signalling protein 1 (Asip1) induces
549 pigment anomalies in flatfish. *PLoS One* **7**, e48526.

550 Guillot, R., Cortés, R., Navarro, S., Mischitelli, M., García-Herranz, V., Sánchez,
551 E., Cal L., Navarro, J.C., Míguez, J.M., Afanasyev, S., Krasnov, A., Cone, R.D.,
552 Rotllant, J. and Cerdá-Reverter, J.M. (2016) Behind melanocortin antagonist
553 overexpression in the zebrafish brain: A behavioral and transcriptomic approach.
554 *Horm Behav* **82**, 87–100.

555 Hirata, M., Nakamura, K., Kanemaru, T., Shibata, Y. and Kondo, S. (2003)
556 Pigment cell organization in the hypodermis of zebrafish. *Dev Dyn* **227**, 497–503.

557 Hirata, M., Nakamura, K.I. and Kondo, S. (2005) Pigment cell distributions in
558 different tissues of the zebrafish, with special reference to the striped pigment
559 pattern. *Dev Dyn* **234**, 293–300.

560 Jackson, I.J., Budd, P.S., Keighren, M., McKie, L. (2007)
561 Humanized MC1R transgenic mice reveal human specific receptor function. *Hum*
562 *Mol Genet.* **16**(19):2341-8.

563 Kelsh, R.N. (2004) Genetics and evolution of pigment patterns in fish. *Pigment*
564 *Cell Res* **17**, 326–336.

565 Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F.
566 (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253–310.

567 Kobayashi, Y., Mizusawa, K., Saito, Y. and Takahashi, A. (2012) Melanocortin

568 systems on pigment dispersion in fish chromatophores. *Front Endocrinol* 3(9), 1-
569 6.

570 Kottler, V.A., Künstner, A. and Schartl, M. (2015) Pheomelanin in fish? Pigment
571 *Cell Melanoma Res* 28, 355–356.

572 Le Pape, E., Passeron, T., Giubellino, A., Valencia, J.C., Wolber, R. and Hearing,
573 V.J. (2009) Microarray analysis sheds light on the dedifferentiating role of agouti
574 signal protein in murine melanocytes via the Mc1r. *Proc Natl Acad Sci U S A* 106,
575 1802–1807.

576 Levesque, M.P., Krauss, J., Koehler, C., Boden, C. and Harris, M.P. (2013) New
577 Tools for the Identification of Developmentally Regulated Enhancer Regions in
578 Embryonic and Adult Zebrafish. *Zebrafish* 10, 21–29.

579 Linnen, C., Poh, Y., Peterson, B., Barret., R, Larson, J., Jensen, J. and Hoekstra,
580 H.E. (2013) Adaptive evolution of multiple traits through multiple mutations at a
581 single gene. *Science* 339, 1312–1316.

582 Michaud, E.J., Bultman, S.J., Stubb,s L.J, and Woychik, R.P. (1993) The
583 embryonic lethality of homozygous lethal yellow mice [AylAy] is associated with
584 the disruption of a novel RNA-binding protein. *Genes Dev.* 7, 1203–1213.

585 Miller, M.W., Duhl, D.M., Vrieling, H., Cordes, S.P., Ollmann, M.M., Winkes,
586 B.M. and Barsh, S. (1993) Cloning of the mouse agouti gene predicts a secreted
587 protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes*
588 *Dev* 7, 454–467.

589 Montague, T.G., Cruz, J.M., Gagnon, J., Church, G.M. and Valen, E. (2014)
590 CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic*
591 *Acids Res* **42**:401-7. doi: 10.1093/nar/gku410

592 Ollmann, M.M., Lamoreux, M.L., Wilson, B.D., Barsh, G.S. (1998) Interaction of
593 Agouti protein with the melanocortin 1 receptor in vitro and in vivo. *Genes Dev* **12**
594 (3): 316-30.

595

596 Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N. and Engeszer, R.E.
597 (2009) Normal table of postembryonic zebrafish development: Staging by
598 externally visible anatomy of the living fish. *Dev Dyn* **238**, 2975–3015.

599 Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Reh fuss, L.,
600 Baack, E., Mountjoy, K.G. and Cone, R.D. (1993) Pigmentation phenotypes of
601 variant extension locus alleles result from point mutations that alter MSH receptor
602 function. *Cell* **72**(6):827–34.

603 Sánchez, E., Rubio, V.C. and Cerdá-Reverter, J.M. (2009) Characterization of the
604 sea bass melanocortin 5 receptor: a putative role in hepatic lipid metabolism. *J Exp*
605 *Biol* **212**, 3901–3910.

606 Sánchez, E., Rubio, V.C. and Cerdá-Reverter, J.M. (2010) Molecular and
607 pharmacological characterization of the melanocortin type 1 receptor in the sea
608 bass. *Gen Comp Endocrinol* **165**, 163–169.

609 Schiöth, H.B., Haitina, T., Ling, M.K., Ringholm, A., Fredriksson, R., Cerdá-

610 Reverter, J.M. and Klovins, J. (2005) Evolutionary conservation of the structural,
611 pharmacological, and genomic characteristics of the melanocortin receptor
612 subtypes. *Peptides* **26**, 1886–1900.

613 Singh, A.P., Dinwiddie, A., Mahalwar, P., Schach, U., Linker, C., Irion, U. and
614 Nüsslein-Volhard, C. (2016) Pigment Cell Progenitors in Zebrafish Remain
615 Multipotent through Metamorphosis. *Dev Cell* **38**, 316–330.

616 Sviderskaya, E.V., Hill, S.P., Balachandar, D., Barsh, G.S. and Bennett, D.C.
617 (2001) Agouti signaling protein and other factors modulating differentiation and
618 proliferation of immortal melanoblasts. *Dev Dyn* **221**, 373–379.

619 Vrieling, H., Duhl, D.M., Millar, S.E., Miller, K. and Barsh, G.S. (1994)
620 Differences in dorsal and ventral pigmentation result from regional expression of
621 the mouse agouti gene. *Proc Natl. Acad. Sci. U S A.* **91**, 5667–5671.

622 Wei, R., Yuan, D., Zhou, C., Wang, T., Lin, F., Chen, H., Wang, Y., Liu, J., Gao,
623 Y. and Li, Z. (2013) Cloning, distribution and effects of fasting status of
624 melanocortin 4 receptor (MC4R) in *Schizothorax prenanti*. *Gene* **532**, 100–107.

625 Westerfield, M. (2007) *The Zebrafish Book. A Guide for the Laboratory Use of*
626 *Zebrafish (Danio rerio)*. 5th edn. University of Oregon Press, Eugene

627 Zemel, M.B., Kim, J., Woychikt, R.P., Michaudt, E.J., Kadwellt, S., Patel, I.R.
628 and Wilkison, W.O. (1995) Agouti regulation of intracellular calcium : Role in the
629 insulin resistance of viable yellow mice. *Proc Natl Acad Sci* **92**, 4733–4737.

630

631 **FIGURE LEGENDS**

632 **Figure 1. CRISPR/Cas9-induced mutations in the zebrafish *mc1r* gene.** (A)

633 Scheme of the *mc1r* single exon gene showing the target sites (black arrowheads: T1,
634 T2). Coding region (CDS) are represented as white boxes and 5' UTR and 3'UTR are
635 shown as black boxes. (B) Sequence of induced mutations in the *mc1r* gene. The first
636 and fourth lines show the wild-type target sequences (T1 and T2). Black arrowheads
637 label the protospacer-adjacent motif (PAM). The following lines show different
638 induced mutations. Italic lower case letters represent inserted new sequence. The
639 number of deleted (-) and inserted (+) bases are marked in the right side of each
640 sequence. Selected mutations for further analysis are labeled by white arrowheads. (C)
641 Predicted amino acid sequence encoded by generated *mc1r* variants. The first line
642 shows the wild type protein, and following lines show the potential protein sequence
643 of each selected mutation. Grey boxes show part conserved with the wild type (WT)
644 sequence. Asterisk represents the stop codon. Mutation M2 generates the shortest
645 predicted protein.

646

647 **Figure 2. The adult dorsal-ventral countershading pattern is disrupted in *mc1r^{K.O}***

648 Lateral (A, B), anterior-lateral (C, D), ventral-head (E, F), ventral-belly (G, H) and
649 dorsal (I, J) views of 210 dpf WT and *mc1r^{K.O}* zebrafish. (A, B) The pigment pattern
650 of WT zebrafish is a striped pigment pattern with dark stripes and light interstripes.
651 Each dark stripe has a standard nomenclature: two primary stripes are called 1D and
652 1V, and the two secondary stripes are named 2D and 2V. The *mc1r^{K.O}* phenotype is
653 characterized by a darker ventrum than WT. (C, D) The striped pigment pattern looks
654 almost unaltered in *mc1r^{K.O}* fish. The only perceptible modification is that the *mc1r^{K.O}*
655 mutant fish showed a thickened 2V-stripe and a fully-developed 3V-stripe in the

656 ventrum compared to WT. The darker ventrum of *mc1r^{K.O}* than WT fish is clearly
657 evident. (E, F) In WT, the melanophores appear in reduced numbers around the jaws
658 and branchiostegals; however, branchiostegal, jaw and operculum regions are
659 hyperpigmented in *mc1r^{K.O}*. (G, H) Melanophores on the WT belly are virtually
660 absent; thus, their ventral region shows a bright white color as a result of high numbers
661 of iridophores in the abdominal wall. However, *mc1r^{K.O}* shows hyperpigmentation,
662 with increased numbers of melanophores and xanthophores in ventral skin, and the
663 abdominal wall seems to be also affected because it appears overall more yellowish
664 than WT. (I, J) The dorsal region is also affected in *mc1r^{K.O}*: the WT dorsum shows
665 more melanophores than the *mc1r^{K.O}* dorsum. Scale bar: (A,B) 5 mm, (C,D, E, F, G,
666 H) 1 mm and (I, J) 0.5 mm. Abbreviation: br, branchiostegal.

667

668 **Figure 3. Dorsal-ventral distribution of melanophores during metamorphosis.**

669 (A) Distribution and density of melanophores in WT and *mc1r^{K.O}*. 15dpf fish. At this
670 stage, *mc1r^{K.O}* shows significantly lower density of melanophores in the dorsal region,
671 lateral region and in the belly. (B) Distribution and density of melanophores in WT
672 and *mc1r^{K.O}* 30 dpf fish. At this stage, *mc1r^{K.O}* shows significantly lower density of
673 melanophores in the dorsal region of the head, in the dorsal region, in the lateral region,
674 and in the belly region. Data are the mean±SEM, n=6 fish. Asterisks indicate
675 significant differences between WT and *mc1r^{K.O}* fish. Scale bar: (A) 200 µm, (B) 500
676 µm.

677

678 **Figure 4. Dorsal-ventral distribution of melanophores and xanthophores in WT**

679 **and *mc1r^{K.O}* adult fish.** (A) Lateral view of zebrafish showing the body regions
680 selected for melanophore and xanthophore count at 210dpf. (B) Ventral view of bellies
681 of WT and *mc1r^{K.O}* zebrafish at 210 dpf. (C) Distribution and density of melanophores
682 in 60 dpf WT and *mc1r^{K.O}* fish. At this stage, *mc1r^{K.O}* shows significantly lower density
683 of melanophores in dorsal head, dorsal region, and black stripes 2D, 1D and 1V.

684 However, *mc1r^{K.O}* shows significantly higher density of melanophores in the ventral
685 region of the head. (D) Density of xanthophores in dorsal and ventral skin of WT and
686 *mc1r^{K.O}* 60 dpf fish. At this stage, *mc1r^{K.O}* shows significantly higher density of
687 xanthophores in the belly region. (E) Distribution and density of melanophores in WT
688 and *mc1r^{K.O}* 210 dpf fish. At this stage, *mc1r^{K.O}* shows significantly lower density of
689 melanophores in dorsal head, dorsal region and black stripes 2D and 1D; conversely,
690 *mc1r^{K.O}* shows significantly higher density of melanophores in black stripe 3V, ventral
691 head and belly. (F) Number of xanthophores in dorsal and ventral skin of WT and
692 *mc1r^{K.O}* 210 dpf fish. The *mc1r^{K.O}* fish showed highly significant lower density of
693 xanthophores in dorsal regions and significantly higher density of xanthophores in
694 belly region than WT. Data are the mean \pm SEM, n=7. Asterisks indicate significant
695 differences between WT and *mc1r^{K.O}* fish. Scale bar (A,C,E) 1mm, (B) 100 μ m.

696

697 **Figure 5. Detection of transgenically-labelled pigment cells in WT and *mc1r^{K.O}***
698 **fish.** (A) Ventral view of 210 dpf WT belly. (B) Belly of 210 dpf WT fish carrying
699 Tg(Kita:GalTA4;UAS:mCherry) transgene shows no melanophores in ventral skin.
700 (C) Ventral view of 210 dpf *mc1r^{K.O}* belly. (D) Belly of 210 dpf *mc1r^{K.O}* fish carrying
701 Tg(Kita:GalTA4;UAS:mCherry) transgene shows higher number of melanophores in
702 ventral skin (white arrow). (E) Internal view of 210 dpf fish abdominal wall. WT
703 ventral abdominal wall shows a white sheet of iridophores with few internal
704 melanophores (black arrow). (F) Ventral abdominal wall of 210 dpf WT fish carrying
705 Tg(TDL358:GFP) transgene displays a uniform sheet of iridophores. (G) Ventral
706 abdominal wall of 210 dpf *mc1r^{K.O}* shows a discontinuous sheet of iridophores with
707 high number of melanophores. (H) Ventral abdominal wall of 210 dpf *mc1r^{K.O}* fish

708 carrying Tg(TDL358:GFP) transgene exhibits a broken sheet of iridophores. Scale
709 bars: 100 μ m.

710

711 **Figure 6. Adult *mc1r*^{K.O} dorsal and ventral scales displayed an anomalous color**
712 **pattern.** (A) A typical 210 dpf WT dorsal scale exhibit a pattern of melanophores
713 (black arrowheads) and xanthophores (yellow arrowheads). (B) 210 dpf *mc1r*^{K.O} dorsal
714 scale exhibits a strong reduction of melanophores number (black arrowheads). (C) A
715 typical 210 dpf WT ventral scale does not exhibit any chromatophores. (D) 210 dpf
716 *mc1r*^{K.O} ventral scales exhibit a pattern of melanophores (black arrowheads) and
717 xanthophores (yellow arrowheads). Scale bars: 100 μ m.

718

719 **Figure 7. Interaction between *asip1* overexpression phenotype and *mc1r*^{K.O} fish.**
720 Lateral (A, E, I, M), anterior-lateral (B, F, J, N), ventral-belly (C, G, K, O) and dorsal
721 (D, H, L, P) views of 100 dpf WT, *mc1r*^{K.O}, *asip1*-Tg zebrafish and *mc1r*^{K.O}+*asip1*-
722 Tg. (A) The pigment pattern of WT zebrafish shows (B) normal striped pattern, (C)
723 light belly, and (D) dark dorsum. (E) The pigment pattern of *mc1r*^{K.O} fish shows (F)
724 almost normal striped pattern with a dark stripe 3V more developed and the dark stripe
725 2D less developed than WT fish, (G) hyperpigmented belly, and (H) a lesser pigmented
726 dorsum compared to WT. (I) The pigment pattern of *asip1*-Tg fish shows (J) almost
727 normal striped pattern, but without dark stripe 2D, (K) light belly, and (L)
728 hypopigmented dorsum. (M) The *mc1r*^{K.O}+*asip1*-Tg phenotype shows the same
729 phenotype as the *mc1r*^{K.O} phenotype. Almost normal striped pattern with a dark stripe
730 3V (N) more developed and the dark stripe 2D less developed than WT fish, (O)
731 hyperpigmented belly and (P) a lesser pigmented dorsum. (Q-T) Density of
732 melanophores in dorsal and the ventral region of WT (Q), *mc1r*^{K.O} (R), *asip1*-Tg (S)

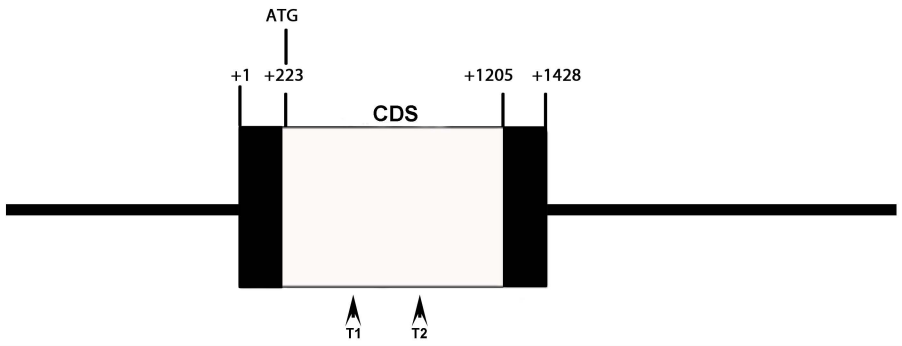
733 and *mc1r^{K.O.}+asip1-Tg* (T) adult fish. Data are the mean±SEM, n=6 fish. Scale bar: (A,
734 E, I, M) 2 mm, (B, C, D, F, G, H, J, K, L, N, O, P) 1 mm.

735

736 **Figure 8: Schematic representation of melanophore dorsal phenotypes of WT,**
737 ***mc1r^{K.O.}, asip1-Tg* and *mc1r^{K.O.}+asip1-Tg* fish.** We propose three different
738 melanophore cohorts in the dorsal skin of adult fish: Mc1r-free melanophores (⊗, also
739 called Asip1-independent), melanophores expressing Mc1r (⊙, also call Mc1r-
740 dependent), melanophores expressing Mc1r but also expressing a second Mcr (●, also
741 called Mc1r-independent melanophores). The sum of melanophore populations
742 expressing only Mc1r together with the melanophores that express Mc1r together with
743 another Mcr are called Asip-1 dependent melanophores (see text for more
744 information).

SUPPLEMENTARY DATA

Figure S1. Functional *Asip1/Mc1r* interaction on dorsal (upper row) and ventral (lower row) scales color pattern in adult fish. (A) *mc1r^{K.O}* dorsal scales exhibits a reduction of melanophores and xanthophore number compared to the typical adult WT fish dorsal scales. (B) *asip1-Tg* dorsal scale exhibits a substantial reduction of melanophores and xanthophore number compared to the *mc1r^{K.O}* dorsal scales and the typical adult WT dorsal scales color pattern. (C) *mc1r^{K.O}+asip1-Tg* dorsal scales exhibits a melanophores and xanthophore number similar to that shown by the *mc1r^{K.O}* dorsal scales. (D) *mc1r^{K.O}* ventral scales exhibit a pattern of melanophores and xanthophores. (E) *Asip1-Tg* ventral scale does not exhibit any chromatophores, similar to the typical ventral scale of adult WT fish. (F) *mc1r^{K.O}+asip1-Tg* ventral scales exhibit a pattern of melanophores and xanthophores similar to that shown by the *mc1r^{K.O}* ventral scales. Scale bars: 100 μm .

A**B**

Target(T¹)

WT> CGGTGGCAGACATGCTAGTGAGCGTCAGTA---ATGTGGTGGAGACGTTGTTTATG
 M1> CGGTGGCAGACATGCTAGTGAGCGTCAGTA*tca*ATGTGGTGGAGACGTTGTTT (+3)
 M2> CGGTGGCAGACATGCTAGTGAGCGTCAGTA*ggtgtggtgga* GGAGACGTTGTTT (-7;+11)

Target(T²)

WT> GGTTCGTACCTGCACA-----TG TTCATCTTGGCCCATGTCCACTCAA
 M3> GGTTCGTACCTGCA-----TG TTCATCTTGGCCCATGTCCACTCAA (-2)
 M4> GGTTCGTACCTGC*Atctgctcataaaaatc*TG TTCATCTTGGCCCATGTCCACTCAA (-2;+16)
 M5> GGT-----CA-----TG TTCATCTTGGCCCATGTCCACTCAA (-11)
 M6> GGTTCGTACCTG-----TTCATCTTGGCCCATGTCCACTCAA (-6)

C

WT> MNDSSRHHFSMKHMDYMYNADNNITLNSNSTASDINVTGIAQIMIPQELFLMLG
 M2> MNDSSRHHFSMKHMDYMYNADNNITLNSNSTASDINVTGIAQIMIPQELFLMLG
 M3> MNDSSRHHFSMKHMDYMYNADNNITLNSNSTASDINVTGIAQIMIPQELFLMLG
 M4> MNDSSRHHFSMKHMDYMYNADNNITLNSNSTASDINVTGIAQIMIPQELFLMLG

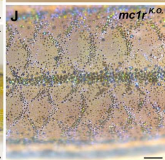
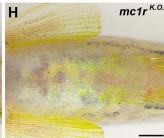
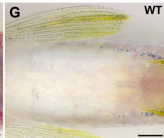
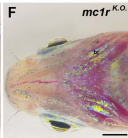
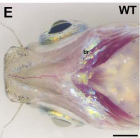
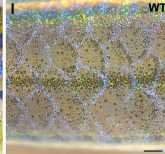
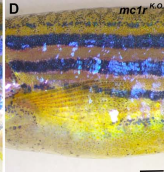
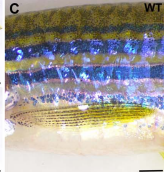
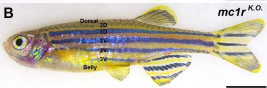
WT> LISLVENILVVVAIIKRNRLHSPMYFICCLAVADMLVSVSNVVETLFMLLTEHGLLL
 M2> LISLVENILVVVAIIKRNRLHSPMYFICCLAVADMLVSVSRGGGDVVVYVTDGAW
 M3> LISLVENILVVVAIIKRNRLHSPMYFICCLAVADMLVSVSNVVETLFMLLTEHGLLL
 M4> LISLVENILVVVAIIKRNRLHSPMYFICCLAVADMLVSVSNVVETLFMLLTEHGLLL

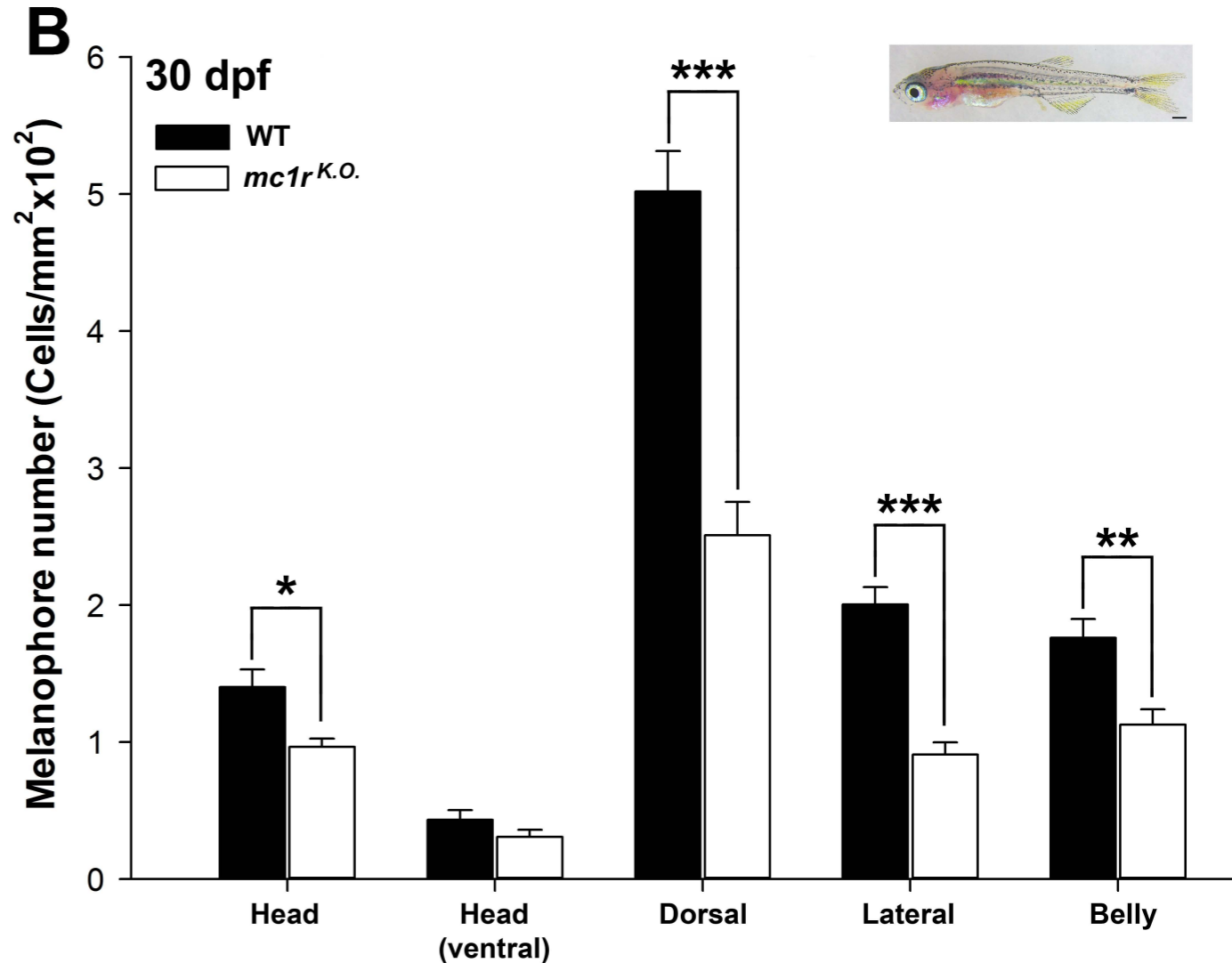
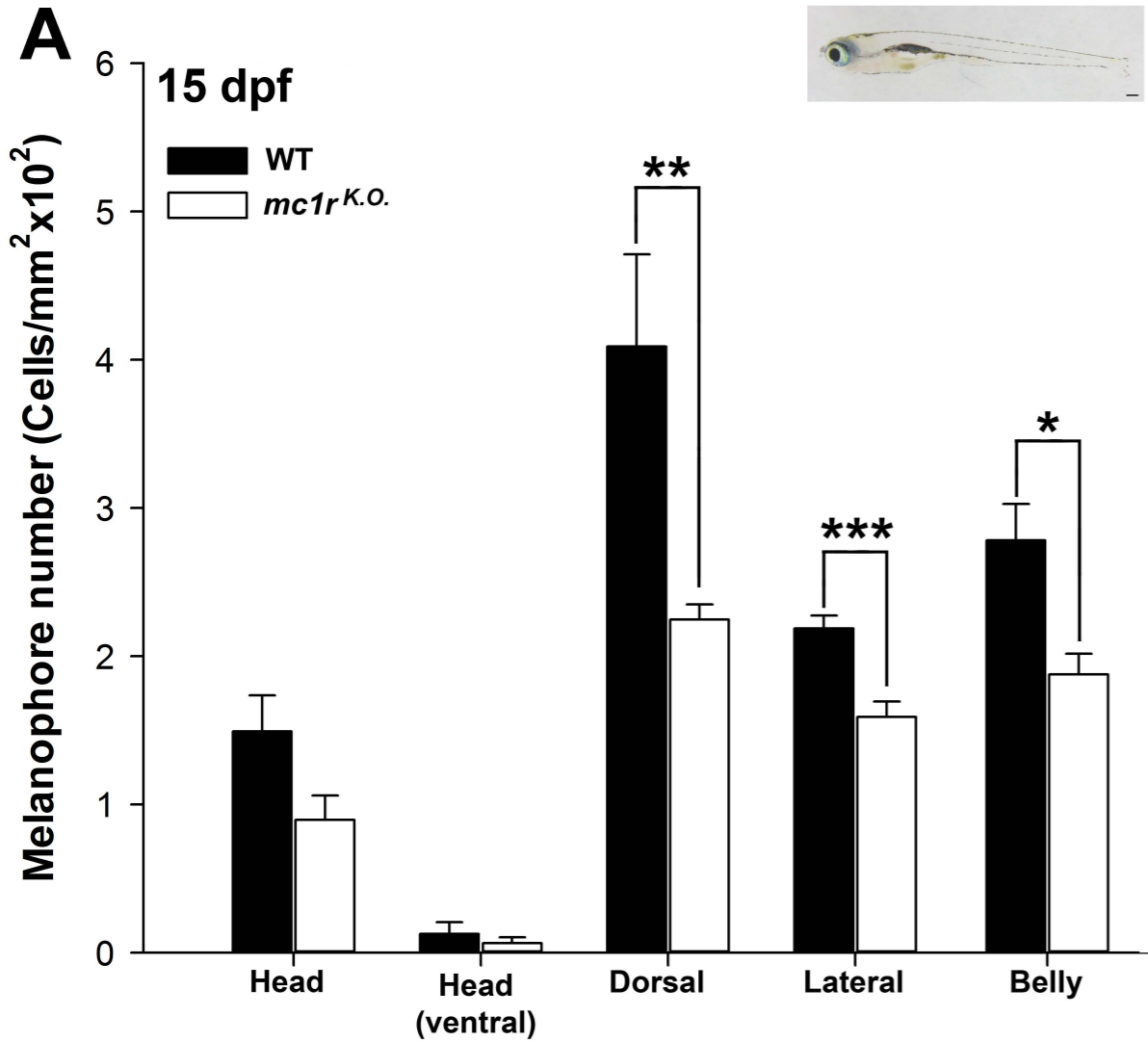
WT> VTAKMLQHLDNVIDIMICSSVSSLSFLCTIAADRYITIFYALRYHSIMTTQRAVGIIIL
 M2> ATAGHRKNVAALR*-----
 M3> VTAKMLQHLDNVIDIMICSSVSSLSFLCTIAADRYITIFYALRYHSIMTTQRAVGIIIL
 M4> VTAKMLQHLDNVIDIMICSSVSSLSFLCTIAADRYITIFYALRYHSIMTTQRAVGIIIL

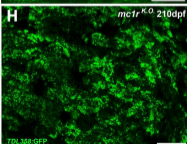
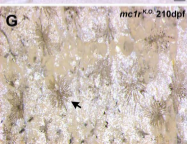
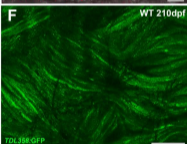
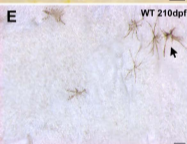
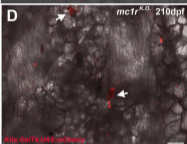
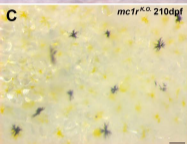
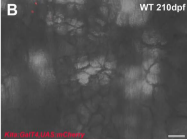
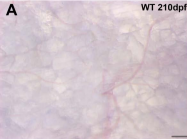
WT> VVWLASITSSSLFIVYHTDNAVIACLVTFFGVTLVFTAVLYLHMFILAHVHSRRITAL
 M2> -----
 M3> VVWLASITSSSLFIVYHTDNAVIACLVTFFGVTLVFTAVLYLHVHLGPCPLKTHHGS
 M4> VVWLASITSSSLFIVYHTDNAVIACLVTFFGVTLVFTAVLYLHLLIKICSSWPMSTQD

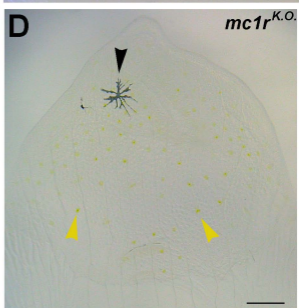
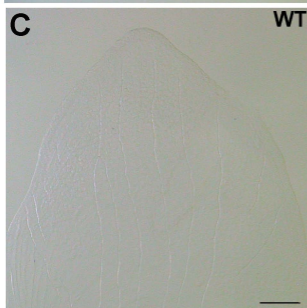
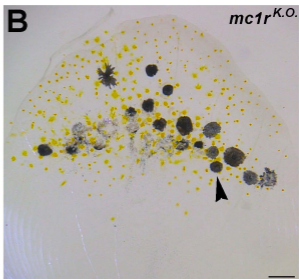
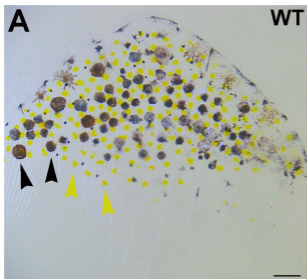
WT> HKSRRQTSMKGAITLTILLGVFILCWGPFFLHLILILTCPTNPYCKCYFSHFNLFLI
 M2> -----
 M3> P*-----
 M4> ASRLSIRAVGRPLA*-----

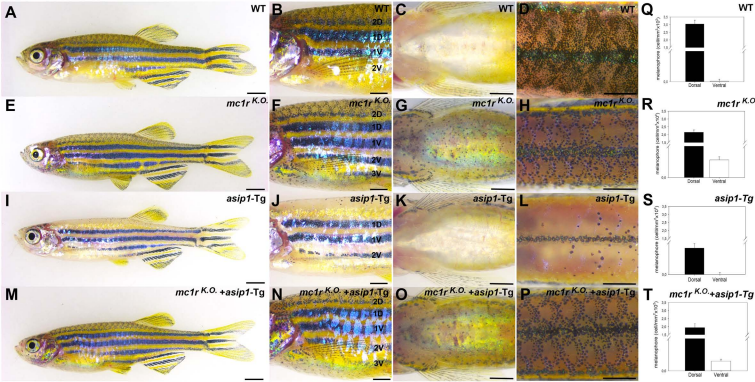
WT> WTLIICNSLIDPLIYAYRSQELRRTLKELIFCSWCFVAV*
 M2> -----
 M3> -----
 M4> -----



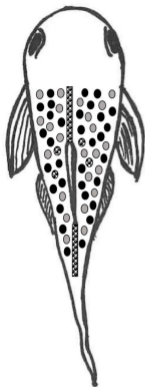








WT

mc1r^{k.o.}*asip1*-Tg*mc1r*^{k.o.} + *asip1*-Tg

- Mc1r-dependent melanophores
- Mc1r-independent melanophores
- ⊖ Asip1-independent melanophores
- ⊕● Asip1-dependent melanophores

