1 **Main manuscript**

2

3 **Title**

4 Evolution of the potassium channel gene *Kcnj13* underlies colour pattern
5 diversification in *Danio* fish

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31

32 **Abstract**

33 The genetic basis of morphological variation provides a major topic in evolutionary
34 biology¹⁻⁶. Colour patterns in fish are among the most diverse of all vertebrates.
35 Species of the genus *Danio* display strikingly different colour patterns ranging from
36 horizontal stripes, to vertical bars or spots⁷⁻¹⁰. Stripe formation in zebrafish, *Danio*
37 *rerio*, oriented by the horizontal myoseptum, is a self-organizing process based on
38 cell-contact-mediated interactions between three types of chromatophores with a
39 leading role of iridophores¹¹⁻¹⁴. We investigated genes known to regulate
40 chromatophore interactions in zebrafish as candidates that might have evolved to
41 produce a pattern of vertical bars in its sibling species, *Danio aesculapii*^{8,10}. Using
42 gene editing¹⁵⁻¹⁷ we generated several mutants in *D. aesculapii* that demonstrate a
43 lower complexity in the interactions between chromatophores in this species, as well
44 as a minor role of iridophores in patterning. Complementation tests in interspecific
45 hybrids^{18,19} identified *obelix/Kcnj13*, which encodes an inwardly rectifying potassium
46 channel (Kir7.1)²⁰, as a gene evolved between *D. rerio* and *D. aesculapii* as well as
47 in two of seven more *Danio* species tested. Our results demonstrate that the
48 CRISPR/Cas9-system allows straightforward genetic tests also in non-model
49 vertebrates to identify genes that underlie morphological evolution.

50

51 **Main**

52 Colour patterns are common features of animals and have important functions in
53 camouflage, as signals for kin recognition, or mate choice. As targets for natural and
54 sexual selection, they are of high evolutionary significance²¹⁻²⁴. The zebrafish, *Danio*
55 *rerio*, has emerged as a model system to study colour pattern development in a
56 vertebrate^{14,25-29}. A fair number of genes have been identified that are required for
57 the formation of the pattern^{28,29}, which is composed of a series of horizontal light and
58 dark stripes on the flank of the fish as well as in the anal and tail fins (Fig. 1a). The
59 adult pattern is created by three different types of pigment cells (chromatophores) in
60 the skin, black melanophores, blue or silvery iridophores and yellow
61 xanthophores^{13,30-32}. The chromatophores producing this pattern mainly originate

from multipotent neural crest–derived stem cells located at the dorsal root ganglia of the peripheral nervous system³³⁻³⁷. Signalling pathways, e.g. *Csf1* or *Edn3*, control proliferation and spreading of chromatophores³⁸⁻⁴⁰. During metamorphosis, the period when adult form and colour pattern are established, assembly into the striped pattern is controlled by interactions between the three cell types. Several genes are autonomously required in the chromatophores for these heterotypic interactions^{11,12,20,41-43}. These genes typically encode integral membrane proteins such as adhesion molecules⁴², channels²⁰, or components of cellular junctions, some of which mediate direct cell contacts^{43,44}. In *Meox1* (*choker*) mutants, lacking the horizontal myoseptum as anatomical landmark, the horizontal orientation is lost, but stripes form of normal width and composition (Fig. 1b), indicating that stripe formation is a process of self-organization of the pigment cells¹¹.

Danio species show an amazing variety of colour patterns, which range from horizontal stripes in *D. rerio* (Fig. 1a), over vertical bars in *D. aesculapii*, *D. choprae* or *D. erythromicron* (Fig. 1c, g, m) to spotted patterns in *D. tinwini* or *D. margaritatus* (Fig. 1d, h). The *Danio* species diversified for at least 13 million years in Southeast Asia and their spatial distributions only partially overlap today^{10,45}. Hybrids between *D. rerio* and other *Danio* species can be produced in the laboratory by natural matings or by *in vitro* fertilization¹⁹. They invariably display colour patterns similar to the stripes in *D. rerio*, thus, horizontal stripes appear to be dominant over divergent patterns (Fig. 1e, f, i, j)¹⁹; whether this is due to a gain-of-function in striped species or losses in the other species is an open question^{25,28,29}. The hybrids are virtually sterile impeding further genetic experiments, like QTL mapping, but they allow interspecific complementation tests¹⁹. Using this approach, we identified the potassium channel gene *Kcnj13* as repeatedly evolved in the *Danio* genus.

Prevalent pattern orientation by the horizontal myoseptum

To reconstruct the history of colour pattern evolution we first investigated how pattern orientation is inherited in hybrids (see phylogenetic relationships depicted on the left of Fig. 4). The horizontal orientation of the stripes in *D. rerio* depends on the horizontal myoseptum (Fig. 1a, b)^{11,13}. The closest sibling species to *D. rerio*,

D. aesculapii, shows a very different pattern of vertically oriented dark bars (Fig. 1c)⁸. Similar barred patterns are exhibited by the more distantly related *D. choprae* and *D. erythromicron* (Fig. 1g, m). These patterns clearly do not use the horizontal myoseptum for orientation. In all three cases, hybrids with *D. rerio* show a pattern that resembles the horizontal *D. rerio* stripes (Fig. 1e, i, n)²⁹. Strikingly, hybrids between *D. aesculapii* and *D. choprae* display a barred pattern (Fig. 1k). This indicates that the cues for horizontal orientation are lacking and that the pattern develops in a similar manner in both barred species. In contrast, hybrids between *D. aesculapii* and *D. erythromicron* develop highly variable patterns without any clear orientation (Fig. 1o; Extended Data Fig. 1). Therefore, the vertical bars must develop in a different manner in *D. erythromicron* compared to *D. aesculapii* and *D. choprae*.

Two *Danio* species display spotted patterns: *D. tinwini* has dark spots on a light background (Fig. 1d)⁹, whereas *D. margaritatus* shows light spots on a dark background (Fig. 1h)⁷. In both cases, hybrids with *D. rerio* show a stripe pattern similar to *D. rerio* (Fig. 1f, j)²⁹. Hybrids between the two spotted species also develop a pattern of horizontal stripes, albeit with some interruptions and irregularities (Fig. 1l). These results indicate that the horizontal myoseptum functions to orient the pattern in the hybrids between *D. tinwini* and *D. margaritatus*, and therefore in at least one of the two parental species. It seems likely that this is the case in *D. tinwini*, as the spots show some horizontal orientation reminiscent of interrupted stripes. Hybrids between *D. aesculapii* and *D. margaritatus* develop meandering patterns that do not resemble either of the parental species and lack a clear horizontal or vertical orientation (Fig. 1p). Based on the most recent phylogeny¹⁰, we hypothesize an evolutionary history, in which the horizontal orientation of the pattern in the *D. rerio* group was gained from an ancestral ambiguous pattern and lost again in *D. aesculapii*. Two other species, *D. erythromicron* and *D. choprae*, independently might have acquired a vertical orientation from this ancestral pattern. The patterns of the hybrids between *D. aesculapii* and *D. erythromicron* or *D. margaritatus*, which are without clear orientation, might resemble such an ancestral pattern. These patterns are much more variable than the species patterns (Extended Data Fig. 1) suggesting that the ancestral patterns did not function as recognition signals but rather provided camouflage.

127

128 **Chromatophore interactions in stripes and bars**

129 To investigate the developmental and genetic basis for the differences in pattern
 130 formation, we focussed on the sibling species *D. rerio* and *D. aesculapii*, which
 131 display horizontal stripes and vertical bars, respectively (Fig. 1a, c). In *D. rerio*,
 132 during early metamorphosis, iridophores emerge along the horizontal myoseptum to
 133 form the first light stripe (Extended Data Fig. 2a)¹¹⁻¹³. In contrast, in *D. aesculapii*
 134 iridophores appear more scattered over the flank and only during later stages
 135 (Extended Data Fig. 2b, d). This indicates that it is not the physical presence of the
 136 horizontal myoseptum, which exists in both species, but specific guidance signals
 137 directing the cells into the skin in *D. rerio*, which lack in *D. aesculapii*. Later, when
 138 iridophores, covered by compact xanthophores, have formed the first contiguous
 139 light stripe with adjacent melanophore stripes in *D. rerio* (Extended Data Fig. 2c, e),
 140 in *D. aesculapii* melanophores and xanthophores intermix broadly (Extended Data
 141 Fig. 2f); they sort out loosely into vertical bars of low contrast without coherent
 142 sheets of dense iridophores between the melanophore bars during later stages
 143 (Extended Data Fig. 2h). Our observations suggest that the different patterns in
 144 these sibling species are produced by the presence or absence of guidance signals
 145 for iridophores along the horizontal myoseptum as well as by cellular interactions
 146 that prevent mixing of melanophores and xanthophores in *D. rerio* but not in *D.*
 147 *aesculapii*.

148

149 To address the role of the different cell types, we used the CRISPR/Cas9 system to
 150 generate mutants lacking individual chromatophore types in *D. aesculapii*. Whereas
 151 in *D. rerio* vestiges of the striped pattern form in the absence of one chromatophore
 152 type (Fig. 2a, b, c)¹¹, loss of either melanophores (Fig. 2d) or xanthophores (Fig. 2e)
 153 completely abolishes the patterning in *D. aesculapii*. This indicates that the repulsive
 154 interactions between melanophores or xanthophores and iridophores, which account
 155 for the residual patterns in *D. rerio*^{11,12}, are absent in *D. aesculapii*. In contrast,
 156 eliminating iridophores in *D. aesculapii* still permits some melanophore bar formation
 157 (Fig. 2f). This indicates that iridophores, which play a dominant role for stripe

formation in *D. rerio*, are dispensable for the formation of vertical bars in
D. aesculapii.

Next, we analysed genes with known functions in heterotypic interactions between chromatophores. In *D. rerio* loss-of-function mutations in the gap junction genes *Cx39.4 (luchs)*^{44,46} and *Cx41.8/Gja5b (leopard)*^{41,46,47} as well as mutations in *Igsf11 (seurat)*⁴², which codes for a cell adhesion molecule, lead to melanophore spots (Fig. 2j, Extended Data Fig. 3a, c, e), whereas mutations in *Kir7.1/Kcnj13 (obelix/jaguar)* result in fewer and wider stripes with some mixing of melanophores and xanthophores (Fig. 2g)^{20,48}. So far, only dominant alleles of *Kcnj13* have been described^{41,46,49}. We used the CRISPR/Cas9 system to generate a *Kcnj13* loss-of-function allele in *D. rerio*, which is recessive (Fig. 2h, Extended Data Fig. 3e, g, Supplementary Information). To investigate the functions of these four genes in *D. aesculapii*, we generated mutants in the orthologs. In all of them we find an even distribution of melanophores (Fig. 2i, Extended Data Fig. 3b, d, f, h) indicating that the interactions mediated by these genes are essential to generate the melanophore bars in *D. aesculapii*. The complete loss of a pattern in single mutants in *D. aesculapii* is different from *D. rerio* where this occurs only in double mutants (Fig. 2k)⁴⁶. In concert with predictions of agent-based models of patterning⁵⁰, this indicates that the robust formation of horizontal stripes in *D. rerio* is due to a gain in complexity based on partially redundant chromatophore interactions. These are dominated by iridophores and oriented by an as yet unidentified signal along the horizontal myoseptum. *D. aesculapii* might have secondarily lost the dominance of iridophores leading to a pattern based primarily on interactions between xanthophores and melanophores and thus of lower complexity.

The ability to generate loss-of-function mutations in both species allowed us to generate interspecific hybrids, which carry loss-of-function alleles from both parental species. These hybrids are very similar to the respective *D. rerio* mutants (Fig. 2l, Extended Data Fig. 3i, j), indicating that these genes have the same functions during stripe formation in *D. rerio* and the hybrids.

***Kcnj13* evolved repeatedly in the *Danio* genus**

Next, we generated reciprocal heterozygotes, i.e. interspecific hybrids carrying a loss-of-function allele from either one of the parental species in an otherwise identical genetic background¹⁸. This powerful genetic test to identify evolved genes has not been applied in vertebrates, so far. We expect similar patterns in these hybrids if the gene function can be fully provided by either wild-type allele. A qualitatively altered hybrid pattern would reveal that one of the wild-type alleles cannot complement the loss-of-function of the other, therefore indicating functional changes during evolution. We found that heterozygous hybrids with the loss-of-function allele of *Kcnj13* from *D. rerio* display a pattern of spots or interrupted stripes whereas a striped pattern forms with the mutant allele from *D. aesculapii* (Fig. 3a, b, Extended Data Fig. 4g, h). This indicates that the wild-type allele from *D. aesculapii* cannot compensate for the loss of the *D. rerio* allele. In contrast, in the case of heterozygous hybrids with *Cx39.4*, *Cx41.8* and *Igsf11* striped patterns indistinguishable from wild-type hybrids are formed regardless whether the wild-type allele stems from *D. rerio* (Extended Data Fig. 4b, d, f) or the other species (Extended Data Fig. 4a, c, e). These reciprocal heterozygosity tests indicate that *Cx39.4*, *Cx41.8* and *Igsf11* provide similar functions in both species, whereas the function of *Kcnj13* has evolved between the two species.

To investigate if *Kcnj13* underlies the pattern variation more broadly across the *Danio* genus, we tested seven additional species (Fig. 4a, top to bottom). As mentioned above, wild-type hybrids between *D. rerio* and all other *Danio* species display horizontal stripes, resembling the *D. rerio* pattern, with slight defects in *D. albolineatus* (Fig. 4b). Strikingly, not only *D. rerio Kcnj13* k.o. / *D. aesculapii* hybrids (Fig. 4c, highlighted in magenta) but also *D. rerio Kcnj13* k.o. / *D. tinwini* hybrids developed patterns of spots or interrupted stripes indicating that the *Kcnj13* function must have evolved compared to *D. rerio* (Fig. 4c, highlighted in yellow). This pattern, which is qualitatively different from all wild-type hybrids and also from the *D. rerio Kcnj13* mutant pattern, is similar to the parental pattern of *D. tinwini*, where dense iridophores interrupt the dark melanophore stripes (Fig. 1d, Fig. 4c). *D. rerio Kcnj13* k.o. / *D. choprae* hybrids also developed patterns that resemble interrupted

stripes (Fig. 4c, highlighted in cyan), similar to the *D. rerio Kcnj13* k.o. / *D. aesculapii* hybrid pattern (Fig. 4c). No qualitative differences were detected between wild-type hybrids (Fig. 4b) and hybrids heterozygous for *D. rerio Kcnj13* in the case of *D. kyathit*, *D. nigrofasciatus*, *D. albolineatus*, *D. erythromicron* and *D. margaritatus* (Fig. 4c). This indicates that the alleles from these species complement the loss of the *D. rerio Kcnj13* allele. Functional changes of *Kcnj13* occurred in *D. aesculapii*, *D. tinwini* and *D. choprae* compared to *D. rerio*, however, heterozygous hybrids did not develop pure *D. rerio Kcnj13* mutant patterns indicating that the orthologs still provide some function for patterning across all species tested. The separated positions of the three species with the different functions of *Kcnj13* in the phylogenetic tree (graph on the left of Fig. 4)¹⁰ indicate a repeated and independent evolution of the same gene.

Kcnj13

Potassium channels have important roles in tissue patterning⁵¹, notably in the regulation of allometric growth of fins in *D. rerio*^{52,53}. *Kcnj13* encodes an inwardly rectifying potassium channel (Kir7.1) conserved in vertebrates (Extended Data Fig. 5). Mutations are known to cause defects in tracheal development in mice⁵⁴ and two rare diseases in humans leading to visual impairment⁵⁴⁻⁶⁰. During colour pattern formation in *D. rerio* its function is autonomously required in melanophores⁴⁸, and in *ex vivo* studies it was shown that the channel is involved in the contact-dependent depolarisation of melanophores upon interaction with xanthophores leading to a repulsion between these cells⁴³. Evolution in *Kcnj13* in *D. aesculapii*, *D. tinwini* and *D. choprae* might therefore cause differences in heterotypic chromatophore interactions between species. The protein coding sequences of *Kcnj13* orthologs are highly conserved in all *Danio* species with only very few diverged sites in the cytoplasmic N- and C-terminal parts of the protein (Extended Data Fig. 6). Whether any of these amino acid changes might affect the function of the channel and/or if changes in gene expression are the basis for the repeated evolution of *Kcnj13* will require further experiments.

In contrast to mammals and birds, basal vertebrates retained several chromatophore types providing a substrate for the development of elaborate colour patterns. Their rapid and extensive evolutionary diversification is most likely if the number of underlying genes is small⁶¹. Several patterning genes have been repeatedly identified in genetic screens in *D. rerio*^{41,46,49}. These genes provide candidates that might have evolved to contribute to patterning differences between *Danio* species. Evolved genes have been identified in *D. albolineatus* and *D. nigrofasciatus*, where changes in two signalling pathways, Csf1 or Edn3, likely underlie patterning variations by differentially promoting xanthophore or iridophore development, respectively^{19,36,62}. We focused on genes regulating heterotypic interactions between chromatophores as a potential genetic basis for colour pattern evolution. Using interspecific mutant complementation tests we identified the potassium channel gene *Kcnj13* as contributing to patterning divergence in multiple *Danio* species. We have shown that this genus offers the opportunity to identify evolved genes and to reconstruct the evolution of biodiversity.

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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Fish husbandry

Zebrafish, *D. rerio*, were maintained as described earlier¹. If not newly generated (Table 4, Supplementary Information), the following genotypes were used: wild-type Tuebingen/TU, *nacre*^{w2}/*nac*/*Mitfa*², *pfeffer*^{tm236}/*pfe*/*Csf1ra*³, *transparent*^{b6}/*tra*/*Mpv17*⁴, *leopard*^{t1}/*leo*/*Cx41.8*^{5,6}, *luchs*^{t37ui}/*luc*/*Cx39.4*⁷, *obelix*^{tXG6}/*obe*/*Kcnj13*⁷.

D. aesculapii and *D. albolineatus* were treated identical to *D. rerio*. For the other *Danio* species, *D. kyathit*, *D. tinwini*, *D. nigrofasciatus*, *D. choprae*, *D. margaritatus* and *D. erythromicron* individual pair matings were not successful. Therefore, the fish were kept in groups in tanks containing boxes lightly covered with Java moss (*Taxiphyllum barbieri*), which resulted in sporadic matings and allowed us to collect fertilized eggs.

Interspecific hybrids were either obtained by natural matings or by in vitro fertilizations⁸. Heterozygous or trans-heterozygous mutant hybrids were identified by PCR and sequence analysis using specific primer pairs (Table 1 and 3, Supplementary Information).

All species were staged according to the normal table of *D. rerio* development⁹. All animal experiments were performed in accordance with the rules of the State of Baden-Württemberg, Germany, and approved by the Regierungspräsidium Tübingen.

CRISPR/Cas9 gene editing

The CRISPR/Cas9 system was applied either as described in¹⁰ or according to the guidelines for embryo microinjection of IDT. Briefly, oligonucleotides were cloned into pDR274 to generate the sgRNA vector (Table 2, Supplementary Information). sgRNAs were transcribed from the linearized vector using the MEGAscript T7

Transcription Kit (Invitrogen). Alternatively, target-specific crRNAs and universal tracrRNAs were purchased from IDT. sgRNAs or crRNA:tracrRNA duplexes were injected as ribonucleoprotein complexes with Cas9 proteins into one-cell stage embryos. The efficiency of indel generation was tested on eight larvae at 1 dpf by PCR using specific primer pairs and by sequence analysis as described previously (Table 1 and 3, Supplementary Information)¹¹. The remaining larvae were raised to adulthood. Mature F0 fish carrying indels were outcrossed. Loss-of-function alleles in heterozygous F1 fish were selected to establish homozygous or trans-heterozygous mutant lines (Table 4, Supplementary Information).

Image acquisition

Anesthesia of adult fish was performed as described previously¹¹. A Canon 5D Mk II camera was used to obtain images. Juvenile fish were either embedded in low melting point agarose or fixed in 4% formaldehyde/0.08% glutaraldehyde and then photographed under a Leica MZ1 stereomicroscope (Extended Data Fig. 2). Images were processed using Fiji¹², Adobe Photoshop and Adobe Illustrator CS6.

RNA-Sequencing and transcriptome analysis

Skin of *Danio* species

Adult fish (n=5 each for *D. rerio* (TU), *D. aesculapii*, *D. kyathit*, *D. nigrofasciatus*, *D. tinwini*, *D. albolineatus*, *D. choprae*, *D. erythromicron*, *D. margaritatus*) were euthanized by exposure to buffered 0.5 g/L MS-222 (Tricaine). Skin tissues were dissected in ice-cold PBS and collected using TRIzol (Life Technologies). RNA integrity and quantity were assessed by Agilent 2100 Bioanalyzer. Library preparation (TruSeq stranded mRNA, Illumina; 200 ng per sample) and sequencing (NovaSeq 6000, 2 x 100 bp) were performed by CeGaT GmbH (Tübingen, Germany). RNA-Seq analysis was carried out using the *Danio rerio* GRCz11 genome build for all *Danio* species and STAR aligner with default settings¹³. Differential expression analysis was then carried out using DESeq2¹⁴. The actual commands used can be found here: <https://github.com/najasplus/STAR-deseq2>.

519

520 Sequence analysis

521 We found SNPs in the coding region of *Kcnj13* and considered other resources¹⁵,
 522 including the latest zebrafish reference genome assembly (GRCz11) and the ENA
 523 deposition Zebrafish Genome Diversity (PRJEB20043, Wellcome Trust Sanger).
 524 Also, all identified SNPs in the *kcnj13* coding sequence from the Zebrafish Mutation
 525 Project were incorporated¹⁶. The variant calling pipeline for all *Danio* species
 526 consisted of GATK 3.8 and 4 and picard¹⁷ from STAR-aligned bam files based on
 527 GATK Best-Practices pipeline. The full commands used can be found here:
 528 https://github.com/najasplus/rnaseq_variant_calling. Furthermore, variants were also
 529 called and checked using SAMtools, mpileup and bcftools¹⁸. The protein sequence
 530 alignment was produced using T-coffee¹⁹ and refined using BOXSHADE (developed
 531 by Kay Hofmann and Michael D. Baron, unpublished) and Microsoft Word.

532

533 Methods References

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591

592 **Data and code availability**

593 The dataset generated during this study is available at The European Nucleotide
594 Archive (ENA) accession number: pending.

595

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611 Contributions

612 All authors were involved in the design of the experiments. M.P., U.I. and H.G.F.
 613 performed the experiments. U.I., C.N.V., M.P., H.G.F. and C.D. analysed the data
 614 with support of A.E. M.P. made the figures with contributions from U.I. and C.N.V.
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619

620 **Ethics declaration**

621 Competing interests

622 The authors declare no competing interests.

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Figures and Figure Legends

Fig. 1: Colour patterns in *Danio* fish and interspecific hybrids.

a, Colour pattern of zebrafish, *D. rerio* (*D.re*). **b**, *D. rerio* *Meox1* (*choker*) mutants, which lack a horizontal myoseptum. **c**, *D. aesculapii* (*D.ae*). **d**, *D. tinwini* (*D.ti*). **e**, Interspecific hybrid between *D. rerio* and *D. aesculapii* and, **f**, between *D. rerio* and *D. tinwini*. **g**, *D. choprae* (*D.ch*). **h**, *D. margaritatus* (*D.ma*). **i** Interspecific hybrid between *D. rerio* and *D. choprae*, **j**, and between *D. rerio* and *D. margaritatus*. **k**, Interspecific hybrid between *D. aesculapii* and *D. choprae*. **l**, Interspecific hybrid between *D. tinwini* and *D. margaritatus*. **m**, *D. erythromicron* (*D. er*). **n**, Interspecific hybrid between *D. rerio* and *D. erythromicron*. **o**, Interspecific hybrid between *D. aesculapii* and *D. erythromicron*. **p**, Interspecific hybrid between *D. aesculapii* and *D. margaritatus*.

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652

653 **Fig. 2: Mutant phenotypes in *D. rerio*, *D. aesculapii* and their hybrids.**

654 In *D. rerio* loss of one type of pigment cell, **a**, melanophores in *Mitfa* (*nacre*) mutants,
655 **b**, xanthophores in *Csf1ra* (*pfeffer*) mutants, or **c**, iridophores in *Mpv17* (*transparent*)
656 mutants, still permits rudimentary aggregation of dense iridophores (a) or
657 melanophores (b, c). In *D. aesculapii*, **d**, loss of melanophores in *Mitfa* mutants or **e**,
658 loss of xanthophores in *Csf1ra* mutants, abrogate any residual pattern formation.
659 However, **f**, bars still form in *Mpv17* mutants, despite the absence of iridophores. **g**,
660 *D. rerio* heterozygous for the dominant allele *Kcnj13*^{tXG6}/*obelix*. **h**, *D. rerio*
661 homozygous for the loss-of-function allele *Kcnj13*^{t24ui}. **i**, *D. aesculapii* homozygous
662 for a *Kcnj13* loss-of-function allele. **j**, *D. rerio* homozygous for the loss-of-function of
663 *Cx39.4*. **k**, in *D. rerio* double mutants for *Cx39.4* and *Kcnj13* patterning is completely
664 absent. **l**, interspecific hybrids between *D. rerio* and *D. aesculapii* that are both
665 mutant in *Kcnj13*.

Fig. 3: A reciprocal heterozygosity test to identify *Kcnj13* evolution.

Two interspecific hybrids between *D. rerio* and *D. aesculapii*, which are heterozygous for a *Kcnj13* loss-of-function mutation. **a**, stripes are interrupted in hybrids carrying the mutant allele from *D. rerio* (nick in the blue line representing the zebrafish genome). **b**, hybrids carrying the mutant allele from *D. aesculapii* (nick in the magenta line, representing the *D. aesculapii* genome) are indistinguishable from wild-type hybrids (Fig. 1e).

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678 **Fig. 4: Repeated *Kcnj13* evolution.**

679 **a**, From top to bottom wild-type *Danio* colour patterns (*D. rerio* species group: barred

680 *D. aesculapii*, *D. ae*, the closest relative to *D. rerio*; striped *D. kyathit*, *D. ky*; striped

681 *D. nigrofasciatus*, *D.ni*; spotted *D. tinwini*, *D.ti*;) unpatterned *D. albolineatus*, *D.al*; (*D.*
682 *choprae* species group: barred *D. choprae*, *D.ch*; barred *D. erythromicron*, *D.er*,
683 spotted *D. margaritatus*, *D.ma*). The phylogenetic tree to the left depicts the
684 relationships between the species¹. The asterisk denotes an uncertainty in the
685 phylogenetic relationships within the *D. rerio* species group. **b**, Hybrids between
686 *D. rerio* (*D.re*) and eight other danios (from top to bottom *D. aesculapii*, *D.ae*,
687 *D. kyathit*, *D.ky*, *D. nigrofasciatus*, *D.ni*, *D. tinwini*, *D.ti*, *D. albolineatus*, *D.al*,
688 *D. choprae*, *D.ch*, *D. erythromicron*, *D.er*, *D. margaritatus*, *D.ma*). **c**, Interspecific
689 hybrids carrying the *Kcnj13* k.o. allele from *D. rerio* and the wild-type allele in the
690 eight species. Pattern defects occur in three cases, *D. rerio Kcnj13* k.o. / *D.*
691 *aesculapii* (magenta box), *D. rerio Kcnj13* k.o. / *D. tinwini* (yellow box) and *D. rerio*
692 *Kcnj13* k.o. / *D. choprae* (cyan box). In the other five cases the patterns in
693 heterozygous hybrids do not differ from the striped patterns of wild-type hybrids.

Extended Data Figures, Tables and Legends

Extended Data Fig. 1: Variability of patterns in interspecific hybrids between *D. aesculapii* and *D. erythromicron*

Interspecific hybrids between *D. aesculapii* and *D. erythromicron*, **a - e**, show a range of patterns without clear horizontal or vertical orientation.

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704 **Extended Data Fig. 2: Development of colour patterns in *D. rerio* and *D.***
705 ***aesculapii*.**

706 **a**, *D. rerio* fish at stage PR. Iridophores (arrowhead) emerge along the horizontal
707 myoseptum (asterisk) to form the first light stripe. **b**, *D. aesculapii* fish at stage PR. **c**,
708 *D. rerio* at stage SP. The first light stripe is flanked dorsally and ventrally by dark
709 stripes **d**, *D. aesculapii* at stage SP. Iridophores emerge in a scattered fashion. **e**, *D.*
710 *rerio* at stage J++. Light stripes are covered by compact xanthophores **f**, *D.*
711 *aesculapii* at stage J++. Melanophores and xanthophores broadly intermix. **g**, *D.*
712 *rerio* at stage JA. **h**, *D. aesculapii* at stage JA. Melanophores and xanthophores sort
713 out loosely into vertical bars of low contrast; no dense iridophores are visible
714 between the dark bars. a-d: incident light illumination to highlight iridophores, e-h:
715 bright field illumination to visualise xanthophores and melanophores. Staging
716 according to². PB (pectoral fin bud, 7.2 mm SL). SP (squamation posterior, 9.5 mm

717 SL). J++ (juvenile posterior, 16 mm SL). JA (juvenile-adult, >16 mm SL). Scale bars
718 correspond to 250 μ m.
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722 **Extended Data Fig. 3: Mutant phenotypes in *D. rerio* and *D. aesculapii* of genes**
723 **required for heterotypic interactions.**

724 **a, *D. rerio* (*D.re*) Cx39.4 knock-out (k.o.) mutant³. b, *D. aesculapii* (*D.ae*) Cx39.4 k.o.**
725 **mutant. c, *D.re* Cx41.8 mutant^{4,5}. d, *D.ae* Cx41.8 k.o. mutant. e, *D.re* lgsf11 k.o.**

726 mutant⁶. **f**, *D. ae lgsf11* mutant. **g**, *D. rerio Kcnj13* k.o. mutant^{3,4,7}. **h**, *D. ae Kcnj13*
727 k.o. mutant. **i**, Interspecific hybrids between *D. re Cx41.8* and *D. ae Cx41.8* mutants. **j**,
728 Interspecific hybrids between *D. re Kcnj13* and *D. ae Kcnj13* mutants. In all cases,
729 loss-of function (k.o.) alleles were created using CRISPR/Cas9 gene editing.
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733 Extended Data Fig. 4: Reciprocal heterozygosity tests identify *Kcnj13* 734 evolution.

735 Interspecific heterozygous hybrids carrying a mutant allele (nicked bar) from either
736 parental species (blue: *D. rerio*, *D.re*, or magenta: *D. aesculapii*, *D.ae*) in an
737 otherwise identical genetic background. In the cases of **a/b**, *Cx39.4*, **c/d**, *Cx41.8* and

e/f, *lgsf11* both hybrids show identical phenotypes. In the case of **g/h**, *Kcnj13*, the two hybrids show different phenotypes: Interrupted stripes and spots in those carrying the mutant allele from *D. rerio* (g) and a striped pattern in those carrying the mutant allele from *D. aesculapii* (h).

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746

747 **Extended Data Fig. 5: Kcnj13 sequence alignment of vertebrate orthologues.**

748 Sequence alignment of Kcnj13 orthologues from different vertebrate species. The
749 two transmembrane domains (M1/M2) are shaded in light grey, the P-Loop (H5) in
750 dark grey. Dominant (d) or recessive (r) mutations are indicated for zebrafish^{3,4,7,8}

(blue) and human⁹⁻¹³ (purple). Positions that are different between *D. rerio* and *D. aesculapii* (magenta), *D. tinwini* (yellow), *D. choprae* (cyan) and polymorphic positions in *D. rerio* (dark grey) are highlighted. Kcnj13 sequences of *Danio rerio* (zebrafish, NP_001039014.1), *Lepisosteus oculatus* (spotted gar, XP_006638004.1), *Xenopus tropicalis* (tropical clawed frog, NP_001096437.1), *Anolis carolinensis* (green anole, XP_016847621.1), *Geospiza fortis* (medium ground finch, XP_005430275.1), *Gallus gallus* (chicken, XP_015132697.1), *Mus musculus* (house mouse, NP_001103697.1) and *Homo sapiens* (human, NP_002233.2).

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761

762 **Extended Data Fig. 6: Sequence alignment of Kcnj13 orthologues from *Danio***
763 **species.**

764 Kcnj13 sequences from *D. rerio* (*D.re*), *D. aesculapii* (*D.ae*), *D. kyathit* (*D.ky*),
765 *D. nigrofasciatus* (*D.ni*), *D. tinwini* (*D.ti*), *D. albolineatus* (*D.al*), *D. choprae* (*D.ch*),
766 *D. margaritatus* (*D.ma*), *D. erythromicron* (*D.er*). Amino acids evolved between *D.re*
767 and *D.ae* (magenta), *D.ti* (yellow) and *D.ch* (cyan). Dominant (d) or recessive (r)

mutations in *D.re Kcnj13*^{3,4,7,8} (blue). Amino acid polymorphisms in *D.re* (dark grey).
Transmembrane domains (M1/M2) (light grey blocks) and the P-loop (H5) (dark grey
block).

Figure References

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Supplementary Information and Legends

Supplementary Tables

Table 1 | List of targeted genes.

target	CRISPR target sequence (5'-3')	genotyping
<i>D. aesculapii csf1ra</i>	GGCCTTTAACCTGGTCGGTC	T2143, T2144
<i>D. aesculapii cx39.4</i>	GGACTCACAGCCGGGCTGTT	T2145, T2146
<i>D. aesculapii cx41.8</i>	GAACCTTTCTAGAAGAAGTCC	MP92, MP318
<i>D. aesculapii igsf11</i>	GCTGAAAGTACAGGGCAAGA	MP330, MP 331
<i>D. aesculapii kcnj13</i>	TGCTGTATTATGGTACCTGC	T963, T964
<i>D. aesculapii mitfa</i>	GGAGCGCTGGCTCCGGGTCC	T2147, T2148
<i>D. aesculapii mpv17</i>	GGTGCTTTTCTGGGAATAAC	T2149, T2150
<i>D. rerio igsf11</i>	GGACGCAATATAGGAGTGAT	T1449, T1450
<i>D. rerio kcnj13</i> (1)	GGCAAGCAGCGCGATGGCAG	T2139, T2140
<i>D. rerio kcnj13</i> (2)	GGCTGGCGCTACGGTGGCGG	T963, T964

Table 2 | Primer pairs used for the generation of sgRNAs.

target	forward	reverse
<i>D. aesculapii csf1ra</i>	AAACGACCGACCAGGTTAAAGG	TAGGCCTTTAACCTGGTCGGTC
<i>D. aesculapii cx39.4</i>	AAACAACAGCCCGGCTGTGAGT	TAGGACTCACAGCCGGGCTGTT
<i>D. aesculapii mitfa</i>	AAACGGACCCGGAGCCAGCGCT	TAGGAGCGCTGGCTCCGGGTCC
<i>D. aesculapii mpv17</i>	AAACGTTATTCCCAGAAAAGCA	TAGGTGCTTTTCTGGGAATAAC
<i>D. rerio csf1ra</i>	AAACGACCGACCAGGTTAAAGG	TAGGCCTTTAACCTGGTCGGTC
<i>D. rerio igsf11</i>	AAACATCACTCCTATATTGCGT	TAGGACGCAATATAGGAGTGAT
<i>D. rerio kcnj13</i> (1)	AAACCCGCCACCGTAGCGCCAG	TAGGCTGGCGCTACGGTGGCGG
<i>D. rerio kcnj13</i> (2)	AAACCTGCCATCGCGCTGCTTG	TAGGCAAGCAGCGCGATGGCAG

Table 3 | Primers used for genotyping.

primer name	sequence (5'-3')
MP318	AGCTGTGCCCAAGCAAGA
MP330	CCCCCATGCATTTTATTTGACCA
MP331	CTGAATTCAGAAAGGAGGAGGT
MP92	CTCCCTTCCATTCACACTACC
T963	GAAACTATTCTTGCCGTGACTTG
T964	TCAAACAAACCTGGGTGTGGAC

T1449	TCATCTACCAGAGTGGTCAG
T1450	CCTAAACTTTTGCAGCACAG
T2139	TCAATGGAGACCTGGATGTC
T2140	TGGACCAAAGTGTGAAAGC
T2143	TGCCTGTGTTTATGTGTCTG
T2144	AATGACCAAGAAGGATGAGC
T2145	GCCTCTAGGAACATGATTGG
T2146	GCTTCTCATTCTAGCCCTC
T2147	GGCAACATTGGCGTTATCTC
T2148	TCTCACAGCATTCTGGCAC
T2149	CTGCCGTTTATATCTCCACAG
T2150	GGCTGAAAATTGGCTGATTG

827

828 Table 4 | List of generated mutants.

mutant	mutation	description according to ¹
<i>D. aesculapii</i> <i>Csf1ra</i> ^{t31ui}	4 bp deletion	recessive, c.1500_1503delGGTC p.Gly502LysfsX35
<i>D. aesculapii</i> <i>Cx39.4</i> ^{t15ui}	8 bp deletion	recessive, c.175_182delAAACAGCC p.Lys59_ArgfsX2
<i>D. aesculapii</i> <i>Cx39.4</i> ^{t59ui}	21 bp deletion	recessive, c.168_188delCAACACCAAACAGCCCGGCTG p.Asn57_Cys63del
<i>D. aesculapii</i> <i>Cx41.8</i> ^{t18mp}	18 bp deletion	recessive, c.41_58delTCCAGGAGCATTCAACCT p.Val14AlaGlu15_Ser20del
<i>D. aesculapii</i> <i>Igsf11</i> ^{t10mp}	1 bp insertion	recessive, c.130dupT p.Leu44PhefsX12
<i>D. aesculapii</i> <i>Igsf11</i> ^{t19mp}	11 bp deletion	recessive, c.129_139delCTTGCCCTGTA p.Leu44PhefsX8
<i>D. aesculapii</i> <i>Kcnj13</i> ^{t11mp}	4 bp deletion	recessive, c.260_263delACCT p.Tyr87CysfsX27
<i>D. aesculapii</i> <i>Mitfa</i> ^{t30ui}	7 bp deletion	recessive, c.194_200delGACCCGG

		p.Gly65GlufsX22
<i>D. aesculapii</i> <i>Mpv17</i> ^{32ul}	4 bp deletion	recessive, c.321_324delAATA p.Ile108LeufsX6
<i>D. rerio</i> <i>Igsf11</i> ^{t35ul}	17 bp deletion	recessive, c.412_428delGTGATCGGCCTGACGGT p.Ile138AlafsX19
<i>D. rerio</i> <i>Kcnj13</i> ^{dt58ul}	6 bp deletion	dominant, c.190_195delTGGCGG p.Trp64Arg65del
<i>D. rerio</i> <i>Kcnj13</i> ^{124ul}	14 bp insertion	recessive, c.436_437insGATGGAAGATGCTT p.Ala146GlyfsX28

829

830 Supplementary Discussion

831 *Kcnj13* functions as a tetramer (Fig. 4d, e), where each subunit contributes two
832 transmembrane helices (M1 and M2, Extended Data Fig. 4) to the formation of the
833 channel pore and a short extracellular loop that folds back to form the pore lining ion
834 selectivity filter (P-loop or H5, Extended Data Fig. 4). The N- and C-termini of the
835 subunits reside in the cytoplasm, where they also contribute to the ion pore, but are
836 mainly involved in gating of the channel (reviewed in²). Four dominant *Kcnj13* alleles
837 have been identified in *D. rerio* in several independent forward genetic screens
838 (Extended Data Fig. 4)³⁻⁵. All of them show broad stripes with irregular interruptions
839 when heterozygous (Fig. 2g) and strong pattern aberrations with fewer, wider and
840 interrupted dark stripes and some mixing of melanophores and xanthophores when
841 homozygous or trans-heterozygous. Three of them carry point mutations affecting
842 H5 or M2^{3,4,6}, one is the result of a C-terminal truncation (Extended Data Fig. 4)⁵.
843 The point mutations lead to proteins that do not produce functional channels⁶ and it
844 has been suggested that the dominant phenotype is caused by a dosage-dependent
845 effect, i.e. haploinsufficiency⁷. To test this possibility we used the CRISPR/Cas9
846 system and generated a loss of function allele of *Kcnj13* in *D. rerio* which is
847 recessive. The 14 base pair insertion near the end of the first coding exon leads to
848 an early truncation of the protein. Homozygous mutants show a phenotype similar to
849 homozygous mutants for the dominant alleles (Fig. 2h)³⁻⁷. This shows that the
850 dominant alleles are dominant-negatives, where the mutant proteins inhibit the

function of the wild-type protein in heterozygotes. The generation of this recessive allele allowed us to perform the reciprocal heterozygosity test with *D. aesculapii* as well as interspecific complementation with seven other *Danio* species⁸.

Supplementary Information References

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