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DOI: 10.1111/pcmr.12272
Volume 27, Issue 5, Pages 827–830

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A dominant mutation in tyrp1A leads to melanophore death in zebrafish

Jana Krauss, Silke Geiger-Rudolph, Iris Koch, Christiane Nüsslein-Volhard and Uwe Irion

Max Planck Institute for Developmental Biology, Tübingen, Germany

CORRESPONDENCE Uwe Irion, e-mail: uwe.irion@tuebingen.mpg.de

KEYWORDS zebrafish/pigmentation/melanophore/tyrosinase-related protein 1/melanin

PUBLICATION DATA Received 17 January 2014, revised and accepted for publication 30 May 2014, published online 2 June 2014
doi: 10.1111/pcmr.12272

Summary
Melanin biosynthesis in vertebrates depends on the function of three enzymes of the tyrosinase family, tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrp1), and dopachrome tautomerase (Dct or Tyrp2). Tyrp1 might play an additional role in the survival and proliferation of melanocytes. Here, we describe a mutation in tyrp1A, one of the two tyrp1 paralogs in zebrafish, which causes melanophore death leading to a semi-dominant phenotype. The mutation, an Arg->Cys change in the amino-terminal part of the protein, is similar to mutations in humans and mice where they lead to blond hair (in melanesians) or dark hair with white bases, respectively. We demonstrate that the phenotype in zebrafish depends on the presence of the mutant protein and on melanin synthesis. Ultrastructural analysis shows that the melanosome morphology and pigment content are altered in the mutants. These structural changes might be the underlying cause for the observed cell death, which, surprisingly, does not result in patterning defects.

Melanin is the most prevalent pigment in animals and the only pigment synthesized in mammals. It is responsible for much of the body colouration in vertebrates and has important functions in photoprotection, camouflage, and mate selection (Hubbard et al., 2010). Melanin is derived from L-tyrosine through several steps catalyzed by enzymes of the tyrosinase family. In vertebrates, this family consists of three members: tyrosinase (Tyr) and tyrosinase-related proteins, Tyrp1, and Tyrp2 (Godin and Setaluri, 2006). Tyr is the rate-limiting enzyme, catalyzing the conversion of L-tyrosine to L-dopaquinone. All three enzymes are involved in downstream reactions leading to melanin polymers (Solano and Garcia-Borron, 2006). The enzymatic activities of Tyrp1 are not as well understood as those of Tyr and Tyrp2. It has been suggested that Tyrp1 could also fulfill a structural role in melanosomes, either by complexing and stabilizing Tyr in melanosome membranes (Kobayashi and Hearing, 2007) or by enhancing melanocyte proliferation and maturation (Sarangarajan et al., 2000).

All these enzymes are highly expressed in melanocytes and malignant melanomas; TYRP1 expression levels in melanoma correlate negatively with patient survival (El Hajj et al., 2013). In humans, mutations in TYR cause oculocutaneous albinism type1 (OCA1), while OCA3, Rufous albinism, is

Significance
Tyrp1 is highly expressed in melanocytes and a potential target for treatment of melanomas. We identified a mutation in tyrp1A in zebrafish that leads to melanophore death and continual regeneration. We find that tyrosinase activity is required for melanophore death to occur in the mutants. However, because zebrafish contain another functional Tyrp1 paralog, the phenotype cannot simply be due to the buildup of toxic intermediates of melanin synthesis caused by the interruption of the synthetic pathway. Rather our data suggest that the structural integrity of melanosomes is affected in the mutants.
due to mutations in TYRP1 (Dessinioti et al., 2009); a specific change, Arg93 to Cys, is responsible for blond hair in melanesians (Kenny et al., 2012). The corresponding locus in mouse is the brown (b) gene (Zdarsky et al., 1990) leading to brown coat color in homozygous mutants; the allele $b^{Light}$ displays a dominant phenotype (Johnson and Jackson, 1992).

In zebrafish (Danio rerio), melanin is produced by melanophores, the black cells responsible for the characteristic dark stripes. Mutations in tyr/sandy (sdy) lead to the absence of melanin and an albino phenotype (Kelsh et al., 1996). The zebrafish albino (alb) gene, however, encodes a solute carrier (SLC45A2) necessary for pH homeostasis (Dooley et al., 2013). Mutations for tyrp1 or tyrp2 have not been described in zebrafish, so far. For tyrp1, this is likely due to redundancy after gene duplication (tyrp1A and tyrp1B) so that loss-of-function of one paralog does not cause a phenotype (Braasch et al., 2009).

Here, we describe VA6, a tyrp1A mutant in zebrafish. The mutation causes a semi-dominant effect with heterozygous fish usually showing a milder and more variable phenotype than homozygotes. In the mutants, melanophores are present and pigment normally at around 28 h post-fertilization (hpf). But from about 3 days post-fertilization (dpf) onwards, the melanophores disappear and only their remnants remain visible as pigmented detritus. Simultaneously, new, lighter pigmented, melanophores reappear, demonstrating their regeneration (Figure 1A–F') (Yang and Johnson, 2006). The fish stay lighter pigmented for several months (Figure 1G–L). Ultrastructural analysis showed that the melanosomes in the homozygous mutants are less regular in shape and contain a lower amount of pigment than in wild type; in heterozygous mutants, the melanosomes are not clearly distinguishable from wild type (Figure 1M–O). From about 3 to 4 months onwards, the fish regain the normal pigmentation (Figure 1P–U). The formation of larval and adult pigmentation pattern is essentially normal in the mutants, suggesting that the cells survive long enough to fulfill their roles in this process.

We mapped the mutation to chromosome 7 (Figure 2A) close to the gene tyrp1A and identified a C to T transition in its second exon at position 436 of the coding sequence (see Supporting Information, Data S1 for methods). This leads to the exchange of a conserved arginine to cysteine at position 146 between the N-terminal EGF-like domain and the tyrosinase domain of the protein (Figure 2B,C).

In zebrafish larvae, tyrp1A expression can be detected from about 24 hpf onwards in melanophores (Figure 2F).

Figure 1. tyrp1A<sup>VA6</sup> leads to melanophore loss. Lateral (A–C) and dorsal (D–F) views of 5 dpf larvae showing the strong reduction of melanophores in heterozygous (B, E) and homozygous (C, F) mutant larvae compared to wild type (A, D). Magnifications of D–F (D′–F′). The pigment detritus of dead melanophores and the lightly pigmented regenerating melanophores are visible in the mutants. Lateral views of adult fish (approx. 10 weeks, G–I) and magnifications of the striped region (J–L). Melanophore density in the stripes is lower in heterozygous (H, K) and homozygous (I, L)3 mutants, compared to wild-type fish (G, J). Electron micrographs (M–O) show irregular melanosomes with less electron dense pigment in the homozygous mutants (O). There is no clear difference visible between heterozygous mutants (N) and wild type (M). Magnifications of the striped regions of 14-week-old fish (P–R) and 18-week-old fish (S–U). Melanophore density and stripe pigmentation recovers in the mutants.
Figure 2. Mapping of VA6 and expression of \textit{tyrp1A} and \textit{tyrp1B} in larvae. The mutant phenotype is 100% linked to z21714 and z22628 flanking \textit{tyrp1A} at the telomeric end of chromosome 7 (\textit{A}). A mutation in the second exon of this gene (\textit{B}, introns are not drawn to scale), marked with an asterisk in (\textit{D}), leads to the exchange of Arg146 to Cys in the protein. Schematic representation (\textit{C}) of the protein showing signal peptide (SP), EGF-like and tyrosinase domains and transmembrane region (TM). Mutations in the human protein with the respective phenotypes (blond or OCA3) and two mutations in the mouse protein are indicated. In (\textit{E}) partial amino acid sequences from zebrafish (Drer), mouse (Mmus), cat (Fcat), and human (Hsap) proteins are compared. The numbering refers to \textit{tyrp1A\textendash}VA6. Expression of \textit{tyrp1A} (\textit{F, H}) and \textit{tyrp1B} (\textit{G, I}) detected by \textit{in situ} hybridization in 24 hpf (\textit{F, G}) and 48 hpf (\textit{H, I}) alb/l48 larvae. Both orthologs are expressed in body melanophores; \textit{tyrp1A} is hardly detectable in the RPE at 48 hpf.

Figure 3. Melanophore death depends on the mutant protein and pigment synthesis. Dorsal views of larvae homozygous for \textit{tyrp1A\textendash}VA6 (\textit{A–F}), uninjected controls (\textit{A, C, E}), and injected with 400 \textmu M \textit{tyrp1A} morpholino (\textit{B, D, F}) shown at 4 dpf (\textit{A, B}), 5 dpf (\textit{C, D}), and 7 dpf (\textit{E, F}). Melanophore death is suppressed by the morpholino. Dorsal views of head regions of larvae at 84 hpf (\textit{G–J}). After treatment with PTU, unpigmented melanophores are present in wild type (\textit{I}) and mutant larvae (\textit{J}) in normal numbers, visualized by expression of \textit{tyrp1B}. In untreated larvae (\textit{G, H}), melanophores are visible by their pigment; melanophore death is apparent in the mutants (\textit{H}). In double mutants for \textit{albino} and \textit{tyrp1A\textendash}VA6 (\textit{K}) and \textit{sandy} and \textit{tyrp1A\textendash}VA6 (\textit{L}), melanophore death is suppressed; melanophores are visualized by their expression of \textit{tyrp1B}.
It persists during larval development but appears somewhat weaker than tyrp1B, and we find that tyrp1A is hardly detectable in the retinal pigment epithelia (RPE), where tyrp1B is strongly expressed (compare Figure 2H, I). This is consistent with the fact that the mutants show no visible defects in melanophores of the RPE.

Morpholino-induced knockdown of either tyrp1A or tyrp1B has no phenotypic effect; only the double knockdown results in hypo-pigmented melanophores (Braasch et al., 2009). This suggests that the 3A6 mutation represents a neomorphic allele where the mutant protein interferes with some essential process in melanophores, thereby causing cell death. Indeed, we find that a splice morpholino targeting tyrp1A suppresses melanophore death in homozygous mutant larvae and restores normal pigmentation (Figure 3A–F). This effect lasts up to 7 days when the morpholino loses its effect, and the mutant phenotype becomes visible again.

To test whether melanophage death in the mutants requires melanin production, we blocked pigment synthesis in the larvae using phenylthiourea (PTU) (Tryon and Johnson, 2012). We did not find any difference in melanophores between mutant and wild-type larvae at 84 hpf (Figure 3G–J). Similar results were obtained in double mutants with sandy (Tyr) or albino (SLC45A2) indicating that melanin synthesis is indeed required for cell death to occur in tyrp1A^VA6 mutants (Figure 3K,L).

Mutations in tyrp1 are known in a variety of different species; loss-of-function generally leads to brown instead of black pigmentation. Dominant alleles have been described in mice, b^T (Johnson and Jackson, 1982), and pigeons, Ash-red (B^A) (Domyan et al., 2014). In the case of mice carrying the Light allele of Tyrp1, it has been suggested that an Arg to Cys mutation in close proximity to the N-terminal EGF-like domain of the protein might interfere with its functions in the melanosome membrane ultimately leading to the disruption of melanosome integrity and leakage of toxic melanin precursors into the cytoplasm. The semi-dominant mutation we identified in zebrafish also leads to an Arg to Cys exchange in the N-terminal part of the protein and could have a similar effect. This notion is supported by the fact that the melanosomes in the mutants appear less regular in size and shape, and they seem to have a lower pigment content (Figure 1M–O).

**Acknowledgements**

We thank H.-G. Frohnhöfer and C. Söllner for discussions, C. Weiler for technical assistance. This work was supported by the Max-Planck-Gesellschaft.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Methods used and References for Methods.