Supporting Online Material for

Pigment Pattern Formation by Contact-Dependent Depolarization
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Published 10 February 2012, Science 335, 677 (2012)
DOI: 10.1126/science.1212821

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Materials and Methods

Zebrafish stock

Wild-type zebrafish and albino mutant were obtained from the zebrafish stock center at the Max Planck Institute (Tübingen, Germany). jaguar<sup>h230</sup> mutants were derived from stocks provided by the Johnson lab (Washington University School of Medicine, St. Louis, Missouri, USA).

Cell preparation

Adult zebrafish were anesthetized with 0.02% tricaine. Next, their tail or anal fins were cut. All the subsequent steps were performed at 28°C. The fins were incubated in a trypsin solution containing 2.5 mg/ml trypsin, 1.2 mg/ml bovine serum albumin, and 1 mM EDTA for 60 min. After the incubation, the fins were washed with 1× PBS for 5 min at 1,000 rpm. To remove epidermal cells from the fins, the wash step was repeated five times. In the next step, the fins were incubated with stirring in a collagenase solution containing 0.1 mg/ml collagenase I, 0.1 mg/ml DNase I, 0.1 mg/ml soybean trypsin inhibitor, and 1.2 mg/ml bovine serum albumin for 60 min at 1,000 rpm. The dissociated tissue was filtered through 25µm-pore nylon mesh to remove fin rays, and the filtrate was centrifuged at 800 rpm for 8 min. From the pellet containing the pigment cells, the melanophores and the xanthophores were isolated by density gradient centrifugation on 35–70% liner gradients of Percoll or the JSAN cell sorter (Bay bioscience, Kobe, Japan), respectively.

Gene expression analysis

Total RNA was isolated from 5 × 10<sup>4</sup> pigment cells (melanophores or xanthophores) using the RNeasy kit (Qiagen, Tokyo, Japan). Reverse transcription into cDNA was conducted using the SuperScript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA, USA). qPCR reactions were performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All qPCR experiments were conducted in triplicate. RNA expression levels were normalized to expression of the β-actin gene. Kir7.1 was amplified with forward primer 5′-GGAGACGCAAICTCAGTGGG-3′ and reverse primer 5′-AATAGGATCCCGTCACATCG-3′. β-actin was amplified with forward primer
5′-CGGTTTTGCTGGGAGATGATG-3′ and reverse primer 5′-CGTGCTCAATGGGGTGTTTG-3′.

### Plasmid construction and generation of transgenic fish

To induce stable gene expression in adult zebrafish, we used the Tol2 transposon-based transgenesis system. To express the Kir genes only in melanophores, a 1.3-kb fragment [(with a nacre promoter (10)], including the translation initiation codon of the nacre gene, was amplified by PCR and subsequently inserted into a pDsRed-Expression-N1 vector (Clontech Lab, Mountain View, CA, USA). The DNA for the nacre promoter-DsRed was subcloned into the Tol2 vector, pT2AL200R150G (11), following the removal of EF1α promoter-EGFP. The resulting plasmid was designated as pTol2-nacre-DsRed. To identify the zebrafish ortholog of the mammalian Kir2.1 gene (ENSDARE00000163109), we searched the Sanger zebrafish database. Total RNA was isolated from zebrafish brain using the RNeasy kit (Qiagen). cDNA was generated from RNA using the SuperScript III First-Strand Synthesis system (Invitrogen). Kir7.1 was amplified with forward primer 5′-GCTGAAGCTTGCCACCATGCCTACCACCATGACAAA-3′ and reverse primer 5′-TGCAGTCGACGACTACTCCACGCGGTCGCTGC-3′. Kir2.1 was amplified with forward primer 5′-GCTGAAGCTTGCCACCATGGAAGTGTGCGGGCC-3′ and reverse primer 5′-CAGTGGATCCTCATATTTCAGATTCCCG-3′. Kir7.1 and Kir2.1 fragments were then inserted into the HindIII-SalI or HindIII-BamHI sites of the pTol2-nacre-DsRed plasmid, respectively.

Transgenic fish were generated by co-injection of plasmid DNA (20 ng/µl) with Tol2 mRNA (25 ng/µl) into fertilized eggs at the one-cell stage. The eggs were raised to adulthood as founder fish and they were outcrossed with wild-type or jaguar mutant fish. Their progeny were used to evaluate the effect of the transgene expression on pigment pattern formation in this report (fig. S1, B-G).

### Cell culture

To detect the fluorescence signal of DiBAC4(3) from melanophores, we used melanin-synthesis mutants (12): albino mutant (alb/alb), golden mutant and double mutant (alb/alb: jag/jag). The pigment cells were collected by the same protocol used in gene expression analysis, except for the isolating steps with Percoll gradient centrifugation and
use of a cell sorter. Since albino melanophores were not isolated by Percoll gradient centrifugation, we used cell suspensions containing non-pigment cells for cell culture. The pigment cells were suspended in serum-free L-15 medium (Invitrogen), and this cell suspension was then plated onto 35 mm glass-bottomed dishes (Iwaki, Tokyo, Japan) that had been coated with 0.05 mg/ml type-I collagen. The pigment cells were incubated for 24 h at 28°C before imaging of membrane potential. In this procedure, the pigment cells from wild-type zebrafish and the jaguar mutant were incubated in 2 separate dishes.

**Confocal imaging of membrane potential**

Pigment cells were stained with 200 nM *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] (Dojindo, Kumamoto, Japan) (13) in L-15 medium with 10% FBS in the dark for 30 min at 28°C. To prevent the evaporation of water, 2 ml mineral oil was loaded on the culture medium. Images were recorded using a FV 300 confocal laser scanning microscope (Olympus, Tokyo, Japan). The dye was excited by an argon laser (488 nm) and imaged under the FITC filter.

**Image processing**

To compare the fluorescence intensity of DiBAC₄(3) between wild-type and the jaguar mutant, the mean intensity within a cellular region was measured using ImageJ software (NIH). Statistical analyses were performed with the Steel-Dwass test (fig. S2). The change in fluorescence intensity of melanophores (Fig. 1B) was calculated as

\[ \Delta F/F_0 = (F_n - F_0)/F_0, \]

where \( F_n \) is the mean fluorescence intensity of a melanophore at time frame \( n \), and \( F_0 \) is the mean fluorescence intensity of a melanophore just before contact with a xanthophore.
Figure S1. Function of Kir7.1, K⁺ transport, is required only in melanophores. 
(A) Kir7.1 expression in melanophores is higher than in xanthophores. Melanophores and xanthophores were isolated from adult wild-type fish using Percoll density gradient centrifugation and cell sorter, respectively. Relative expression (normalized to β-actin) of Kir7.1 was revealed by qPCR experiments. See “Gene expression analysis” in materials and methods. Error bars represent SD.
(B-G) Recovery of pigment pattern abnormality of jaguar mutant by the Kir channel genes. 
(B and C) The homozygous jaguar mutant rescued by BAC plasmid containing the genomic kir7.1 sequence (5) (jaguar; BAC-kir7.1). (D and E) The homozygous jaguar mutant rescued by expressing functional Kir7.1 in melanophores (jaguar; nacre-Kir7.1). (F and G) The homozygous jaguar mutant rescued by expressing Kir2.1 in melanophores (jaguar; nacre-Kir2.1). These data (B-G) imply that the activity of K⁺ transport is sufficient to rescue the pigment pattern abnormality. Black cells and yellow cells are melanophores and xanthophores, respectively. Blue lines indicate the edge of each group of pigment cells. Scale bars in overall view, 1 cm; enlarged view, 100 µm. See “Plasmid construction and generation of transgenic fish” in materials and methods.
Figure S2. Detection of the melanophore depolarization *in vitro*. (A and B) DiBAC$_4$(3) fluorescence in wild type (A) and the *jaguar* mutant (B) are shown as pseudo-color images. Outlined cells and small cells show melanophores and fibroblasts, respectively. (C) The fluorescence intensity of wild-type melanophores was higher than those of *jaguar* ones ($P=7.1 \times 10^{-8}$). (D) The fluorescence intensity of fibroblasts between wild-type and *jaguar* mutant did not show a significant difference ($P=0.99$). Scale bar, 50 µm. See “Image processing” in materials and methods.
**Movie S1.** DiBAC₄(3) image of a melanophore (M) before and after contact with a xanthophore (X) or a fibroblast (F) in wild-type fish. Scale bar is 50 µm, frames are 3 min apart.

**Movie S2.** DiBAC₄(3) image of a melanophore (M) before and after contact with a xanthophore (X) in the homozygous *jaguar* mutant. Scale bar is 50 µm, frames are 3 min apart.

**Movie S3.** DiBAC₄(3) image of cell behavior during the interaction between a melanophore (M) and a xanthophore (X) in wild-type fish. Scale bar is 50 µm, frames are 3 min apart.

**Movie S4.** DiBAC₄(3) image of cell behavior during the interaction between a melanophore (M) and a xanthophore (X) in the homozygous *jaguar* mutant. Scale bar is 50 µm, frames are 3 min apart.
References


4. G. Takahashi, S. Kondo, Melanophores in the stripes of adult zebrafish do not have the nature to gather, but disperse when they have the space to move. Pigment Cell Melanoma Res. 21, 677 (2008). doi:10.1111/j.1755-148X.2008.00504.x Medline


