Supplementary Information

Spot pattern of Leopard Danio is caused by the mutation in zebrafish connexin41.8 gene

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Supplementary text:

Fish

Zebrafish mutant strains *leo*^{t1}, *leo*^{tw28} and *leo*^{tq270} were obtained from the stock center of the Max-Plank Institute (Tuebingen, Germany). Zebrafish were maintained and bred as described in *The Zebrafish Book* (Westerfield, 1995).

Positional cloning of *leopard* gene

We started positional cloning of *leopard* gene using microsatellite markers mapped onto linkage group 1 (LG1) of zebrafish because the *leopard* gene was first mapped near the microsatellite marker z9704 on LG1 (Johnson *et al.*, 1995). We used *leo*^{t1} fish as the mutant strain and Tu, AB and india as wild type strains for meiosis mapping. After intercrossing of heterozygote F1 fish, we obtained 1,738 F2 fish that had the homozygous phenotype of *leo*^{t1}. These 1,738 F2 fish resulted from three cross experiments: 433 F2 fish were obtained from the cross using india (WT) and *leo*^{t1} as F0 generations, 610 F2 fish resulted from the cross experiment using Tu (WT) and *leo*^{t1}, and 695 F2 fish resulted from the cross experiment using AB (WT) and *leo*^{t1}. We mapped the *leopard* locus within a ~0.2-cM interval, which was calculated from the recombinant frequency of the 7/1,738 F2 homozygotes, upstream from the z9704 microsatellite marker on LG1. In addition, the microsatellite z-marker z21548 mapped upstream of the *leopard* locus with

a ~2-cM interval from this locus (data not shown). Because the z-marker z9704 was situated closer to the *leopard* locus (Figure 2A), we identified single nucleotide polymorphisms using primers (listed in Table S1) around this region for further analysis. We used these 1,738 individuals for genetic mapping analysis of the region downstream of the *leopard* locus including loci z9704, 106K4T, 74G19T QC and D4D. We also used these 1,738 individuals for D4K**,** a locus upstream of the *leopard* region, but used 962 individuals for loci 276127F06R06 and 276127F03R03 (Figure 2A) because there is no identified polymorphic sequence between AB and leo^{t1} fishes at these loci. The distances from the markers to the *leopard* locus were calculated as follows; 0.03 cM from QC marker to *leopard* locus, and 0.05 cM from 276127F06R06 marker to *leopard* locus. The genome map of the *leopard* critical region was constructed using WGS assembly ver.3, ver.4 and ver.5 of the *Danio rerio* Sequence Project at the Sanger Institute (http://www.sanger.ac.uk/). The position of D4K was estimated by sequence data from the Sanger Institute. In this *leopard* critical region we ultimately identified two candidate genes, *bcl9* and connexin41.8. We confirmed by PCR that the BAC clone DKEY-53o8 harbors these two genes in its insert fragment (data not shown) and also confirmed by microinjection of this BAC clone that this clone contains a factor for the development and/or pattern formation of pigment cells (Figure S2). The BAC clone DKEY-53o8 was purchased from RZPD (http://www.rzpd.de/).

HeLa cell transfection

Patch-clamp experiments were performed with clones of HeLa cells that were transfected with cDNA constructs containing the coding sequence of WT or mutant *zfCx41.8*. The *zfCx41.8* sequence was amplified with a pair of primers, zfCx40xhoF01 (5'-TCCACTCGAGGCTCCTCTGAATAGGCATG-3') and zfCx40SalR01 (5'-ACAGTCGACTGGCCAGTGCTATACCGCA-3'). The amplified fragments were ligated into a cloning vector, pGEM-T (Promega, Madison, WI). The plasmids were digested with the restriction enzymes, *Xho* I and *Sal* I, and the subsequent fragments were subcloned into the expression vector pcDNA3 (Promega). HeLa cells were transfected with 5 μg recombinant zfCx41.8-pcDNA plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after the transfection, the medium, Opti-men (Invitrogen) was changed to D-MEM (Invitrogen) containing 10% FBS and 0.5 mg/ml G-418; clones grown in that medium were selected after two weeks, and mRNA expression was determined by RT-PCR. The cells were seeded onto sterile glass coverslips placed in 12-well dishes and used within 24–48 h after plating.

Hemichannel current measurements

Patch pipettes were filled with normal pipette solution: 120 mM potassium aspartate, 10 mM

NaCl, 3 mM Mg^{2+}-ATP, 5 mM HEPES, pH 7.2, and 10 mM EGTA, filtered through a 0.22 µm pore filter. Bathing solution contained 120 mM potassium aspartate, 10 mM NaCl, 5 mM HEPES, pH 7.4, 5 mM glucose, 2 mM CsCl, 2 mM $BaCl_2$, and 2 mM tetraethyl ammonium. Patch pipettes were pulled from glass capillaries, coated with sylguard and then fire polished. When filled with solution, the pipettes had DC resistances of 5–7 MΩ. Experiments were carried out on single cells attached to a pipette. After establishing a G seal, the membrane patch was disrupted, enabling whole-cell recording. The channel current was recorded using a patch clamp amplifier, Axon200B (Axon Instrument, CA), and analyzed with commercially available software, pCLAMP9 (Axon Instrument). Results are shown as mean values, and error bars represent ± SEM.

RNA isolation, cDNA synthesis, sequence analysis, and RT-PCR analysis

Total RNA was isolated from adult tissues of zebrafish strains Tu, AB, *leo*^{t1}, *leo*^{tw28} and *leo*^{tq270} after lysis in TRIzol reagent (Invitrogen), and first-strand cDNAs were synthesized with Superscript III (Invitrogen) and oligo $d(T)_{18}$ primer. A full-length *zfCx41.8* orf was obtained by PCR using the upstream primer zfCx40F01 (5'-CATTCACACTACCTCTTATTTC-3') and the downstream primer zfCx40R01 (5'-ACAATAGAACAACCCAATTTCC-3'). PCR products were sequenced using a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

ZfCx41.8 expression was detected by reverse transcription-PCR (RT-PCR) using the upstream primer zfCx40F02 (5'-GACTTCAACCAATGCTTGAC-3') and the downstream primer zfCx40R01. β-actin expression was also detected by RT-PCR using the upstream primer zfbactF01 (5’-GGAGAAGAGCTATGAGCTGC-3’) and the downstream primer zfbactR01 (5’-ACCTCCAGACAGCACTGTGT-3’). The cDNA equivalent of 100ng total RNA was used as template for PCR reactions (20 µl total volumes). Because the orf sequence of zebrafish connexin41.8 gene consists of one exon, we constructed primers for RT-PCR experiment as shown above. To ensure that the PCR signals were not the result of contaminating genomic DNA, control samples containing RNA in which the reverse transcriptase was omitted from the cDNA synthesis step were run in parallel. An initial denaturation step at 95°C for 5 min was followed by 35 amplification cycles (30 s at 95°C, 30 s at 50°C, 30 s at 72°C) and a final extension period of 2 min at 72°C.

Supplementary Figure S1: Alignment of Zebrafish connexin41.8 gene.

Amino acid sequence alignment of zebrafish connexin41.8 and mutants. Red characters indicate amino acid substitutions detected in the *leo*^{tw28} and *leo*^{tq270} alleles. A red asterisk at position 68 in the *leo*^{t1} allele indicates a nonsense substitution. M1–M4 indicate the predicted transmembrane regions. Gray characters indicate the untranslated region of the *leo*^{t1} allele, which results from the nonsense mutation.

Supplementary Figure S2: Complementation of connexin41.8.

Among ~500 of fertilized egg of homozygous *leo*^{t1} mutant into which the BAC clone zK53o8 was injected, larger spots of melanophores (arrows) were observed on the skin of two fish, although these fish did not show a stripe pattern form of melanophores. This observation is similar to that obtained by transplanting WT cell extracts into mutant eggs (Maderspacher and Nusslein-Volhard, 2003), suggesting that this BAC clone contributes to development and/or pattern formation of melanophores and actually contains the *leopard* gene. The plasmid clone was purified with the Qiagen Large-Construct Kit and used for microinjection into the fertilized egg of *leo*^{t1} homozygotes at the one- or two-cell stage. Final concentration of the plasmid was 10 ng/μl.

Microinjection of the plasmid harboring the β-actin promoter upstream of the connexin41.8 gene was lethal for the egg or embryo (data not shown). Morpholino-oligos or antisense-oligos were not applicable for this experiment because it takes more than one month to form the stripe pattern on zebrafish skin.

Supplementary Figure S3: Gene expression of zebrafish connexin41.8 gene.

To confirm the result in Figure 4 that connexin41.8 is expressed in both the dark and light portions of zebrafish fin and to exclude the possibility of cross contamination of pigment cells in each fraction, we used the zebrafish mutants, *nacre* and *panther*, for RT-PCR experiments. (A) The caudal fins of WT, *leopard*, *nacre* and *panther* zebrafish are shown. *nacre* is a zebrafish mutant that lacks melanophores, which is caused by a mutation in *mitf*, causing a yellow fin phenotype (Lister *et al*., 1999). On the other hand, *panther* is lacks xanthophores, which is caused by a mutation in *fms*, causing a dark fin phenotype (Parichy *et al.*, 2000). (B) The result of RT-PCR indicates that both mutants express the connexin41.8 gene. Lanes 1 and 5, WT; lanes 2 and 6, leo^{t1}; lanes 3 and 7, *nacre*; lanes 4 and 8, *panther*, lanes 1–4, +RT; lanes 5–8, –RT (negative control); upper panel, connexin41.8; lower panel, β-actin. RNA was extracted from caudal fin of each strain. (C) We next examined whether pigment cells, melanophores, express the connexin41.8 gene. Lanes 1 and 5, zebrafish fin-derived fibroblast-like cell line BRF41, which was obtained from RIKEN Cell Bank, (RIKEN, Japan). Lanes 2 and 6, zebrafish embryo-derived fibroblast-like cell line ZEM2S, which was obtained from ATCC (ATCC, VA). Lanes 3 and 7, zebrafish fin excluding melanophores but containing a few xanthophores. Lanes 4 and 8, zebrafish melanophores. Lanes 1–4, +RT, Lanes 5–8, -RT (negative control); upper panel, connexin41.8; lower panel, β-actin. Fifteen hundred cells of each cell type were used for the

reverse transcription experiment. Specimens for lanes 3 and 4 were prepared as follows. Melanophore regions of fin were collected and then treated with Reagent A (2 mM epinephrine, 25 mM EDTA, in PBS) for 10 minutes at room temperature. Next, the tissues were treated with Reagent B (1 mg/ml collagenaseIII, 0.05 mg/ml DNaseI, 1 mg/ml trypsin, 100 μg/ml epinephrine, in PBS) twice at 37°C for 20 minutes, and then treated with Reagent C (1 mg/ml collagenaseIII, 0.05 mg/ml DNaseI, 0.01% trypsin, 100 μg/ml epinephrine, in PBS) at 37°C for 15 minutes. Segregated cells were filtrated with a cell strainer (BD Falcon, 60 μm mesh) and collected by centrifugation, 150 x g for 10 min at 4°C in 15-ml tube. Cells dissolved in PBS were applied to the 0–60% Percoll gradient in a 15-ml disposable tube and then centrifuged at 1,000 × *g* at 4°C for 20 min. Melanophores were collected as precipitates and subjected to RT-PCR. Other cells were collected from around the 30% Percoll layer, in which we found a few contaminating xanthophores, but almost all tissues were not pigmented cells. These cells were subjected to RT-PCR, and the result is shown in lanes 3 and 7. Reagents were purchased from Sigma-Aldrich (USA) or WAKO (Japan).

This panel indicates that zebrafish connexin41.8 is expressed in melanophores but not in other components of the dark stripe in fin. Taken together with the results in Figure 4 that demonstrate that connexin41.8 is expressed in the light stripe portion of fin, and in Figures S3B and S3C that show that fibroblast-like cultured cells or other components of the dark stripe of fin

excluding melanophores do not express connexin41.8, we concluded that connexin41.8 is expressed in both melanophore and xanthophore.

References

Johnson SL, Africa D, Horne S, Postlethwait JH (1995) Half-tetrad analysis in zebrafish: mapping the *ros* mutation and the centromere of linkage group I. *Genetics* **139**: 1727-1735.

Lister JA, Robertson CP, Lepage T, Johnson, SL, Raible DW (1999) nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development*, **126**: 3757-3767.

Maderspacher F, Nusslein-Volhard C (2003) Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development* **130**: 3447-57

Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL (2000) An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. *Development*, **127**: 3031-3044.

Shimoda N, Knapik EW, Ziniti J, Sim C, Yamada E, Kaplan S, Jackson D, de Sauvage F, Jacob H, Fishman MC (1999) Zebrafish genetic map with 2000 microsatellite markers. *Genomics* 58: 219-32.

Westerfield M. (1995) *The Zebrafish Book, Edition 3*. University of Oregon Press, Eugene, Oregon.

Table S1 Marker name and primer sequence used for positional cloning

Marker	Primer sequence-F	Primer sequence-R
276127F03R03	GTGATTGGGTTAATCGTTAC	TGAAAACTCATTAATATGGA
276127F06R06	CAAATAGTAAAACTGTGAAC	CTTCATTTTGAAATAAGCTG
D4K	TTATCGACTAGCTGCAATGC	TCACAGTTTCTTCTGGAGAA
D4D	CGGTGAGAGCGCCGAATCCT	CATGATTATATGCTGGTCAT
QC	CCAGAAAGGTGATACATTGT	TTGATCGCTATATGAAGATC
74G19T	ATGAATCATGTGCTTGATTT	AGTTCTGATTTCAAGAGTTC
106K4T	TCAGATTCTCCTACCTCCCA	CAGTCTGAGGTAAAGAGCAG
z9704	CACGATGGCACAAAAATCTG	AAATCTTGGATGCGTATCGG

276127F03R03, 276127F06R06, D4K, D4D and QC sequences were developed from WGS assembly ver.3 and ver.4 by Sanger Institute. 74G19T and 106K4T were derived from end sequences of DKEY BAC clone, DKEY-74G19 and DKEY-106K4, respectively. z9704 was from Shimoda et al.

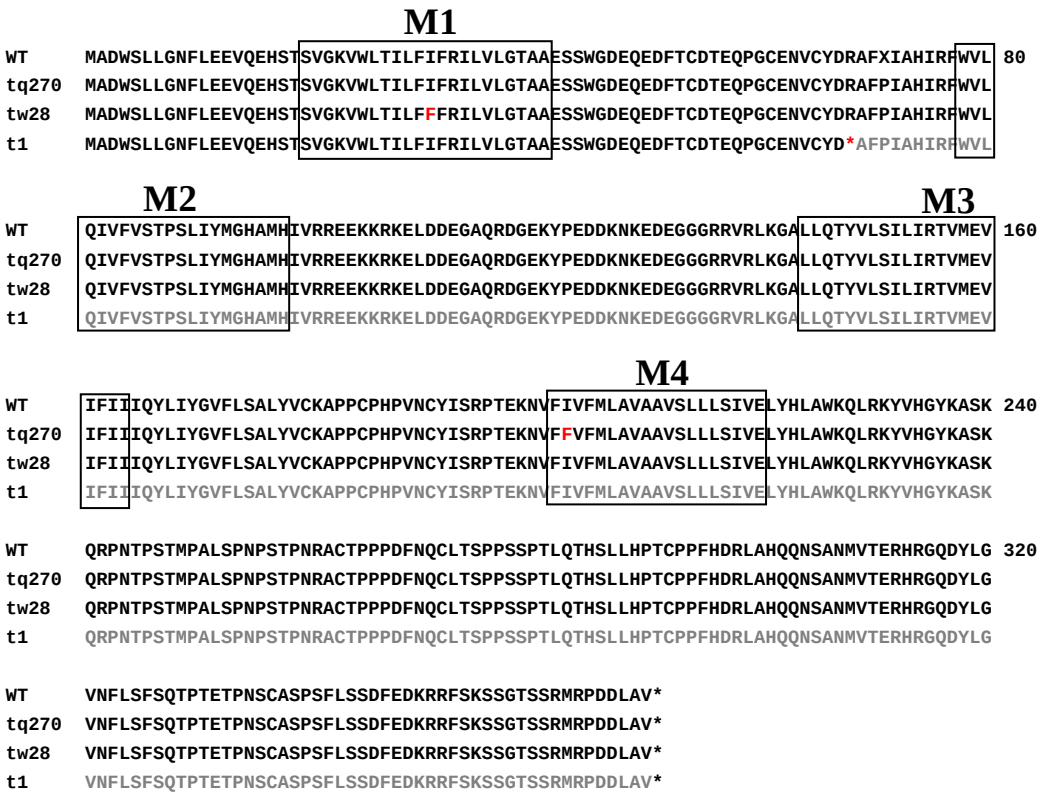
M1
WT MADWSLLGNFLEEVQEHSTSVGKVWLTILFIFRILVLGTAAESSWGDEQEDFTCDTEQPGCENVCYDRAFXIAHIRFWVL 80
tq270 MADWSLLGNFLEEVQEHSTSVGKVWLTILFIFRILVLGTAAESSWGDEQEDFTCDTEQPGCENVCYDRAFPIAHIRFWVL
tw28 MADWSLLGNFLEEVQEHSTSVGKVWLTILFFRILVLGTAAESSWGDEQEDFTCDTEQPGCENVCYDRAFPIAHIRFWVL
t1 MADWSLLGNFLEEVQEHSTSVGKVWLTILFIFRILVLGTAAESSWGDEQEDFTCDTEQPGCENVCYD*AFPIAHIRFWVL
M2
M3
WT QIVFVSTPSLIYMGHAMHIVRREEKKRKELDDEGAQRDGEKYPEDDKNKEDEGGGRRVRLKGALLQTYVLSILIRTVMEV 160
tq270 QIVFVSTPSLIYMGHAMHIVRREEKKRKELDDEGAQRDGEKYPEDDKNKEDEGGGRRVRLKGALLQTYVLSILIRTVMEV
tw28 QIVFVSTPSLIYMGHAMHIVRREEKKRKELDDEGAQRDGEKYPEDDKNKEDEGGGRRVRLKGALLQTYVLSILIRTVMEV
t1 QIVFVSTPSLIYMGHAMHIVRREEKKRKELDDEGAQRDGEKYPEDDKNKEDEGGGRVRLKGALLQTYVLSILIRTVMEV
M4
WT IFIIIQYLIYGVFLSALYVCKAPPCPHPVNCYISRPTEKNVFIVFMLAVAAVSLLLSIVELYHLAWKQLRKYVHGYKASK 240
tq270 IFIIIQYLIYGVFLSALYVCKAPPCPHPVNCYISRPTEKNVFFVFMLAVAAVSLLLSIVELYHLAWKQLRKYVHGYKASK
tw28 IFIIIQYLIYGVFLSALYVCKAPPCPHPVNCYISRPTEKNVFIVFMLAVAAVSLLLSIVELYHLAWKQLRKYVHGYKASK
t1 IFIIIQYLIYGVFLSALYVCKAPPCPHPVNCYISRPTEKNVFIVFMLAVAAVSLLLSIVELYHLAWKQLRKYVHGYKASK
WT QRPNTPSTMPALSPNPSTPNRACTPPPDFNQCLTSPPSSPTLQTHSLLHPTCPPFHDRLAHQQNSANMVTERHRGQDYLG 320
tq270 QRPNTPSTMPALSPNPSTPNRACTPPPDFNQCLTSPPSSPTLQTHSLLHPTCPPFHDRLAHQQNSANMVTERHRGQDYLG
tw28 QRPNTPSTMPALSPNPSTPNRACTPPPDFNQCLTSPPSSPTLQTHSLLHPTCPPFHDRLAHQQNSANMVTERHRGQDYLG
t1 QRPNTPSTMPALSPNPSTPNRACTPPPDFNQCLTSPPSSPTLQTHSLLHPTCPPFHDRLAHQQNSANMVTERHRGQDYLG
WT VNFLSFSQTPTETPNSCASPSFLSSDFEDKRRFSKSSGTSSRMRPDDLAV*
tq270 VNFLSFSQTPTETPNSCASPSFLSSDFEDKRRFSKSSGTSSRMRPDDLAV*
tw28 VNFLSFSQTPTETPNSCASPSFLSSDFEDKRRFSKSSGTSSRMRPDDLAV*
t1 VNFLSFSQTPTETPNSCASPSFLSSDFEDKRRFSKSSGTSSRMRPDDLAV*

Figure S1

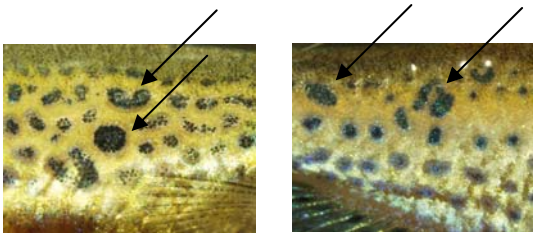

Figure S2

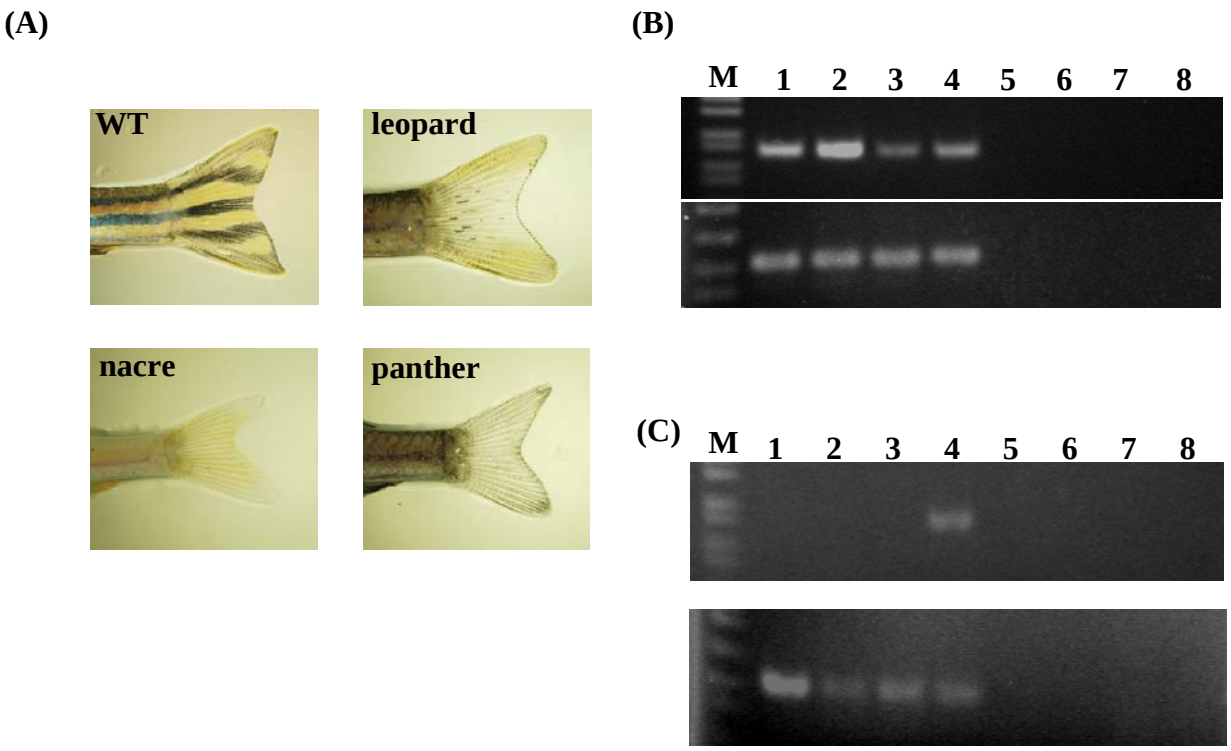

Figure S3