



Published in final edited form as:

Pigment Cell Melanoma Res. 2014 March ; 27(2): 190–200.

Tetraspanin 3c requirement for pigment cell interactions and boundary formation in zebrafish adult pigment stripes

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Summary

Skin pigment pattern formation in zebrafish requires pigment-cell autonomous interactions between melanophores and xanthophores, yet the molecular bases for these interactions remain largely unknown. Here, we examined the *dali* mutant that exhibits stripes in which melanophores are intermingled abnormally with xanthophores. By *in vitro* cell culture, we found that melanophores of *dali* mutants have a defect in motility and that interactions between melanophores and xanthophores are defective as well. Positional cloning and rescue identified *dali* as *tetraspanin 3c* (*tspan3c*), encoding a transmembrane scaffolding protein expressed by melanophores and xanthophores. We further showed that *dali* mutant Tspan3c expressed in HeLa cell exhibits a defect in *N*-glycosylation and is retained inappropriately in the endoplasmic reticulum. Our results are the first to identify roles for a tetraspanin superfamily protein in skin pigment pattern formation and suggest new mechanisms for the establishment and maintenance of zebrafish stripe boundaries.

Keywords

Zebrafish; Stripe pattern; Tetraspanin; melanophore; xanthophore

Introduction

Animals exhibit a variety of striking and colorful pigment patterns that are appealing not only for their aesthetics but also for the fundamental questions they raise about the diversification and generation of form. A useful model organism in which to address these issues is the zebrafish, which has a distinctive and stereotyped adult pigment pattern of several dark stripes that alternate with light interstripes. Stripes comprise black melanophores and iridescent iridophores, whereas interstripes comprise yellow–orange xanthophores and iridophores; all of these cell types arise from latent precursors of neural

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crest origin during the larval-to-adult transformation (Johnson et al., 1995; Parichy and Turner, 2003b).

The development of zebrafish stripes and interstripes involves dynamic and reciprocal interactions amongst pigment cell classes. During early stages of adult pigment pattern development, iridophores develop in the prospective interstripe region and contribute to specifying the development of xanthophores at this site, as well as the localization of melanophores further dorsally and ventrally (Frohnhofer et al., 2013; Parichy, 2009; Patterson and Parichy, 2013). Short and long-range interactions between melanophores and xanthophores are then essential for establishing and maintaining boundaries between stripes and interstripes (Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003a; Yamaguchi et al., 2007). Remarkably, these interactions conform to the expectations of reaction–diffusion models of pattern formation (Kondo and Asai, 1995; Kondo and Shirota, 2009; Miyazawa et al., 2010; Nakamasu et al., 2009) that rely on mechanisms of local activation and long-range inhibition (Turing, 1952); such patterning systems may be widespread in animal development (Jung et al., 1998; Miura et al., 2009; Miura and Shiota, 2000; Nakamura et al., 2006; Sheth et al., 2012). These pigment cell-autonomous interactions are capable of generating a wide variety of patterns, and changes in these interactions may have contributed to the diversification of patterns among species (Miyazawa et al., 2010; Quigley et al., 2005; Watanabe and Kondo, 2012).

The molecular mechanisms of pigment cell development and pattern formation are beginning to be elucidated, and several genes have been identified for their requirements in the development of particular pigment cell lineages (Budi et al., 2008; Krauss et al., 2013; Lister et al., 1999; Lopes et al., 2008; Minchin and Hughes, 2008; Parichy et al., 2000a; Parichy et al., 2000b; Parichy et al., 1999) or for the acquisition of cell-type specific pigments (Dooley et al., 2013; Kelsh et al., 2000; Le Guyader et al., 2005; Ziegler, 2003). By contrast, phenotypes of several zebrafish mutants identify genes required for pattern formation itself. For example, mutations in *connexin 41.8* (*cx41.8*) and *igsf11* result in the development of spots rather than stripes in *leopard* and *seurat* mutants, respectively (Eom et al., 2012; Watanabe et al., 2006; Watanabe and Kondo, 2012). A qualitatively different pattern phenotype is found in *jaguar* mutants with mutations in *potassium inwardly-rectifying channel, subfamily J, member 13* (*kcnj13*; previously, *kir7.1*), in which melanophores and xanthophores are no longer confined to stripes and interstripes but are instead intermingled with one another in stripes that are abnormally broad, demonstrating a requirement for *kcnj13* in the development of stripe–interstripe boundaries (Iwashita et al., 2006; Maderspacher and Nusslein-Volhard, 2003). More recently, a role has been found for Kcnj13 in mediating melanophore–xanthophore interactions *in vitro*. When melanophores and xanthophores are placed adjacent to one another, melanophores migrate away and xanthophores follow. This “escape behavior” of melanophores was preceded by a change in their membrane potential following contact by xanthophores. Melanophores derived from *jaguar* mutants, however, were constantly depolarized and failed to retreat from xanthophores, indicating that Kcnj13 sets the resting membrane potential of melanophores (Inaba et al., 2012) and suggesting that loss of this repulsive interaction in *jaguar* mutants contributes to a failure of sorting-out *in vivo*.

In this study, we analyzed the *dali* mutant, which shows a phenotype similar to that of *jaguar*, namely, broad stripes consisting of xanthophores as well as melanophores. We show that *dali* promotes melanophore motility and is essential for melanophore–xanthophore interactions *in vitro*, similar to Kcnj13, yet is not required for melanophore depolarization. We further show that *dali* encodes Tetraspanin 3c, a member of the tetraspanin superfamily (Hemler, 2003; Hemler, 2005; Zoller, 2009) expressed by melanophores and xanthophores. Tetraspanins act as scaffolds to anchor proteins at cell and organelle membranes (Baldwin et al., 2008; Kobayashi et al., 2000; Xu et al., 2004) within tetraspanin-enriched microdomains, and contribute to cell adhesion (Shigeta et al., 2003; Spring et al., 2013), migration (Baldwin et al., 2008; Liu et al., 2007), proliferation (Tiwari-Woodruff et al., 2001), and cell–cell fusion (Chen et al., 1999; Tachibana and Hemler, 1999). We show that intermingled melanophores and xanthophores in the *dali* mutant result from a missense substitution in the first transmembrane domain of Tspan3c that leads to abnormal endoplasmic reticulum (ER) retention and incomplete *N*-glycosylation. These are the first data to implicate a tetraspanin in pigment pattern formation and suggest novel mechanisms underlying the melanophore–xanthophore interactions and the development of boundaries during zebrafish adult stripe development.

Results

***dali* promotes boundary formation between melanophores and xanthophores**

In wild-type zebrafish, melanophores and xanthophores are found in stripes and interstripes, respectively (Figure 1A). To identify genes underlying the formation of this pattern, we undertook a forward genetic screen for ENU-induced mutant phenotypes having defects in stripe–interstripe boundaries. One such mutant is *dali*, which exhibits distinct heterozygous and homozygous phenotypes. Heterozygotes exhibit broken stripes and retain clear boundaries between melanophores and xanthophores (Figure 1B). Homozygotes, however, have fewer, broader stripes that contain not only melanophores but intermingled xanthophores as well (Figure 1C). The defect in stripe number and width is evident by the juvenile stage and the intermingled pigment cells phenotype is apparent in the adult (data not shown). All experiments described below used pigment cells isolated from homozygous mutants. Thus, *dali* is essential for the development of stripes and the segregation of melanophores and xanthophores into discrete spatial domains. These phenotypes are strikingly similar to those of mutants for *jaguar*, encoding Kcnj13 (Figure 1D, 1E), raising the possibility that *dali* and *jaguar* might affect some of the same processes.

***dali* promotes melanophore migration**

Stripe development and regeneration involve the directional migration of melanophores (Parichy et al., 2000b; Parichy and Turner, 2003b). To test if *dali* promotes melanophore migration, we assessed the motility of pigment cells *in vitro* over 24 hours (Eom et al., 2012). These analyses revealed that total distances migrated by melanophores isolated from *dali* mutants were half those of melanophores from wild-type fish (Figure 2A, 2B, 2E-left). Net (rectilinear) displacements of *dali* mutant melanophores were, likewise, only a quarter those of wild-type melanophores (Figure 2E-right; Supplementary Videos S1, S2). In contrast to melanophores, isolated xanthophores migrated little *in vitro* (but see below) and

we failed to detect differences between wild-type and *dali* mutant backgrounds (Figure 2C–E; Supplementary Videos S2, S3).

***dali*-dependent interactions between melanophores and xanthophores**

Repulsive interactions between melanophores and xanthophores play critical roles in organizing and maintaining stripes and interstripes (Inaba et al., 2012; Madarspacher and Nusslein-Volhard, 2003; Nakamasu et al., 2009; Parichy et al., 2000b; Parichy and Turner, 2003a; Yamaguchi et al., 2007). We asked whether such interactions might be defective in *dali* mutants by placing adult melanophores and xanthophores in close proximity to one another *in vitro*. We assessed resulting interactions both by time-lapse imaging and by measuring the migration distances of melanophores during successive time intervals during the 4 hours after each melanophore was first contacted by a xanthophore. To measure both repulsive and attractive behavior of melanophores relative to xanthophores, we used only x-axis values of melanophore migration (Figure 3E). Changes in distance that are positive would indicate a repulsive interaction (red double-headed arrow) in which the melanophore “escapes” from the xanthophore (which might give “chase”); changes in distance that are negative (i.e., if the melanophore moves even closer to the xanthophore) would reflect an attractive interaction (blue double-headed arrow). Measured as displacements along just a single axis, these values indicate directionality but will tend to underestimate total distances migrated. In pairs of wild-type cells, melanophores contacted by xanthophores migrated away and the xanthophores followed, with increasing distances between cell centroids (i.e., melanophores “escaped” despite being “chased” (Supplementary Video S3 and Figure 3A, 3F)), consistent with a repulsive effect of xanthophores on melanophores and with previous observations (Inaba et al., 2012). In contrast, *dali* mutant melanophores and xanthophores failed to migrate and remained in close contact with one another (Supplementary Video S4 and Figure 3B, 3F).

To determine the cellular bases for the lack of responsiveness by *dali* mutant pigment cells, we generated reciprocal melanophore–xanthophore pairings of wild-type and mutant cells. In these experiments, wild-type xanthophores failed to stimulate the migration of *dali* mutant melanophores, whereas *dali* mutant xanthophores failed to stimulate the migration of wild-type melanophores (Supplementary Videos S5, S6; Figure 3C, 3D, 3F). These results indicate that *dali* acts within melanophores to promote their responsiveness to xanthophores, and also acts within xanthophores to promote their repulsive effects on melanophores. Time-lapse videos of reciprocal pairings also revealed a more subtle difference in cell behaviors. After being contacted by wild-type xanthophores, *dali* mutant melanophores retracted somewhat, as evidenced by their strongly asymmetrical shapes, although they failed to “escape”, apparently owing to a defect in directional migration (Supplementary Video S5). By contrast, after being contacted by *dali* mutant xanthophores, wild-type melanophores exhibited neither retraction nor directional escape behaviors (Supplementary Video S6). These observations raise the possibility that Tspan3c has different functions in melanophores and xanthophores.

Finally, because a normal resting membrane potential is required for melanophores to depolarize in response to contacts with xanthophores, we compared the resting membrane

potential of *dali* mutant melanophore to wild-type melanophores. In contrast to the defective membrane potential of *jaguar/kncj13* mutants (Inaba et al., 2012), we found no differences in membrane potential between melanophores of wild-type and *dali* mutants (Supplementary Figure S1).

***dali* encodes Tetraspanin 3c**

To identify the gene responsible for the *dali* phenotype, we mapped the mutant to chromosome 8 between microsatellites z27391 (30.13 Mb) and z11237 (30.99 Mb) using 257 F2 individuals (Figure 4A). Comparisons of wild-type and mutant open reading frames for all 15 genes within this critical interval revealed a non-synonymous substitution (I18R) in *zgc:100919* that segregates with the *dali* mutant phenotype. *zgc:100919* encodes a vertebrate transmembrane 4 superfamily-like protein (tetraspanin; NP_001002748) most closely related to Tetraspanin 3 (Figure 4B; Supplementary Figure S2A). Because teleosts have three *tetraspanin 3* genes, and *tetraspanin 3a* and *3b* have been annotated in the Ensembl database already, we designated the *dali* gene *tetraspanin 3c* (*tspan3c*). Tspan3c is predicted to have four transmembrane domains, one intracellular loop and two extracellular loops; the I18R substitution occurs in the first transmembrane domain (Figure 4C; Supplementary Figure S2B).

To confirm the correspondence of *dali* and *tspan3c*, we tested if wild-type Tspan3c can rescue the *dali* mutant phenotype. We cloned a 4.5 kb fragment upstream of the Tspan3c start codon and used it to drive expression of wild-type Tspan3c cDNA in a stable transgenic line, *Tg(tspan3c:tspan3c)*, constructed in the *dali* mutant background. Transgenic, homozygous *dali* mutants exhibited markedly improved boundaries between melanophores and xanthophores in comparison to non-transgenic *dali* mutants (Figure 1C vs. 1F); persisting stripe breaks in rescued fish, similar to heterozygous *dali* mutants (Figure 1B), presumably reflect the relative dosage of wild-type and mutant alleles. These results demonstrated that *dali* encodes Tspan3c.

***tspan3c* is expressed in melanophores, xanthophores and other tissues**

To examine the expression of *tspan3c* and other *tetraspanin 3* genes, we performed RT-PCR on mRNAs isolated from several adult tissues (Figure 5A). *tspan3a* and *tspan3c* were expressed in all of the tissues examined, whereas *tspan3b* was expressed only in a subset of tissues. To assess expression in melanophores and xanthophores, we enriched for these cell types by differential gradient centrifugation of dissociated adult fins and assayed transcript abundance by RT-PCR (Figure 5B). *tspan3c* mRNA was abundant in both melanophores and xanthophores, whereas *tspan3a* was only weakly expressed and *tspan3b* transcripts were not detectable. In parallel, we examined activity of a 4.5 kb *tspan3c* promoter in wild-type fish transgenic for a Tspan3c-EGFP fusion protein [*Tg(tspan3c:tspan3c(WT)-EGFP)*]. Frozen sections of adult fish revealed EGFP expression in both melanophores and xanthophores (Figure 5C blue arrows and yellow arrows, respectively), consistent with Tspan3c activities in both of these cell types. We note that Tspan3c-EGFP fusion protein is functional because the fusion protein also rescued *dali* phenotype (Supplementary Figure S3) as shown in the rescue experiment in Figure 1F.

We also examined promoter activity using *Tg(tspan3c:EGFP)* fish, in which the EGFP signal was stronger than that of the Tspan3c-EGFP fusion protein in *Tg(tspan3c:tspan3c-EGFP)* fish. We observed EGFP expression in xanthophores (Supplementary Figure S4), and this signal was easily distinguishable from xanthophore autofluorescence (Supplemental figure S4; see also Methods). In addition, we detected EGFP expression in iridophores when pigment cells from *Tg(tspan3c:EGFP)* fish were cultured (Supplementary Figure S5), suggesting the possibility of *tspan3c* expression in this lineage as well.

I18R mutation causes Tspan3c retention in endoplasmic reticulum and incomplete N-glycosylation

To better understand the consequences of the I18R mutation for Tspan3c function, we compared the localization of wild-type and mutant Tspan3c-EGFP fusion proteins in HeLa cells. Because mutations in transmembrane domains can cause retention of tetraspanins within the endoplasmic reticulum (ER) (Tu et al., 2006), we compared Tspan3c-EGFP localization relative to markers of ER (Figure 6A) and Golgi apparatus (Figure 6B) that are DsRed proteins fused with ER and Golgi retention sequences, respectively (see Methods). Tspan3c(WT)-EGFP was localized predominantly to Golgi apparatus (Figure 6B; 7 of 7 cells) and partially to ER (Figure 6A; 9 of 9 cells) as early as 24 hours after transfection and Golgi localization was enhanced after 48 hours (Figure 6B; 10 of 10 cells). By contrast, Tspan3c(I18R)-EGFP was localized almost entirely to ER at 24 hours after transfection (Figure 6A; 12 of 12 cells) and exhibited pronounced Golgi localization only after 48 hours (Figure 6B; 8 of 9 cells). Although the Golgi marker was weakly detectable in intracellular spaces, perhaps owing to partial mislocalization of the marker protein, our observations nevertheless support the idea that the I18R mutation retards Tspan3c translocation from the ER to the Golgi apparatus. Similar subcellular localizations of Tspan3c (WT or I18R) were detected when we performed the same experiments using mouse B16 melanoma cells (Supplementary Figure S6).

Next, to see if defective trafficking is accompanied by defects in Tspan3c maturation, we analyzed the consequences of I18R mutation for N-linked and O-linked glycosylation (Yamamoto et al., 2005). To this end, we expressed Tspan3c-3×FLAG fusion proteins in HeLa cells, then treated cellular lysates with N-glycosidase, O-glycosidase, or both, and compared protein mobilities by Western blot using an anti-FLAG monoclonal antibody (Figure 7). Tspan3c(WT)-3×FLAG was detected between 30–50 kDa and resolved to a fragment of the predicted, 27 kDa size upon treatment with N-glycosidase, demonstrating N-linked glycosylation of the wild-type protein. By contrast, most Tspan3c(I18R)-3×FLAG protein was detected at ~30 kDa, indicating incomplete N-glycosylation of the mutant form. No change in banding patterns was detected following treatment with O-glycosidase.

Discussion

In this study, we found that melanophores of *dali* mutants have impaired motility and that both melanophores and xanthophores exhibit defects in heterotypic interactions *in vitro*. We further demonstrated that *dali* corresponds to *tetraspanin 3c*, and that *dali* mutants have a

missense substitution that causes ER-retention and incomplete glycosylation of Tspan3c protein. Ours are the first data to implicate a tetraspanin in pigment pattern formation.

Tetraspanin superfamily proteins typically anchor other membrane proteins at cell and organelle membranes, and thereby regulate adhesion, migration, proliferation and cell–cell fusion (Baldwin et al., 2008; Chen et al., 1999; Liu et al., 2007; Shigeta et al., 2003; Spring et al., 2013; Tachibana and Hemler, 1999; Tiwari-Woodruff et al., 2001). In human melanocytes, tetraspanin CD63 participates in endosomal sorting during melanogenesis (van Niel et al., 2011) whereas tetraspanins CD9 and CD151 localize at the tips of dendrites and at sites of homotypic intercellular contacts, and their knockdown enhances motility (Garcia-Lopez et al., 2005). In melanoma cells, CD9 expression is reduced compared to normal melanocytes (Fan et al., 2010), yet TSPAN8 expression is associated with enhanced invasiveness (Berthier-Vergnes et al., 2011). These various functions in melanized and other cells illustrate the diversity of tetraspanin activities.

We can envision several ways in which zebrafish Tspan3c promotes stripe patterning. For example, tetraspanins often interact with integrins, and mouse TSPAN3 forms a protein complex with ITGB1 (integrin β 1) that promotes oligodendrocyte motility (Tiwari-Woodruff et al., 2001; Tiwari-Woodruff et al., 2004). Likewise, human CD151 (TSPAN24) promotes motility by modulating integrin internalization by endocytosis, trafficking or both (Liu et al., 2007), and also promotes integrin glycosylation in the Golgi apparatus (Baldwin et al., 2008). Indeed, a role for endocytic regulation of zebrafish Tspan3c and associated proteins is suggested by the presence in the C-terminal cytoplasmic region of a YQPL sequence, corresponding to a canonical YXX ϕ motif recognized by the adaptor protein 2 complex during endocytosis (Aguilar et al., 2001; Boehm and Bonifacino, 2001). In contrast to roles for Tetraspanin–Integrin interactions, complexes between Tetraspanins and IgSF (Shoham et al., 2006; Stipp et al., 2003), MT1-MMP (Takino et al., 2003), BLI-3 (Moribe et al., 2012; Moribe et al., 2004), and EGFR (Odintsova et al., 2000) also have been shown. Distinguishing among these and other hypotheses for Tspan3c activity, and identifying interaction partners *in vivo*, will require further analyses and the generation of new mutants as well as transgenic lines.

As described above, *jaguar* mutants exhibit the same intermingled arrangement of melanophores and xanthophores as does the *dali* mutant. *Kcnj13* sets the resting membrane potential of melanophores, and membrane depolarization is an important feature of the melanophore response to xanthophore contact. Despite the phenotypic similarity of *jaguar* and *dali* mutants both *in vivo* and *in vitro*, we found that *dali* mutant melanophores exhibited normal resting membrane potentials. These findings suggest that Tspan3c may act independently of *Kcnj13* in promoting repulsive effects of xanthophores on melanophores. Whether Tspan3c functions in melanophores are similar to its functions in xanthophores remains to be determined. Reciprocal pairing experiments (Figure 3C, 3D, Supplementary Videos S5, S6) raise the possibility that Tspan3c is required in melanophores for promoting directional migration in response to contact by xanthophores, but is required within xanthophores to stimulate melanophores. In this regard, xanthophores make direct contact with melanophores using filopodia, which trigger the melanophore “escape response”. Some tetraspanin family members initiate filopodial (Bassani et al., 2012; Shigeta et al., 2003) and

nanopodial (Zukauskas et al., 2011) formation. Besides these cell types, iridophores also have important functions during zebrafish stripe development (Frohnhofer et al., 2013; Patterson and Parichy, 2013). We found that iridophores express a 4.5 kb *tspan3c* promoter element *in vitro*, which may reflect *tspan3c* expression in this lineage *in vivo*. Our on-going investigations of Tspan3c should provide new insights into its functional requirements in each of these pigment cell classes.

Finally, our study also provides insights into the trafficking and post-translational modification of tetraspanins. In bovine UPK1B (TSPAN20), the integrity of all four transmembrane domains is required for translocation from the ER to the cell surface (Tu et al., 2006), suggesting the necessity for a tightly packed helix bundle in the translocation of this protein. Similarly, the I18R substitution of *dali* mutant Tspan3c occurs in a transmembrane domain and we observed retention of mutant protein in the ER. Finally, previous studies have shown that tetraspanins are glycosylated *in vivo*. *N*-glycosylation can contribute to regulating tetraspanin interactions (Baldwin et al., 2008; Hemler, 2005), and is, in general, important for protein stability, folding, dimerization, and trafficking (Helenius and Aebi, 2001; Isaji et al., 2006; Kohno et al., 2002; Mitra et al., 2006; Xu et al., 2003), as well as ligand-binding (Ono et al., 2000). In fact, Tspan3c has three putative *N*-glycosylation sites (Supplementary Figure S2) and we found defects in glycosylation of *dali* mutant (I18R) Tspan3c, suggesting that if mutant Tspan3c is expressed at the cell/organelle membrane, its activity or perdurance may be impaired.

In this study, we determined that Tspan3c contributes to melanophore motility *in vitro* and also mediates interactions between melanophores and xanthophores. Further elucidation of Tspan3c function *in vivo* will provide novel insights into adult pigment pattern formation and will enable the assignment of molecular mechanisms to parameters described by Turing models of pattern formation.

Methods

Fish

All experiments in this study were conducted in accordance with guidelines and approved protocols for animal care and use at Osaka University, Japan and the University of Washington, USA. The *dali*^{wpr21e1} mutant allele was isolated in a forward genetic, early pressure screen for *N*-ethyl-*N*-nitrosourea (ENU) induced mutations in the AB^{WP} genetic background.

Genetic mapping

The *dali* mutant was crossed with wild-type strains AB, Tübingen, and WIK in separate mapping panels. F1 heterozygous fish were intercrossed and F2 fish were used for genetic mapping with microsatellite markers. Candidate gene sequencing was performed using a 3130 Genetic analyzer (Applied Biosystems).

Collection of pigment cells

Pigment cells were isolated from fins of adult fish for RT-PCR analyses of gene expression (wild-type), *in vitro* motility and co-culture assays (wild-type, *dali* mutant), for reporter assay [*Tg(tspan3c:EGFP)*] and for analyses of membrane potential [hypopigmented *golden^{bl}* (Lamason et al., 2005) and *golden; dali* double mutants]. Caudal and anal fins were collected from fish anesthetized with methyl methanesulfonate (MMS, Sigma). The fin clips were treated with trypsin solution (2.5 mg/ml trypsin (Worthington), 1.2 mg/ml BSA (Sigma) and 1 mM EDTA (Wako) in PBS) for 1 hour at 28°C. The trypsin solution was then removed, and the tissues were rinsed several times with PBS. Then, fin clips were incubated with collagenase solution (1 mg/ml collagenase I (Worthington), 0.1 mg/ml DNase I (Worthington), 0.1 mg/ml STI (Worthington), 1.2 mg/ml BSA, 100 µM epinephrine (Sigma) in PBS) for 1 hour at 28°C. For RT-PCR and *in vitro* culture experiments, pigment cells were purified by Percoll gradient centrifugation after filtration with 25 µm mesh (see below). For membrane potential assays and EGFP detection in iridophores, pigment cells were collected only by simple centrifugation without Percoll (see below).

Analyses of pigment cell movement and interaction *in vitro*

Filtered pigment cell suspensions were centrifuged, 30× g for 5 minutes at room temperature, and cell pellets were re-suspended with L15 medium (Sigma) containing 1% fetal bovine serum (FBS; Invitrogen). Pigment cells were then picked using glass capillaries under an Olympus IX81 microscope and placed on a 35 mm culture dish coated with collagen IV (BD Biosciences). To investigate the interactions between melanophores and xanthophores, individual cells of each type were placed adjacent to one another. After overnight incubation in L15 medium at 28°C, the culture medium was exchanged with fresh L15 medium containing 10% FBS and the pigment cells were then used in live imaging. Movies were recorded using a DP73 digital camera (Olympus) and Metamorph software (Molecular Devices) interfaced to an IX81 microscope. Data for pigment cells that survived more than 48 hours after the medium change were used for further analysis (Yamanaka and Kondo, submitted). To evaluate the motility, the tracks of melanophore centroids were plotted every one hour from 12 hours to 36 hours after the medium change, using ImageJ software (<http://rsb.info.nih.gov/ij/>). The rectilinear migration length was defined as the net distance between the 12-hour and the 36-hour time points. To compare the repulsive activity of melanophores against xanthophores, the different cell types were placed within 0–50 µm of one another and cell centroids were recorded every 4 hours after melanophores were first contacted by xanthophores. At each time point, the melanophore position was set at the origin and the xanthophore was set to the left of the melanophores on the x-axis (Figure 3E). After 4 hours, the distance of melanophore migration was plotted. To measure both repulsive and attractive behavior of melanophore against xanthophore, we used only x-axis value of melanophore migration for the evaluation. The repulsive activity was evaluated as the average distance of melanophores migration in 4 hours.

Transgenic fish

To confirm the correspondence of *tspan3c* and *dali*, we rescued the *dali* phenotype using the pTol2-tspan3c:tspan3c transposon plasmid. A 4.5 kb fragment upstream from the translation

initiation codon of *tspan3c* and a complete open reading frame of *tspan3c* gene were amplified then cloned into the pT2AL200R150G plasmid (Urasaki et al., 2006). Plasmid (10 ng/ μ l) and transposase mRNA (25 ng/ μ l) were co-injected into fertilized eggs of *dali* homozygous fish at the single-cell stage. The effect of the transgene on skin pattern formation was determined at the F1 generation of the transgenic line. Integration of the transgene into the fish genome was verified by PCR amplification. To analyze activity of the *tspan3c* promoter, EGFP was inserted into the C-terminus of the *tspan3c* fragment in pTol2-tspan3c:tspan3c(WT or I18R), generating pTol2-tspan3c:tspan3c(WT or I18R)-EGFP reporter plasmid in which the linker fragment Gly-Gly-Gly-Gly-Gly-Gly-Leu was introduced between the *tspan3c* and EGFP fragments. pTol2-tspan3c:EGFP reporter plasmid was also produced to analyze promoter activity. These plasmids were injected into zebrafish eggs by the same method as above.

RT-PCR analysis of *tspan3c* expression

After anesthetizing fish with MMS, total RNA was obtained using the RNeasy Protect Mini Kit (Qiagen), which was applied to each organ (eye, brain, heart, skin, caudal fin, testis, and ovary) of zebrafish. cDNAs were synthesized using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Then, 4.4 ng of cDNA (RNA equivalent) obtained from each organ was used in PCR, to detect *tspan3a-c* expression. PCR amplifications were performed for 30 cycles for *tspan3a-c*, 27 cycles for β -actin at 95°C for 30 seconds, at 60°C for 30 seconds, and 72°C for 30 seconds.

To examine *tspan3c* expression in melanophores and xanthophores, each pigment cell type was purified (see above) then cDNAs prepared for RT-PCR. To assay for cross-contamination of melanophores and xanthophores, the expression of *dct* (a melanophore marker) (Kelsh et al., 2000) and *aox3* (a xanthophore marker) (Parichy et al., 2000b) were also examined. PCR amplifications were performed for 32 cycles at 95°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds. Primer sets were designed to span introns: 5'-ATGGGTCAGTGCGGGATTAC-3' and 5'-CAAATACCAGCATAAGAATGATGAC-3' for *tspan3a*, 5'-ATGGGACAATGTGGCGTGAT-3' and 5'-GACCAGAAGCAGCACTGCAGA-3' for *tspan3b*, 5'-GTGTTCCGGCATCATCTACAGG-3' and 5'-GTTGTCGATGGTGCCAGTGAG-3' for *tspan3c*, 5'-ATCAGCCCCGCGTTCACGGTT-3' and 5'-ACACCGAGGTGTCCAGCTCTCC-3' for *dct*, 5'-AGGGCATTGGAGAACCCCCAGT-3' and 5'-ACACGTTGATGGCCCCACGGT-3' for *aox3*, and 5'-CGGTTTTGCTGGAGATGATG-3' and 5'-CGTGCTCAATGGGGTATTTG-3' for β -actin.

Tspan3c protein in HeLa cells and B16 melanoma cells

HeLa cells and B16 melanoma cells were maintained in DMEM medium (Sigma) that contained 10% FBS (Invitrogen). To generate pCMV-tspan3c(WT or I18R)-EGFP plasmids, cDNA fragment of *tspan3c* with an EGFP cassette were subcloned from the pTol2-tspan3c:tspan3c(WT or I18R)-EGFP plasmid, respectively. Marker plasmids for the ER and Golgi apparatus, pCS2-ER-DsRed2 and pCS2-DsRed-Monomer-Golgi were generated using pDsRed2-ER and pDsRed-Monomer-Golgi plasmids (Clontech), which were originally prepared for other purposes. These plasmids were introduced into HeLa cells and B16

melanoma cells using the GeneJuice Transfection Reagent (Merck Millipore). Protein localizations were observed 24 or 48 hours after transfection by using the IX71 microscope and FLUOVIEW FV300 (Olympus).

Western blotting

tspan3c(WT or I18R) was cloned into the pIRES2-AcGFP plasmid (Clontech), in which the 3×FLAG coding fragment was added to produce a Tspan3c-3×FLAG tagged protein. Then, 48 hours after transfection with the plasmid, transfectant cells were rinsed with PBS and lysed with chilled RIPA buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% TRITON X-100, 0.5% Sodium deoxycholate, 0.1% SDS, and 1× Complete Protease Inhibitor Cocktail Tablets (Roche)]. Cell lysates were centrifuged (17,000× g for 10 minutes at 4°C) and then treated with *N*-glycosidase (PNGaseF, NEB) and/or *O*-glycosidase mix (*O*-Glycosidase & Neuraminidase Bundle, NEB). Protein samples were resolved by SDS-PAGE, followed by Western blot analysis using Monoclonal Anti -FLAG M2 antibody (Sigma).

Analysis of membrane potential in melanophores

Membrane potentials were compared between wild-type and *dali* mutant melanophores using DiBAC₄(3) (Inaba et al., 2012). Because melanin granules in melanophores prevent fluorescence detection, we crossed *dali* and *golden* mutants to generate *dali; golden* double homozygous mutants, in which melanophores lack melanin. Pigment cells were partially purified from the fin clips of the *golden* (positive control) and *dali; golden* double mutants (see above). Following centrifugation, the cell pellets were re-suspended with L15 medium and cultured on a 35 mm glass base dish. After overnight incubation at 28°C, the culture medium was changed to fresh L15 medium that contained 10% FBS and 200 nM DiBAC₄(3) (Wako). After 30 minutes of incubation, the cells were photographed by using the IX71 microscope and FLUOVIEW FV300 (Olympus). The Mean Gray Value was analyzed by using the ImageJ as mean fluorescence intensity (MFI).

Phylogenetic analysis

A maximum-likelihood phylogeny for Tspan3-related proteins was constructed using MEGA software (Tamura et al., 2011).

Observation of EGFP fluorescence in pigment cells

EGFP signals in pigment cells *in vivo*, *in vitro* and on frozen section were analyzed using the BZ-8000 microscope (KEYENCE) with OP-66835 BZ filter GFP. For the *in vitro* observation, pigment cells were collected and cultured as the same way for the analysis of membrane potential in melanophores (see above). After overnight incubation at 28°C, the culture medium was changed to fresh L15 medium that contained 10% FBS and 100 μM epinephrine, and the cells were incubated for 1 hour.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Prof. K. Kawakami at the National Institute of Genetics for providing the Tol2 plasmid and Prof. T. Kunisada at Gifu University for providing mouse B16 melanoma cell line. We also thank Ms. Jessica M. Turner for technical assistance and Drs. Dae Seok Eom and Hiroaki Yamanaka for helpful discussions. Supported by NIH R01 GM062182 and NIH R01 GM096906 to D.M.P., KAKENHI (23770247, 25111714) to M.W., and KAKENHI (22127003) and JST, CREST to S. K.

References

- Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, Bonifacino JS. Signal-binding specificity of the mu4 subunit of the adaptor protein complex AP-4. *The Journal of biological chemistry*. 2001; 276:13145–52. [PubMed: 11139587]
- Baldwin G, Novitskaya V, Sadej R, Pochec E, Litynska A, Hartmann C, Williams J, Ashman L, Eble JA, Berditchevski F. Tetraspanin CD151 regulates glycosylation of (alpha)3(beta)1 integrin. *The Journal of biological chemistry*. 2008; 283:35445–54. [PubMed: 18852263]
- Bassani S, Cingolani LA, Valnegri P, Folci A, Zapata J, Gianfelice A, Sala C, Goda Y, Passafaro M. The X-linked intellectual disability protein TSPAN7 regulates excitatory synapse development and AMPAR trafficking. *Neuron*. 2012; 73:1143–58. [PubMed: 22445342]
- Berthier-Vergnes O, Kharbili ME, De La Fouchardiere A, Pointecouteau T, Verrando P, Wierinckx A, Lachuer J, Le Naour F, Lamartine J. Gene expression profiles of human melanoma cells with different invasive potential reveal TSPAN8 as a novel mediator of invasion. *British journal of cancer*. 2011; 104:155–65. [PubMed: 21081927]
- Boehm M, Bonifacino JS. Adaptins: the final recount. *Molecular biology of the cell*. 2001; 12:2907–20. [PubMed: 11598180]
- Budi EH, Patterson LB, Parichy DM. Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation. *Development*. 2008; 135:2603–14. [PubMed: 18508863]
- Chen MS, Tung KS, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC, White JM. Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:11830–5. [PubMed: 10518536]
- Dooley CM, Schwarz H, Mueller KP, Mongera A, Konantz M, Neuhauss SC, Nusslein-Volhard C, Geisler R. Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment cell & melanoma research*. 2013; 26:205–17. [PubMed: 23205854]
- Eom DS, Inoue S, Patterson LB, Gordon TN, Slingwine R, Kondo S, Watanabe M, Parichy DM. Melanophore migration and survival during zebrafish adult pigment stripe development require the immunoglobulin superfamily adhesion molecule Igsf11. *PLoS genetics*. 2012; 8:e1002899. [PubMed: 22916035]
- Fan J, Zhu GZ, Niles RM. Expression and function of CD9 in melanoma cells. *Molecular carcinogenesis*. 2010; 49:85–93. [PubMed: 19777564]
- Frohnhofer HG, Krauss J, Maischein HM, Nusslein-Volhard C. Iridophores and their interactions with other chromatophores are required for stripe formation in zebrafish. *Development*. 2013; 140:2997–3007. [PubMed: 23821036]
- Garcia-Lopez MA, Barreiro O, Garcia-Diez A, Sanchez-Madrid F, Penas PF. Role of tetraspanins CD9 and CD151 in primary melanocyte motility. *The Journal of investigative dermatology*. 2005; 125:1001–9. [PubMed: 16297202]
- Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science*. 2001; 291:2364–9. [PubMed: 11269317]
- Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annual review of cell and developmental biology*. 2003; 19:397–422.
- Hemler ME. Tetraspanin functions and associated microdomains. *Nature reviews Molecular cell biology*. 2005; 6:801–11.

- Inaba M, Yamanaka H, Kondo S. Pigment pattern formation by contact-dependent depolarization. *Science*. 2012; 335:677. [PubMed: 22323812]
- Isaji T, Sato Y, Zhao Y, Miyoshi E, Wada Y, Taniguchi N, Gu J. N-glycosylation of the beta-propeller domain of the integrin alpha5 subunit is essential for alpha5beta1 heterodimerization, expression on the cell surface, and its biological function. *The Journal of biological chemistry*. 2006; 281:33258–67. [PubMed: 16959765]
- Iwashita M, Watanabe M, Ishii M, Chen T, Johnson SL, Kurachi Y, Okada N, Kondo S. Pigment pattern in jaguar/obelix zebrafish is caused by a Kir7.1 mutation: implications for the regulation of melanosome movement. *PLoS genetics*. 2006; 2:e197. [PubMed: 17121467]
- Johnson SL, Africa D, Walker C, Weston JA. Genetic control of adult pigment stripe development in zebrafish. *Developmental biology*. 1995; 167:27–33. [PubMed: 7851648]
- Jung HS, Francis-West PH, Widelitz RB, Jiang TX, Ting-Berret S, Tickle C, Wolpert L, Chuong CM. Local inhibitory action of BMPs and their relationships with activators in feather formation: implications for periodic patterning. *Developmental biology*. 1998; 196:11–23. [PubMed: 9527877]
- Kelsh RN, Schmid B, Eisen JS. Genetic analysis of melanophore development in zebrafish embryos. *Developmental biology*. 2000; 225:277–93. [PubMed: 10985850]
- Kobayashi T, Vischer UM, Rosnoblet C, Lebrand C, Lindsay M, Parton RG, Kruithof EK, Gruenberg J. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Molecular biology of the cell*. 2000; 11:1829–43. [PubMed: 10793155]
- Kohno T, Wada A, Igarashi Y. N-glycans of sphingosine 1-phosphate receptor Edg-1 regulate ligand-induced receptor internalization. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2002; 16:983–92. [PubMed: 12087059]
- Kondo S, Asai R. A Reaction-Diffusion Wave on the Skin of the Marine Angelfish *Pomacanthus*. *Nature*. 1995; 376:765–768. [PubMed: 24547605]
- Kondo S, Shirota H. Theoretical analysis of mechanisms that generate the pigmentation pattern of animals. *Seminars in cell & developmental biology*. 2009; 20:82–9. [PubMed: 18996494]
- Krauss J, Astrinides P, Frohnhof HG, Walderich B, Nusslein-Volhard C. transparent, a gene affecting stripe formation in Zebrafish, encodes the mitochondrial protein Mpv17 that is required for iridophore survival. *Biology open*. 2013; 2:703–10. [PubMed: 23862018]
- Lamason RL, Mohideen MA, Mest JR, Wong AC, Norton HL, Aros MC, Juryne MJ, Mao X, Humphreville VR, Humbert JE, et al. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science*. 2005; 310:1782–6. [PubMed: 16357253]
- Le Guyader S, Maier J, Jesuthasan S. Esrom, an ortholog of PAM (protein associated with c-myc), regulates pteridine synthesis in the zebrafish. *Developmental biology*. 2005; 277:378–86. [PubMed: 15617681]
- Lister JA, Robertson CP, Lepage T, Johnson SL, Raible DW. nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development*. 1999; 126:3757–67. [PubMed: 10433906]
- Liu L, He B, Liu WM, Zhou D, Cox JV, Zhang XA. Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *The Journal of biological chemistry*. 2007; 282:31631–42. [PubMed: 17716972]
- Lopes SS, Yang X, Muller J, Carney TJ, Mcadow AR, Rauch GJ, Jacoby AS, Hurst LD, Delfino-Machin M, Haffter P, et al. Leukocyte tyrosine kinase functions in pigment cell development. *PLoS genetics*. 2008; 4:e1000026. [PubMed: 18369445]
- Maderspacher F, Nusslein-Volhard C. Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development*. 2003; 130:3447–57. [PubMed: 12810592]
- Minchin JE, Hughes SM. Sequential actions of Pax3 and Pax7 drive xanthophore development in zebrafish neural crest. *Developmental biology*. 2008; 317:508–22. [PubMed: 18417109]
- Mitra N, Sinha S, Ramya TN, Suroliya A. N-linked oligosaccharides as outfitters for glycoprotein folding, form and function. *Trends in biochemical sciences*. 2006; 31:156–63. [PubMed: 16473013]

- Miura T, Hartmann D, Kinboshi M, Komada M, Ishibashi M, Shiota K. The cyst-branch difference in developing chick lung results from a different morphogen diffusion coefficient. *Mechanisms of development*. 2009; 126:160–72. [PubMed: 19073251]
- Miura T, Shiota K. TGFbeta2 acts as an “activator” molecule in reaction-diffusion model and is involved in cell sorting phenomenon in mouse limb micromass culture. *Developmental dynamics: an official publication of the American Association of Anatomists*. 2000; 217:241–9. [PubMed: 10741418]
- Miyazawa S, Okamoto M, Kondo S. Blending of animal colour patterns by hybridization. *Nature communications*. 2010; 1:66.
- Moribe H, Konakawa R, Koga D, Ushiki T, Nakamura K, Mekada E. Tetraspanin is required for generation of reactive oxygen species by the dual oxidase system in *Caenorhabditis elegans*. *PLoS genetics*. 2012; 8:e1002957. [PubMed: 23028364]
- Moribe H, Yochem J, Yamada H, Tabuse Y, Fujimoto T, Mekada E. Tetraspanin protein (TSP-15) is required for epidermal integrity in *Caenorhabditis elegans*. *Journal of cell science*. 2004; 117:5209–20. [PubMed: 15454573]
- Nakamasu A, Takahashi G, Kanbe A, Kondo S. Interactions between zebrafish pigment cells responsible for the generation of Turing patterns. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:8429–34. [PubMed: 19433782]
- Nakamura T, Mine N, Nakaguchi E, Mochizuki A, Yamamoto M, Yashiro K, Meno C, Hamada H. Generation of robust left-right asymmetry in the mouse embryo requires a self-enhancement and lateral-inhibition system. *Developmental cell*. 2006; 11:495–504. [PubMed: 17011489]
- Odintsova E, Sugiura T, Berditchevski F. Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. *Current biology: CB*. 2000; 10:1009–12. [PubMed: 10985391]
- Ono M, Handa K, Withers DA, Hakomori S. Glycosylation effect on membrane domain (GEM) involved in cell adhesion and motility: a preliminary note on functional alpha3, alpha5-CD82 glycosylation complex in Id1D 14 cells. *Biochemical and biophysical research communications*. 2000; 279:744–50. [PubMed: 11162423]
- Parichy DM. Animal pigment pattern: an integrative model system for studying the development, evolution, and regeneration of form. *Seminars in cell & developmental biology*. 2009; 20:63–4. [PubMed: 19146966]
- Parichy DM, Mellgren EM, Rawls JF, Lopes SS, Kelsh RN, Johnson SL. Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. *Developmental biology*. 2000a; 227:294–306. [PubMed: 11071756]
- Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL. 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*. 2000b; 127:3031–44. [PubMed: 10862741]
- Parichy DM, Rawls JF, Pratt SJ, Whitfield TT, Johnson SL. Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. *Development*. 1999; 126:3425–36. [PubMed: 10393121]
- Parichy DM, Turner JM. Temporal and cellular requirements for *Fms* signaling during zebrafish adult pigment pattern development. *Development*. 2003a; 130:817–33. [PubMed: 12538511]
- Parichy DM, Turner JM. Zebrafish puma mutant decouples pigment pattern and somatic metamorphosis. *Developmental biology*. 2003b; 256:242–57. [PubMed: 12679100]
- Patterson LB, Parichy DM. Interactions with iridophores and the tissue environment required for patterning melanophores and xanthophores during zebrafish adult pigment stripe formation. *PLoS genetics*. 2013; 9:e1003561. [PubMed: 23737760]
- Quigley IK, Manuel JL, Roberts RA, Nuckels RJ, Herrington ER, Macdonald EL, Parichy DM. Evolutionary diversification of pigment pattern in *Danio* fishes: differential *fms* dependence and stripe loss in *D. albolineatus*. *Development*. 2005; 132:89–104. [PubMed: 15563521]

- Sheth R, Marcon L, Bastida MF, Junco M, Quintana L, Dahn R, Kmita M, Sharpe J, Ros MA. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science*. 2012; 338:1476–80. [PubMed: 23239739]
- Shigeta M, Sanzen N, Ozawa M, Gu J, Hasegawa H, Sekiguchi K. CD151 regulates epithelial cell-cell adhesion through PKC- and Cdc42-dependent actin cytoskeletal reorganization. *The Journal of cell biology*. 2003; 163:165–76. [PubMed: 14557253]
- Shoham T, Rajapaksa R, Kuo CC, Haimovich J, Levy S. Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments. *Molecular and cellular biology*. 2006; 26:1373–85. [PubMed: 16449649]
- Spring FA, Griffiths RE, Mankelov TJ, Agnew C, Parsons SF, Chasis JA, Anstee DJ. Tetraspanins CD81 and CD82 facilitate alpha4beta1-mediated adhesion of human erythroblasts to vascular cell adhesion molecule-1. *PLoS one*. 2013; 8:e62654. [PubMed: 23704882]
- Stipp CS, Kolesnikova TV, Hemler ME. EWI-2 regulates alpha3beta1 integrin-dependent cell functions on laminin-5. *The Journal of cell biology*. 2003; 163:1167–77. [PubMed: 14662754]
- Tachibana I, Hemler ME. Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. *The Journal of cell biology*. 1999; 146:893–904. [PubMed: 10459022]
- Takino T, Miyamori H, Kawaguchi N, Uekita T, Seiki M, Sato H. Tetraspanin CD63 promotes targeting and lysosomal proteolysis of membrane-type 1 matrix metalloproteinase. *Biochemical and biophysical research communications*. 2003; 304:160–6. [PubMed: 12705901]
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*. 2011; 28:2731–9. [PubMed: 21546353]
- Tiwari-Woodruff SK, Buznikov AG, Vu TQ, Micevych PE, Chen K, Kornblum HI, Bronstein JM. OSP/claudin-11 forms a complex with a novel member of the tetraspanin super family and beta1 integrin and regulates proliferation and migration of oligodendrocytes. *The Journal of cell biology*. 2001; 153:295–305. [PubMed: 11309411]
- Tiwari-Woodruff SK, Kaplan R, Kornblum HI, Bronstein JM. Developmental expression of OAP-1/Tspan-3, a member of the tetraspanin superfamily. *Journal of neuroscience research*. 2004; 77:166–73. [PubMed: 15211584]
- Tu L, Kong XP, Sun TT, Kreibich G. Integrity of all four transmembrane domains of the tetraspanin uroplakin Ib is required for its exit from the ER. *Journal of cell science*. 2006; 119:5077–86. [PubMed: 17158912]
- Turing AM. The Chemical Basis of Morphogenesis. *Philos T Roy Soc B*. 1952; 237:37–72.
- Urasaki A, Morvan G, Kawakami K. Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics*. 2006; 174:639–649. [PubMed: 16959904]
- Van Niel G, Charrin S, Simoes S, Romao M, Rochin L, Saftig P, Marks MS, Rubinstein E, Raposo G. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Developmental cell*. 2011; 21:708–21. [PubMed: 21962903]
- Watanabe M, Iwashita M, Ishii M, Kurachi Y, Kawakami A, Kondo S, Okada N. Spot pattern of leopard Danio is caused by mutation in the zebrafish connexin41.8 gene. *EMBO reports*. 2006; 7:893–7. [PubMed: 16845369]
- Watanabe M, Kondo S. Changing clothes easily: connexin41.8 regulates skin pattern variation. *Pigment cell & melanoma research*. 2012; 25:326–30. [PubMed: 22313791]
- Xu H, Lee SJ, Suzuki E, Dugan KD, Stoddard A, Li HS, Chodosh LA, Montell C. A lysosomal tetraspanin associated with retinal degeneration identified via a genome-wide screen. *The EMBO journal*. 2004; 23:811–22. [PubMed: 14963491]
- Xu J, He J, Castleberry AM, Balasubramanian S, Lau AG, Hall RA. Heterodimerization of alpha 2A- and beta 1-adrenergic receptors. *The Journal of biological chemistry*. 2003; 278:10770–7. [PubMed: 12529373]
- Yamaguchi M, Yoshimoto E, Kondo S. Pattern regulation in the stripe of zebrafish suggests an underlying dynamic and autonomous mechanism. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:4790–3. [PubMed: 17360399]

- Yamamoto A, Nagano T, Takehara S, Hibi M, Aizawa S. Shisa promotes head formation through the inhibition of receptor protein maturation for the caudalizing factors, Wnt and FGF. *Cell*. 2005; 120:223–35. [PubMed: 15680328]
- Ziegler I. The pteridine pathway in zebrafish: regulation and specification during the determination of neural crest cell-fate. *Pigment cell research/sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*. 2003; 16:172–82.
- Zoller M. Tetraspanins: push and pull in suppressing and promoting metastasis. *Nature reviews Cancer*. 2009; 9:40–55.
- Zukauskas A, Merley A, Li D, Ang LH, Sciuto TE, Salman S, Dvorak AM, Dvorak HF, Jaminet SC. TM4SF1: a tetraspanin-like protein necessary for nanopodia formation and endothelial cell migration. *Angiogenesis*. 2011; 14:345–54. [PubMed: 21626280]

Significance

How animal pigment patterns form and evolve is an intriguing question. We are tackling this problem using the beautiful stripes of the zebrafish. We have shown that cell–cell interactions between pigment cells are important for stripe formation, yet the underlying molecular mechanisms have been elusive. In this study, we examined a pigment pattern mutant, *dali*, which exhibits intermingled, rather than segregated, melanophores and xanthophores. We identified *dali* as encoding Tetraspanin 3c, which anchors other proteins at the cell surface, in organelle membranes, or both. Our finding will improve our understanding of the molecular bases of skin pigment pattern formation.

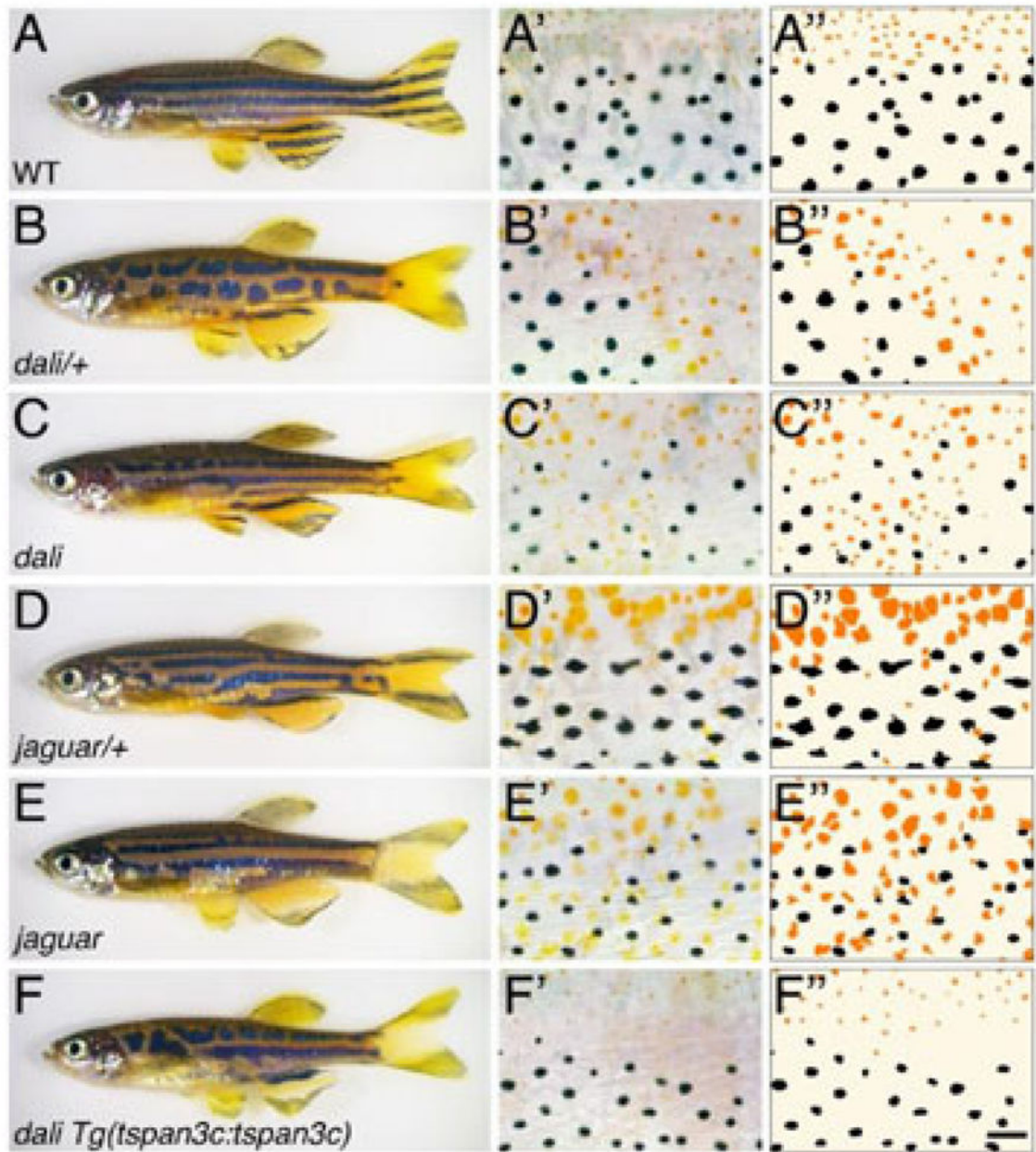


Figure 1.

Adult pigment patterns of wild-type, *dali* and *jaguar* mutants. (A) Wild-type. (B,C) Heterozygous and homozygous *dali* mutants. (D,E) Heterozygous and homozygous *jaguar*^{b230} mutants. (F) Partial rescue of *dali* mutant phenotype by Tspan3c expression (see text for details). (A'–F') Magnified images of middle region between dorsal and anal fins of trunk showing encroachment of xanthophores into melanophore domains in mutants homozygous for *dali* and *jaguar* (C',E'); heterozygous mutants (B',D') as well as homozygous *dali* mutants expressing Tspan3c (F') have disrupted patterns but little or no intermingling of melanophores and xanthophores. (A''–F'') Corresponding tracings

illustrating distributions xanthophores (orange) and melanophores. Scale bar: in (F''), 60 μm for (A'–F').

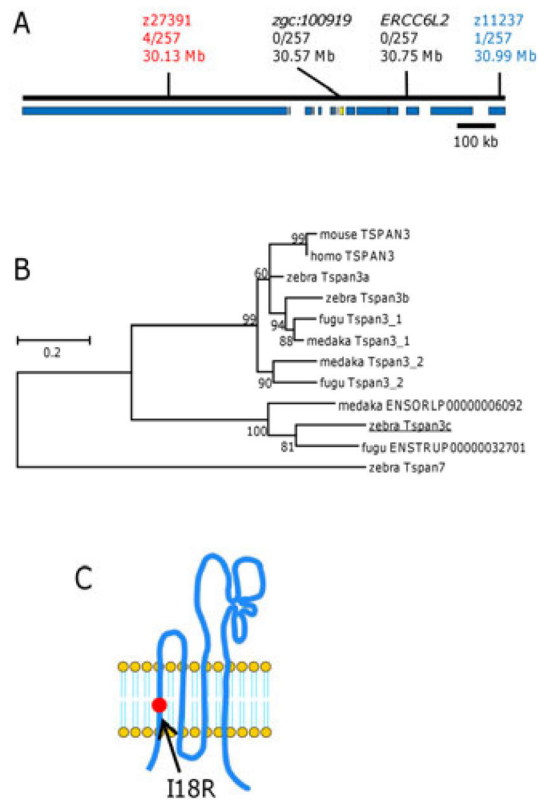


Figure 2. *dali* mutant melanophores have a motility defect *in vitro*. (A,B,E) Distances migrated by wild-type melanophores ($n=10$) and *dali* mutant melanophores ($n=10$) revealed significantly reduced motility of the latter ($*p<0.01$). (C,D,E) Distances migrated by wild-type xanthophores ($n=10$) and *dali* mutant xanthophores ($n=6$) were not significantly different between genotypes. Data were collected from two or more independent experiments.

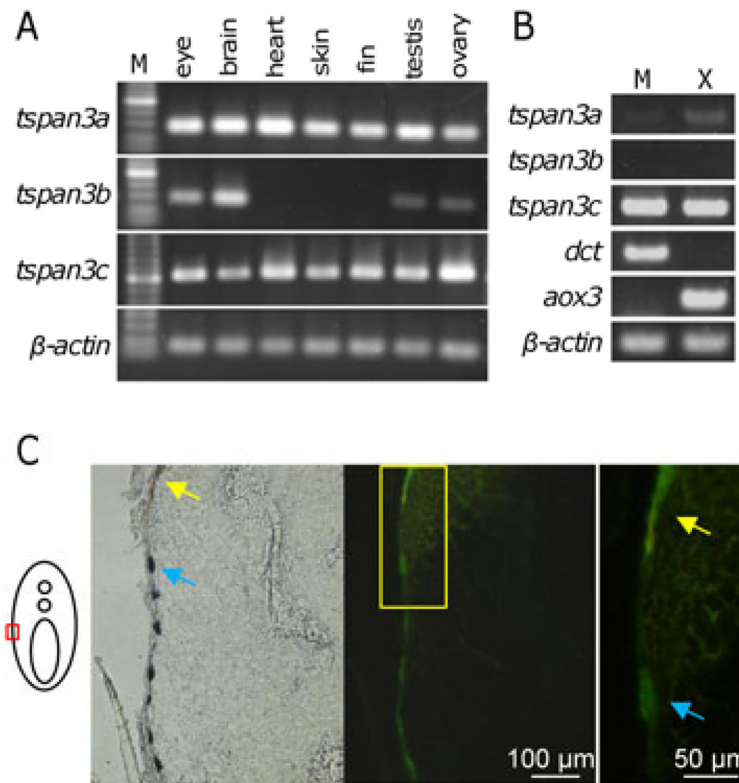


Figure 3.

Pigment cells from *dali* mutant have defects in cell–cell interactions. (A) Contact between wild-type melanophores and wild-type xanthophores results in movement away by the melanophore, and following by the xanthophore (10 of 11 pairs tested). (B) Contact between *dali* mutant melanophores and xanthophores did not result in such behaviors (7 of 7 pairs). (C) Wild-type xanthophores failed to elicit escape responses by *dali* mutant melanophores (7 of 7 pairs). (D) Likewise, *dali* mutant xanthophores failed to elicit escape responses by wild-type melanophores (13 of 15 pairs). Scale bar: 50 μ m. (E) Indicator of repulsive activity of melanophores. X-axis values of melanophore migrations (double-headed arrows) were used to evaluate repulsive (red arrow) or attractive (blue arrow) activities. (F) Significant differences in escape behavior were detected between WT pairs and all others (* p <0.01). Data were collected from two or more independent experiments.

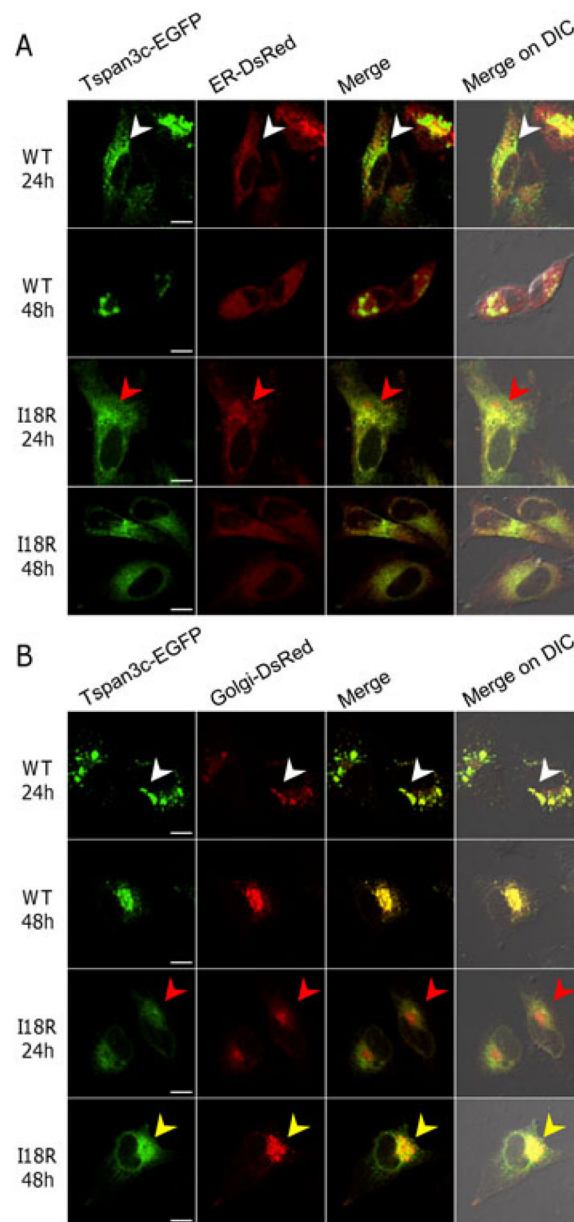


Figure 4.

dali maps to *zgc:100919* encoding Tetraspanin 3c. (A) Meiotic mapping of the *dali* mutant revealed an 860 kb critical genetic interval containing 15 open reading frames (blue bars), of which only the gene-encoding vertebrate transmembrane 4 superfamily-like (*tetraspanin 3c*; abbreviated as *tspan3c*, accession number; NP_001002748) exhibited an ENU-induced lesion (yellow bar) (B) Orthology of zebrafish and other vertebrate Tspan3 proteins. Tspan7, the closest homologous protein to Tspan3, was used as the out group. (C) A schematic of the Tspan3c protein showing the *dali* mutant I18R substitution in the first transmembrane domain.

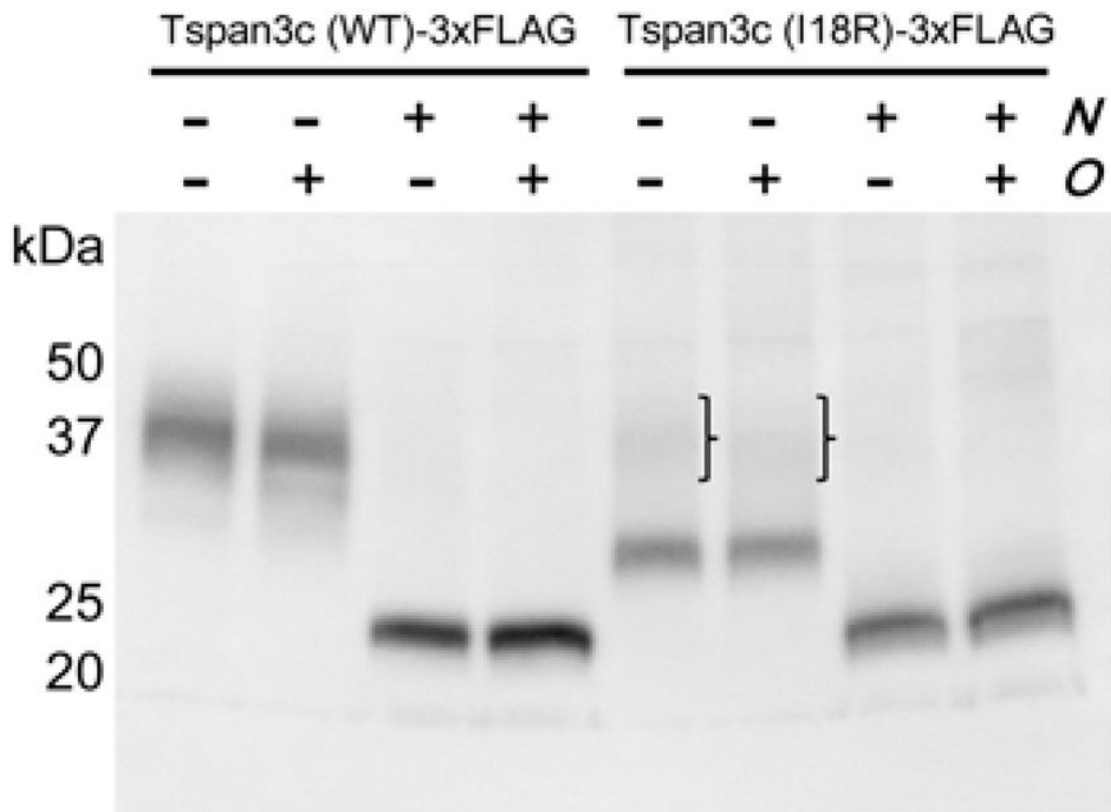


Figure 5.

tspan3c expression by adult melanophores, xanthophores and whole tissues. (A,B) RT-PCR for zebrafish tetraspanin 3 paralogues for tissues as well as populations of isolated melanophores (M) and xanthophores (X). *dct* (*dopachrome tautomerase*) is a marker of melanophores (Kelsh et al., 2000), whereas *aox3* (*aldehyde oxidase 3*) is a marker of xanthophores (Parichy et al., 2000b). (C) EGFP signals driven by the *tspan3c* promoter were detected in adult melanophores (blue arrows) and adult xanthophores (yellow arrows). Right image shows higher magnification view of boxed region in middle.

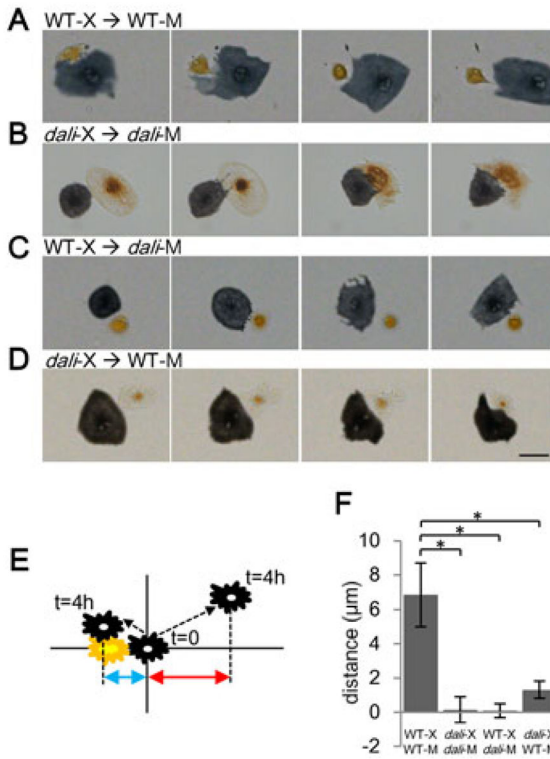


Figure 6.

Tspan3c(I18R) is retained within the ER. Tspan3c(WT or I18R)-EGFP fusion proteins were expressed in HeLa cells and their localization was examined using marker for ER (A) and Golgi apparatus (B) after 24 h and 48 h. Scale bar: 10 μm. Arrowheads indicate marked localization of the Tspan3c protein (see text). Trials were conducted in duplicate.

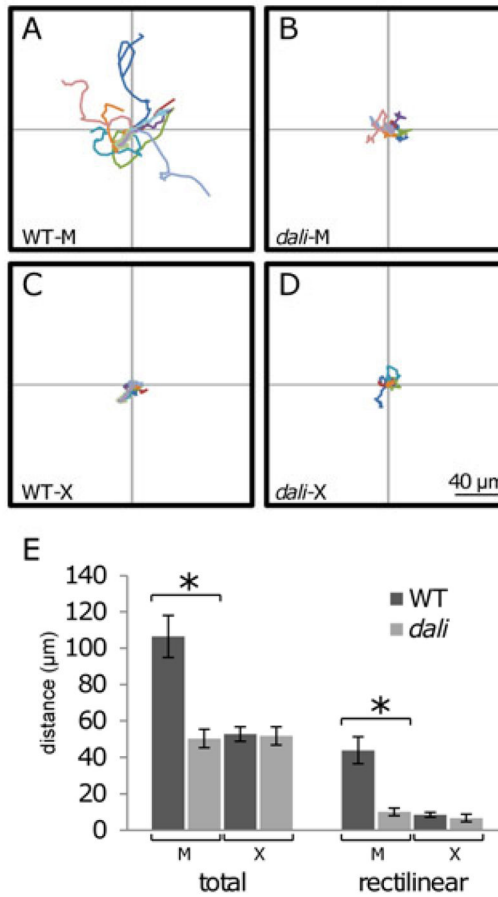


Figure 7.

Reduced *N*-glycosylation of Tspan3c(I18R). Western blot showing Tspan3c(WT or I18R)-3×FLAG tagged proteins expressed in HeLa cells following treatment with *N*-glycosidase (*N*), *O*-glycosidase (*O*), or both. In comparison to Tspan3c(WT), relatively little *N*-glycosylation was evident for Tspan3c(I18R) (brackets). Trials were conducted in triplicate.