Tetraspanin Cd9b and Cxcl12a/Cxcr4b have a synergistic effect on the control of collective cell migration

Katherine S. Marsay1,2,3‡, Sarah Greaves4‡, Harsha Mahabaleshwar5, Charmaine Min Ho6, Henry Roehl4,* Peter N. Monk2, Tom J. Carney3,5, Lynda J. Partridge1

1 Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom, 2 Department of Infection, Immunity and Cardiovascular Science, University of Sheffield, Sheffield, United Kingdom, 3 Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore, Singapore, 4 Department of Biomedical Science, University of Sheffield, Sheffield, United Kingdom, 5 Lee Kong Chian School of Medicine, Experimental Medicine Building, Yunnan Garden Campus, Nanyang Technological University, Singapore, Singapore

‡ KM and SG are joint first authors and contributed equally to this work.

* h.roehl@sheffield.ac.uk

Abstract

Collective cell migration is essential for embryonic development and homeostatic processes. During zebrafish development, the posterior lateral line primordium (pLLP) navigates along the embryo flank by collective cell migration. The chemokine receptors, Cxcr4b and Cxcr7b, as well as their cognate ligand, Cxcl12a, are essential for this process. We corroborate that knockdown of the zebrafish cd9 tetraspanin orthologue, cd9b, results in mild pLL abnormalities. Through generation of CRISPR and TALEN mutants, we show that cd9a and cd9b function partially redundantly in pLLP migration, which is delayed in the cd9b single and cd9a; cd9b double mutants. This delay led to a transient reduction in neuromast numbers. Loss of both Cd9a and Cd9b sensitized embryos to reduced Cxcr4b and Cxcl12a levels. Together these results provide evidence that Cd9 modulates collective cell migration of the pLLP during zebrafish development. One interpretation of these observations is that Cd9 contributes to more effective chemokine signalling.

Introduction

Cells can migrate individually or in groups, the latter is known as collective cell migration. During this process, cells exhibit coordinated behaviour, group polarisation and maintain cell-cell contacts [1]. This mode of migration is employed during embryonic development in the morphogenesis of multiple organ systems and is also important for effective immune responses. This mechanism contributes to several diseases including metastatic cancer and rheumatoid arthritis [2].

The zebrafish lateral line consists of a series of mechanosensory organs (neuromasts), which are distributed along the lateral surface of the zebrafish body and connected to the central nervous system by afferent axons [3]. This arrangement of innervated neuromasts is...
achieved by the collective migration of a placodal primordium of approximately 100 cells, arising just posterior to the otic placode [4]. This posterior lateral line primordium (pLLP), then migrates along the horizontal myoseptum to the tip of the tail. During migration, clusters of cells are deposited from the trailing end of the pLLP, which ultimately differentiate into neuromasts.

Primordium migration is directed by expression of the chemokine Cxcl12a along the horizontal myoseptum, which is received by two chemokine receptors expressed within the primordium [5–7]. Cxcr4b is expressed in the leading two thirds of the primordium, where ligand binding induces Gβ1 signalling and actin polymerisation to promote a migratory phenotype [8]. Cxcr7 is expressed in the anterior-most third and functions as a ligand sink, allowing the formation of a local Cxcl12a gradient. This results in directed migration of the primordium along the Cxcl12a pathway towards the caudal fin [9, 10]. There is evidence that the membrane environment of chemokine receptors, including Cxcr4 and Cxcr7, strongly influences their signalling properties. This includes homodimerisation, oligomerisation and heteromerisation with other membrane receptors [11]. In addition, chemokine receptors have been linked to membrane microdomains where the lipid environment strongly modulates function [12].

Tetraspanins are a large family of small integral membrane proteins, which have been shown to organise neighbouring membrane proteins into complexes called tetraspanin enriched microdomains. This is often referred to as the tetraspanin web, as different interactions build to form dynamic signalling networks that often induce similar functional outcomes [13]. Thus, tetraspanins are associated with a wide variety of cellular functions including signalling and cell migration [14]. For example, the tetraspanin CD9 is downregulated in many human cancers including lung, breast and ovarian, and reduced CD9 expression is related to colon cancer metastasis [15]. In particular, CD9 has been shown to regulate, and be regulated by, CXCL12-CXCR4 signalling [16–18]. CD9 was found in close proximity to CXCR4 on the membrane of B acute lymphoblastic leukaemia cells in vitro, and enhanced their CXCL12 dependent migration [18]. If regulation of CXCL12/CXCR4 signalling by CD9 is conserved, we hypothesised Cd9 would be strongly expressed in the zebrafish pLLP, where it might modulate Cxcr4 signalling and thus pLLP migration. Indeed microarray experiments have localised the zebrafish cd9b parologue to the migrating primordium, whilst a morpholino (MO) experiment indicated loss of cd9b altered primordium structure at 36 hours post fertilisation (hpf) and reduced neuromast number at 48 hpf [2].

In this study we aimed to investigate further the role of zebrafish CD9 orthologues in Cxcr4b-mediated pLLP migration, through use of genetic knockouts. We show expression of both cd9a and cd9b paralogues in the primordium and confirmed the abnormal pLL phenotype seen previously with cd9b knockdown. The cd9b mutants showed delayed pLLP migration but did not fully replicate cd9b morphant pLL phenotypes. We therefore generated cd9a; cd9b double knockouts (cd9 dKOs). These showed both reduced migration of the pLL and increased sensitivity to reduced levels of Cxcr4b and Cxcl12a, supporting a role for CD9 in regulation of Cxcr4 signalling.

Methods

Zebrafish maintenance

Adult wild-type zebrafish (WT), Tg(-8.0cldnb:LY-EGFP)z106 and cd9a/b mutants were housed and bred in a regulated 14:10 hour light:dark cycle under UK Home Office project licence 40/3459 in the Bateson Centre aquaria at the University of Sheffield or in the Singapore IMCB zebrafish facility under the Biological Resource Centre oversight with project license IACUC.
140924. Zebrafish were raised under the standard conditions at 28°C [19]. Ages are expressed as hours (hpf) or days (dpf) post fertilisation.

Morpholino injection

Antisense morpholino oligonucleotides (MO) were obtained from GeneTools LLC and re-suspended in MilliQ H$_2$O to give a stock concentration of 1 mM and injected in one-cell stage embryos. A Flaming/Brown micropipette puller was used to create micro-injection needles from borosilicate glass capillary tubes (0.5 mm inner diameter, Sutter). The PV800 Pneumatic PicoPump, as part of the micro-injection jig, was set up to release the required amount of injection material by adjusting the air pressure and air expulsion time. For the knockdown of cd9b two MOs were designed, a translation blocker with the following sequence (cd9b MO1): 5’-tttatgaggagaaacccaagactga-3’ and a splice site blocker (cd9b i2e3) with the following sequence: 5’-aacccctgaacacagagaaacaaca-3’, whilst the published mismatch MO was used 5’- tttccctgctgcttatacagcgatg -3’ [20]. For knockdown of cxcr4b and cxcl12a, the following sequences were used respectively, 5’-aatgatgctatcgtttgcagtgtgaa-3’ [21].

Zebrafish mutant production

cd9b mutants were created from WT embryos using transcription activator-like effector nucleases (TALEN) and maintained on an WT background. TALEN (ZGene Biotech Inc., Taiwan) were provided in a pZGB4L vector, targeting the cd9b sequence 5’-ttgctctttatcttca-3’. Injected embryos were outcrossed and sequenced to identify mutations. A frameshifting deletion mutation (c.42_49del) was selected that caused premature termination within the first transmembrane domain (cd9b$^{p615}$ allele). CRISPR-Cas9 was used to create cd9a mutants using the gRNA sequence 5’-gagtgtatatcctcattgcgg-3’, which targeted the 3rd exon encoding part of the second transmembrane domain. cd9 dKO mutants were created by injecting the above cd9a gRNA and Cas9 RNA into cd9b$^{p615}$ embryos. These fish were screened for germline transmission by sequencing and backcrossed to cd9b$^{p615}$ mutants. An indel mutation deleting 4bp and inserting 8bp (c.180_187delinsTCGCTAGTATG; cd9a$^{la61}$) generated a frameshift mutation resulting in a premature stop codon in exon 3, which was predicted to truncate the protein before the large extracellular domain. Heterozygous fish of the same genotype were incrossed and adult F2 fish were genotyped to identify homozygous cd9b$^{p615}$; cd9a$^{la61}$ (cd9 dKO).

In situ hybridisation

Embryos were raised at 28°C in petri dishes containing E3 solution. The E3 was changed daily, and any dead embryos removed. At 30–32 hpf, embryos were anaesthetised using tricaine and dechorionated before returned to fresh E3 solution. At the relevant timepoint embryos were fixed overnight at 4°C using 4% (w/v) paraformaldehyde (Sigma-Aldrich, UK) in phosphate buffered saline (PBS). Embryos were washed in PBS/0.05% (v/v) Tween 20 (PBST), then put through a methanol/PBS series using 30%, 60% and 100% (v/v) methanol before being stored in 100% methanol (Sigma-Aldrich) at -20°C. In situ hybridisation (ISH) was carried out as described by [22], except for the embryo digestion with proteinase K, for which 30–32 hpf embryos were digested with 10 mg/ml proteinase K at 20°C for 22 min. Primers used for PCR generation of the in situ probes are given in Table 1 below. The protocol was conducted with the embryos in 1.5 ml microfuge tubes for the first two days, after which they were held in 12-well plates for staining before transferring back to microfuge tubes for storage. Stained embryos were stored in the dark in 80% (v/v) glycerol.
Time lapse imaging

Time-lapse recording was performed using an inverted Zeiss LSM700 Confocal microscope. All larvae were anaesthetised using 0.02% tricaine and then embedded on their side in 1% low melting agarose (Lonza) on a glass bottom dish (MalTEK Corporation) and covered with E3 supplemented with 0.02% tricaine. Separate Z-stack images covering the depth of the horizontal myoseptum were taken at specified intervals over a specified period and assembled into a final movie at a specified frame rate.

Statistics

Data distribution was first assessed for normality using a D’Agostino-Pearson omnibus K2 normality test on the experimental residuals, as well as creating a histogram of residuals. For normally distributed data, an ANOVA with Dunnet’s or Holm's-Sidak multiple comparisons tests were used. For non-normally distributed data non-parametric tests, the Mann-Whitney U test or Kruskal-Wallis with Dunn’s multiple comparisons test, were used.

Results

Both cd9 zebrafish paralogues are expressed in the lateral line

Zebrafish possess two Cd9 paralogues, Cd9a [NP_997784] and Cd9b [NP_998593] which show 60% and 59% identity to human CD9 respectively and 63% identity to each other using multiple sequence comparison by log-expectation (MUSCLE) [23]. Whole mount in situ hybridisation (WISH) using probes against cd9a and cd9b demonstrated expression of both paralogues in the migrating primordium at 36 hpf (Fig 1A–1D). Expression of both cd9a and cd9b was also observed in recently deposited neuromasts and was retained in neuromasts until at least 5 dpf (Fig 1G–1L).

cd9b morphant phenotype

To evaluate if there was a role for either paralogue in pLLP migration, we initially focused on cd9b as its role in the primordium had been previously reported [2]. Two different MOs were designed to target cd9b; cd9b MO1 was a translation blocking MO which targeted the 5’ UTR of cd9b RNA and cd9b i2e3 was a splice blocking MO designed against the intron 2—exon 3 splice site. These MOs were injected independently into 1-cell stage embryos to ensure that they produced the same phenotype. Embryos injected with either cd9b MO1 or cd9b i2e3, but not the mismatch or uninjected embryos, showed a significant decrease in neuromasts deposited (Fig 2). For embryos injected with cd9b MO1, there was a decrease in the percentage of trunk length between the first and last neuromasts (Fig 21). This suggests that the primordium is stalling prematurely or migrating more slowly. This recapitulates and expands on the work by Gallardo et al., 2010 which suggested a role for Cd9b in lateral line development [2].

Table 1. Primers for making ISH probes.

| Probe | Primer name | Sequence (5’-3)      |
|-------|-------------|---------------------|
| claudin b | Claudin b F | aacgaaaaagcatgcatc   |
| claudin b | Claudin b R | gagctgtttaaaacgcttg  |
| cd9a  | CD9a F      | gctcatattcgcgtcgaagt |
| cd9a  | CD9a R      | ctgcgagaaaacaagcaa   |
| cd9b  | CD9b F      | gttgccacaaatgcctgat  |
| cd9b  | CD9b R      | tacatgtactttctctcaaaat |
To confirm this MO result, we created a TALEN mediated knockout of cd9b. A cd9b TALEN pair was designed by ZgeneBio using the program “TEL Effector Nucleotide Targeter 2.0”. The TALEN pair was designed to target Exon 1 and predicted to cut in the 1st transmembrane domain (Fig 3A). The TALEN pair was injected and after 72 hpf a proportion of embryos were genotyped. Injected embryos showed clear mosaicism around the TALEN cut site so embryos were raised to create mosaic adult F0s. F0s transmitting a mutation to offspring were out-crossed with WT fish and the resulting F1 offspring were raised. To create a cd9b homozygous mutant line with a single mutant allele, F1s with a c.42_49del mutation were selected, incrossed and the resulting F1 offspring were raised. To create a cd9b homozygous mutant line with a single mutant allele, F1s with a c.42_49del mutation were selected, incrossed and the resulting offspring raised to adulthood. This allele (cd9b<sup>pg15</sup>) was used as it had the largest deletion and caused the earliest nonsense stop codon. The 8bp deletion in the cd9b allele leads to a frameshift in exon 1, changing codon 15 from TTT (Phe) to CAA (Glu), then 22 aberrant amino acids followed by a stop codon (p.Phe15Glu fsTer22) (Fig 3B). cd9b<sup>pg15</sup> homozygous mutants are viable and showed normal development. Loss of cd9b in situ signal in the cd9b mutants suggested strong nonsense mediated decay (NMD) of the mutated allele (S1 Fig). Due to the phenotypes seen in cd9b morphants, it was expected that a lateral line phenotype would be seen in cd9b mutants with fewer neuromasts deposited. However, no significant difference was found in the number of neuromasts at 52 hpf (Fig 3). As cd9b is expressed throughout pLL development, migration of the primordium as well as lateral line structure was assessed at 36 hpf. Although the pLL shows no structural abnormalities in cd9b mutants at 52 hpf, it is possible that cd9b mutants show a lateral line phenotype earlier in development.

cd9b mutant phenotype

Fig 1. cd9a and cd9b are expressed in the pLLP during zebrafish development. A-F: Micrographs of WISH of cd9a and cd9b at 36 hpf with sense and anti-sense probes. (a-b) Overview shows staining in pLL; (c-d) higher resolution images show expression in the primordium; (e-f) sense probes show no staining. G-J: WISH of cd9a and cd9b at 48 hpf; (g-h) overview shows staining in pLL; (i-j) higher resolution images show expression in a neuromast. K-L: WISH of cd9a and cd9b at 5 dpf in a neuromast. Arrows indicate primordium (black) and neuromasts (red). Scale bar indicates 50 μm (white).

https://doi.org/10.1371/journal.pone.0260372.g001
and have recovered by 52 hpf. At 36 hpf, cd9b mutants show delayed primordium migration, with the percentage of trunk traversed by the primordium reduced in mutants (Fig 3).

**Generation of a cd9a mutant**

To ascertain a role for Cd9a, we targeted the cd9a gene using CRISPR/Cas9. A gRNA was designed to target cd9a in exon 3 (Fig 4A) and injected along with RNA encoding Cas9 into embryos.
Tetraspanin Cd9b and Cxcl12a/Cxcr4b have a synergistic effect on the control of collective cell migration.
**Fig 3.** *cd9b* mutant does not recapitulate morphant phenotype, although primordium migration is delayed at 36 hpf. A: Nature of the *cd9b* mutant allele showing TALEN site location within the intron-exon structure of the gene. B: The TALEN target sequence in exon 1 is shown in blue; the 8bp deletion in the *cd9b* allele is indicated under the WT sequence as dashes. This leads to a frameshift changing codon 15 from TTT (Phe) to CAA (Glu), then 22 aberrant amino acids (red lettering) followed by a stop codon (*). C: Schematic of the *Cd9b* protein with location of premature stop codon given by red arrow. The disulfide bonds between the conserved CCG motif and conserved cysteines are indicated by the dashed lines. EC1/2 = Extracellular domain 1/2, aa = amino acid. D-E: Sequence chromatograms of genomic DNA from (d) WT and (e) *cd9b* KO alleles. Deleted base pairs are underlined in red. F-E: Representative images of WISH of *claudin b* ISH on (f-g) WT and (h-i) *cd9b* mutants at time shown. J-K: Graphs quantifying pLLP measurements in WT and *cd9b* KOs; (j) the migration of the *cd9b* KO primordium at 36 hpf is significantly delayed compared to WT. (k) There is no significance in number of neuromasts deposited at 52 hpf. Unpaired T test on untransformed data. n = minimum 20. *** = p < 0.005.

https://doi.org/10.1371/journal.pone.0260372.g003

WT embryos to generate *cd9a* KOs. After screening for mosaicism, the CRISPR/Cas9 injected embryos were raised to maturity and screened for germline transmission of the *cd9a* mutation. F0s were outcrossed with WT fish to generate F1 *cd9a* single heterozygous mutants carrying an indel mutant allele (c.183_186delinsAT; *cd9a*ic62). This indel led to a frameshift mutation, changing codon 62 from ATT (Iso) to TGC (Cys), then generating 54 aberrant amino acids followed by a premature stop codon before the EC2 domain (p.Iso62Cysfs54Ter) (Fig 4B and 4C). Heterozygous *cd9a*+/ic62 adults were in-crossed to produce the F2 generation. Adult F2s were viable and fertile and were genotyped to identify homozygous *cd9a*ic62 KOs. Loss of *cd9a* in situ signal in the *cd9a* mutants suggested strong NMD of the mutated allele (S2 Fig). The effect of *cd9a* KO on pLL development was investigated but overall, there were no major defects. No significant difference was found in the number of neuromasts deposited at 48 hpf or primordium migration at 36 hpf (Fig 4F–4K).

**Generation of a *cd9a; cd9b* double mutants**

Due to the redundant nature of tetraspanins, and *cd9a* having a similar mRNA expression pattern to *cd9b*, it was speculated that functional redundancy between *Cd9a* and *Cd9b* might be masking stronger phenotypes in the single mutants. Hence, we generated *cd9a; cd9b* double mutants. To do this, the same *cd9a* gRNA as above (Fig 4) and *cas9* mRNA were injected into *cd9b*pg15 embryos. These fish were screened for mutation of *cd9a* as above and then back-crossed to *cd9b*pg15 mutants. An indel mutation in exon 3 was detected (c.180_184delinsTCGC; *cd9a*la61). This indel led to a frameshift mutation and an early stop codon, which truncated the protein before the EC2 domain (p.Leu61Alafs9Ter) (Fig 5A–5D). Heterozygous *cd9a*+/la61 individuals, from now on referred to as *cd9* dKOs; (j) the migration of the *cd9b* KO primordium at 36 hpf is significantly delayed compared to WT. (k) There is no significance in number of neuromasts deposited at 52 hpf. Unpaired T test on untransformed data. n = minimum 20. *** = p < 0.005.

https://doi.org/10.1371/journal.pone.0260372.g003

Trans-heterozygous fish were crossed and adult F2s were genotyped to identify homozygous *cd9a*la61 KOs. Loss of *cd9a* in situ signal suggested strong NMD of the mutated allele (S2 Fig). The effect of *cd9a* KO on pLL development was investigated but overall, there were no major defects. No significant difference was found in the number of neuromasts deposited at 48 hpf or primordium migration at 36 hpf (Fig 4F–4K).

In order to further investigate the migration and organisation of the primordium in *cd9* dKO embryos they were crossed into the *claudin b* gfp transgenic line. Under the *claudin b* promoter GFP is expressed in the lateral line primordium and newly deposited neuromasts [5]. This allowed observation of the primordium migration in real time. Primordium migration
Fig 4. Generation of cd9a mutant does not show any abnormal pLLP development. A: Nature of the cd9a mutant allele showing CRISPR site location within the intron-exon structure of the gene. B: The CRISPR target sequence in exon 3 is shown in blue; the 2bp deletion in the cd9a allele is indicated under the WT sequence as dashes. This leads to...
appeared normal in the cd9 dKO embryos (Fig 6, S1 and S2 Videos). At the leading edge of the primordium filopodia could be seen as well as proliferating cells. Within the migrating primordium rearrangements occurred and rosettes were formed, increasing the length of the primordium. As the trailing cells decelerated and deposited cells as neuromasts, the primordium reduced in size. Quantification of various aspects of the primordium during deposition revealed similar results in WT and mutants (S5 Fig).

Cd9 interacts with the Cxcr4b/Cxcl12a pathway in the migrating zebrafish lateral line primordium

To investigate if cd9 had an effect on cxcr4b expression in the primordium, cxcr4b WISH was performed but no perturbation of cxcr4b expression was seen in the cd9b KOs (S6 Fig). Quantification showed no significant difference between the expression of cxcr4b in WT and cd9b KOs. To investigate if Cd9 was affecting migration in the zebrafish primordium through the Cxcr4b/Cxcl12a pathway, an experiment using MOs was conducted. Cxcr4b and cxcl12a MOs induce premature stalling of the primordium at concentrations of 1.5 mM and 0.5 mM respectively [21]. 100 μM of cxcr4b MO was found to be the highest concentration injected into WT embryos that did not induce a phenotype. However, this concentration of MO resulted in a further delay of the primordium in the cd9 dKOs (Fig 7A). For cxcl12a the highest concentration of MO that did not induce a phenotype in WT embryos was determined to be 12.5 μM but this had no further effect on the primordium migration in cd9 dKOs (S7 Fig). If the cxcl12a MO concentration was raised to 25 μM then the primordium in both WT and cd9 dKOs was delayed although the primordium in cd9 dKOs was significantly more delayed (Fig 7B).

Discussion

In this study we investigated the role of Cd9 in the migration of the pLLP in zebrafish development. We first verified previous data showing cd9b expression in the primordium at 30 hpf [2]. We also noticed cd9b was expressed throughout recently deposited neuromasts at 48 hpf and 5 dpf. Our MO knockdown recapitulated previous results from Gallardo et al., 2010. cd9b morphants showed fewer neuromasts deposited, which suggested a role for Cd9b in the development of the lateral line. A homozygous cd9b mutant was created using TALENs but surprisingly, the morphant phenotype was not recapitulated in cd9b homozygous mutants. This lack of phenotype could be due to several reasons. Firstly, the phenotype seen in cd9b morphants could be due to off target effects of the MOs and not due to cd9b knockdown. However, the fact that two different MOs and researchers induced the same phenotype suggests it was not the lack of specificity [2]. Secondly, the Cd9b truncated protein could have some residual function, although ISH results showed a downregulation of mRNA suggesting NMD was occurring. Also, the cd9b mutation aborts the normal sequence after aa15, before the EC2 domain, so any translated protein would be expected to have minimal function. Finally, the cd9b morphant phenotypes may be specific but tetraspanin redundancy could rescue the phenotype in
Fig 5. Generation of cd9a mutant in the cd9b KO background to create a double cd9 KO mutant. A: cd9a mutant allele sequence from exon 3, showing CRISPR target sequence in blue. The 4bp deletion (dashes) and 12bp insertion (lowercase) results in an 8bp insertion as indicated under the WT sequence. This leads to a frameshift.
Tetraspanin Cd9b and Cxcl12a/Cxcr4b have a synergistic effect on the control of collective cell migration

cd9b mutants. Tetraspanins are well known for their redundancy within the tetraspanin family and mouse knockouts of single tetraspanins often appear healthy and viable with mild phenotypes, whereas double tetraspanin knockout mice often show increased numbers and severity of phenotypes [24–31]. In zebrafish, this redundancy may be amplified due to the occurrence of a fish-specific whole genome duplication in teleost fish. This means that many tetraspanins, for which there is one mammalian ortholog, have two paralogs in zebrafish [32–36]. This is true for CD9, as mammals have a single CD9 whereas zebrafish have Cd9a and Cd9b. We demonstrated cd9a to also be expressed in the pLL in a similar pattern to cd9b. Furthermore, NMD of mutated genes, as we have seen in both our mutants, has been shown to invoke an upregulation of closely related genes as a genetic compensation mechanism [37].

changing codon 61 from CTC (Leu) to GCG (Ala), then 9 aberrant amino acids (red lettering) followed by a stop codon. B: Schematic of the Cd9 protein with location of premature stop codon for Cd9a (red arrow) and Cd9b (green arrow) at a predicted 70 aa and 36 aa respectively. EC1/2 = Extracellular domain 1/2. C-D: Sequence chromatograms of genomic DNA from (c) WT and (d) cd9bmut allele. Deleted base pairs are underlined in red and inserted base pairs in blue. E-F: claudin b expression in WT at (e) 30 hpf and (f) 48 hpf. G-H: claudin b expression in cd9 dKO mutants at (g) 30 hpf and (h) 48 hpf. I-J: Graphs showing pLLP measurements in WT and cd9 dKOs; (i) migration of the cd9 dKO primordium at 36 hpf is significantly delayed compared to WT. (j) There is no significance in number of neuromasts deposited. Signific ance was assessed using an unpaired t test. N = minimum 9, p = <0.001, n1 = neuromast 1 etc. Bars show mean +/- SD.

https://doi.org/10.1371/journal.pone.0260372.g005

Fig 6. Primordium organisation appears normal in cd9 dKO(cldhb:gfp) embryos from 30 hpf. A-B: Still images from a time-lapse recording of a migrating primordium in (a) WT and (b) cd9 dKOs. 0 minutes shows initial deposition as a proneuromast becomes distinct from the primordium and then 2 sequential images show (a’-b’) 20 minutes and (a’’-b’’) 60 minutes later. In the primordium of both WT and cd9 dKOs, filopodia can be seen at the leading edge (white arrow) and the formation of rosettes in the trailing edge (white dashed circle). Representative images from two videos that included two depositions each. Scale bar: 20 μm.

https://doi.org/10.1371/journal.pone.0260372.g006
Following this, tetraspanin cd9a was targeted for knockout using CRISPR technology. Cd9a<sup>h162</sup> homozygous mutants and cd9a<sup>h61</sup> double homozygous mutants were generated. Cd9a KO embryos displayed a normal lateral line phenotype with normal primordium migration and neuromast deposition. Initial analysis of cd9 dKO embryos using the same measurements revealed that the number of neuromasts deposited and spacing were normal yet the distance of primordium migration at 36 hpf was significantly delayed. It was expected that knockout of cd9a would result in a stronger pLL phenotype but cd9 dKOs showed a similar phenotype to the cd9b KOs. Furthermore, when primordium migration of cd9b single KO was compared to that of the cd9 dKOs there was no difference. These results suggest that the different phenotypes seen between the cd9b morphants and mutants are not due to compensation by cd9a. It remains possible that there is compensation by another tetraspanin [38]. Close relations to CD9 are the tetraspanins CD81 and CD63, which have been shown previously to substitute for CD9 in some circumstances. Overexpression of CD81 partially rescued the infertility phenotype seen in CD9 KO mice [39] and it was recently demonstrated that CD9 deletion in human melanoma cells was quickly compensated by CD63 expression upregulation [40]. It would be interesting to perform RNA-seq or SWATH-MS on the cd9 dKOs to identify upregulated RNA and proteins respectively [41].

Videos generated of cd9 dKOs with fluorescent claudin b showed primordium migration and internal organisation were normal. Measurements of the primordium after DAPI stain also revealed the primordium were similar sizes between mutants and WT at 36 hpf. Overall, it seemed the delay in migration at 36 hpf in the cd9 dKO embryos was not due to alterations in the primordium organisation. Therefore, the cd9 dKO phenotype appeared to be a migratory defect that could either be due to a change in the onset of migration, a change in the speed of migration or a combination of the two. CD9 has previously been reported to promote CXCR4b/CXCL12a signalling in mammalian cells so we decided to investigate a potential interference with this chemokine signalling pathway [16–18]. Results from the WISH analysis and quantification showed no obvious perturbation of cxcr4b expression in the cd9 KOs. To postulate if there was some interaction between Cd9 and the Cxcr4b/Cxcl12a signalling pathway a MO experiment was performed. It was theorised that a primordium lacking Cd9 would be more susceptible to disruption by sub-functional doses of either cxcr4b or cxcl12a MO than WT. Injection with a sub-functional concentration of 100 μM cxcr4b MO demonstrated that the cd9 dKO larvae were more sensitive to MO treatment compared to WT with significant

---

Fig 7. cxcr4b and cxcl12a MOs further delay primordium migration at 36 hpf in cd9 dKO embryos. A-B: Distance migrated by the primordium (labelled by claudin b ISH) were recorded for (a) injection of 100 μM cxcr4b MO, and (b) 25 μM cxcl12a MO. Significance was assessed using one-way ANOVA, “*” = p<0.05, “**” = p<0.01, “***” = p<0.001 “****” = p<0.0001. N = minimum 15. Box extends from the 25th to 75th percentile and whiskers from 10th to 90th.

https://doi.org/10.1371/journal.pone.0260372.g007
retardation in primordium migration in the dKO. This suggests that there is some interaction between Cd9 and Cxcr4b within the primordium that promotes migration. Injection with cxcl12a MO was more ambiguous as at a sub-functional concentration of 12.5 μM neither the WT nor the cd9 dKO primordium migration was perturbed. When the dose of cxcl12a MO was increased to 25 μM a migratory delay was induced in both genotypes but the delay seen in the cd9 dKO primordium was significantly worse. Despite disruption in the presence of the MO, the primordium was still able to eventually migrate the full length in the cd9 dKO embryos, suggesting that Cd9 is not essential for maintaining Cxcr4b/Cxcl12a signalling but may play a regulatory or buffering role. A further experiment to check for interaction between Cd9 and Cxcl12a/Cxcr4b in the primordium would be to use immunohistochemistry to investigate co-localisation. Unfortunately, there are no tetraspanin, Cxcl12a or Cxcr4b antibodies available in zebrafish. However, two plasmids are available that encode cxcr4b-egfp or cxcl12a-venus constructs [42]. An interesting experiment would be to inject embryos with these plasmids and visualise the distribution of fluorescent versions of the proteins in the cd9 dKOs.

We mentioned that the migratory defect could be due to a change in the onset of migration. This could be further investigated by performing claudin b ISHs at the onset and earliest stages of development of the posterior lateral line. On the other hand, to try and determine if Cd9b has a role in the speed of migration, ISHs could also be performed at various time points throughout primordium migration and speed could be calculated in a similar method to previous studies [43, 44].

We focused on a connection between Cd9 and Cxcr4b/Cxcl12 signalling in this paper because of previous research linking these proteins. However, Cd9, like other tetraspanins, contributes to cellular processes by organising molecules within the plasma membrane and there are many other signalling pathways involved in primordium development [45, 46]. The fundamental four are FGF, Wnt, Notch and chemokine signalling pathways and their specific and coordinated expression ensures the regular morphogenesis and migration of the pLL [47]. Even though the organisation of the primordium at high resolution appears normal, analysis of the expression of these crucial signalling molecules, through WISH or immunohistochemistry, during primordium development could reveal subtle changes in their organisation that may be affecting migration. Whilst Fgf signalling functions to determine rosette formation and deposition, Wnt signalling drives proliferation in the leading zone [46]. Proliferation analysis with BrdU incorporation could show if there are any changes in cell proliferation within the primordium, specifically in the leading edge. CD9 has also been demonstrated to downregulate expression of Wnt signalling pathways in cell culture [48, 49]. Another important component of collective cell migration is cell adhesion so migratory cells can pull insensitive cells in the same direction. Within the primordium cadherins mediate cell-cell adhesion between primordium cells and are required for directed and robust migration [50]. Cadherin-dependent intercellular adhesion regulated by tetraspanins plays an important role in tumour invasion and metastasis [51, 52]. CD9 has been associated with keratinocyte motility by regulating E-cadherin-mediated cell-cell contacts [53].

The use of computational models has been a valued tool to integrate analysis of the lateral line primordium acquired through microscopic, cellular, molecular and genetic analysis [47, 54]. These models allow researchers to edit parameters and observe their influences on the migrating primordium. This could help us choose the most appropriate avenue to explore next by checking what modifications are able to regenerate the phenotype we observe in the cd9 dKO embryos.

In conclusion, we propose that within the primordium Cd9b is functioning to compartmentalise signalling molecules, like Cxcr4b, to orchestrate and amplify signalling upon ligand
binding but is not essential. This would explain why only subtle differences are seen between WT and cd9 dKOs.

Supporting information

S1 Fig. cd9b is significantly decreased in cd9b KO embryos. A-B: Representative images of cd9b WISH at 36 hpf in (a) WT and (b) cd9b homozygous embryos. (TIF)

S2 Fig. cd9a is significantly decreased in cd9a KO embryos. A-D: Representative images of cd9a WISH on (a-b) WT and (c-d) cd9a homozygous embryos at time shown. (TIF)

S3 Fig. cd9a and cd9b are both significantly decreased in cd9 dKO embryos. A-B: Representative images of cd9a ISH on (a) WT and (b) cd9 dKO mutants at time shown. C-D: Representative images of cd9b ISH on (c) WT and (d) cd9 dKO mutants at time shown. (TIF)

S4 Fig. cd9a is not compensating for cd9b in the primordium. Graph showing the distance migrated by the primordium at 36 hpf is the same in cd9b KO and cd9 dKO embryos. Significance was assessed using an unpaired t test, N = minimum 17. Bars show mean +/- SD. (TIF)

S5 Fig. Primordium shape is normal in the cd9 dKO embryos at 36 hpf. A: Representative image of a DAPI-stained primordium from a WT zebrafish at 36 hpf with red outline to show measured area. B-E: Graphs showing measurements of the primordium (b) height, (c) width, (d) width to height ratio, and (e) area in WT and cd9 KO embryos. The height was measured three times at equal points along the primordium and then averaged. The width of primordium was measured from the two furthest points along the middle of the primordium. Width was then divided by height to generate a ratio. The area was circled using the freehand selection and measured. Cells were counted using the multipoint tool on Image J software. Significance was assessed using an unpaired t test, p = <0.05, N = minimum 11. Bars show mean +/- SD. (TIF)

S6 Fig. cxc4rb expression is not altered in the pLLP of cd9 dKO embryos at indicated stages. A-D: Representative images of cxc4rb ISH in the primordium of (a,c) WT and (b,d) cd9 dKO mutants at time shown. Scale bar: 50 μm. E-F: Graphs showing measurements of (e) length and (f) height of cxc4rb expression in the primordium of WT and cd9 KO embryos at indicated stages. Length measurements were taken along the middle of the embryo between the two furthest points of expression within the primordium. Height measurements were taken between the two highest points of expression within the primordium. Significance was assessed using an unpaired T test. N = minimum 13 for 24 hpf and 11 for 30 hpf. Bars show mean +/- SD. (TIF)

S7 Fig. 12.5 μM of cxc12a MO does not further delay primordium migration at 36 hpf in the cd9 dKO embryos. Distance migrated by the primordium (labelled by claudin b ISH) was recorded for injection of 12.5 μM cxc12a MO. Significance was assessed using one-way ANOVA. N = minimum 13. Box extends from the 25th to 75th percentile and whiskers from 10th to 90th. (TIF)
S1 Video. WT primordium migration. *WT*(cldnb* gfp) from 30–38 hpf. Images taken every 1 minute with a 20x objective. Movie length: 420 min.
(MP4)

S2 Video. cd9 dKO primordium migration. *cd9* dKO(cldnb* gfp) from 30–36 hpf. Images taken every 1 min with a 20x objective. Movie length: 360 min.
(MP4)

Acknowledgments
The Bateson Centre aquarium at the University of Sheffield, the zebrafish facility at IMCB Singapore, Dr Freek van Eden for his advice on creating zebrafish knockouts and Kunal Chopra for his support raising the TALEN line.

Author Contributions
Conceptualization: Tom J. Carney, Lynda J. Partridge.
Data curation: Katherine S. Marsay, Sarah Greaves.
Formal analysis: Katherine S. Marsay, Sarah Greaves.
Funding acquisition: Henry Roehl.
Investigation: Harsha Mahabaleshwar, Charmaine Min Ho.
Project administration: Tom J. Carney, Lynda J. Partridge.
Resources: Henry Roehl, Tom J. Carney, Lynda J. Partridge.
Supervision: Henry Roehl, Peter N. Monk, Tom J. Carney, Lynda J. Partridge.
Visualization: Katherine S. Marsay, Sarah Greaves.
Writing – original draft: Katherine S. Marsay.
Writing – review & editing: Katherine S. Marsay, Tom J. Carney, Lynda J. Partridge.

References
1. Olson HM, Nechiporuk AV. Using Zebrafish to Study Collective Cell Migration in Development and Disease. Front cell Dev Biol. 2018 Aug 17; 6:83. https://doi.org/10.3389/fcell.2018.00083 PMID: 30175096
2. Gallardo VE, Liang J, Behra M, Elkahloun A, Villablanca EJ, Russo V, et al. Molecular dissection of the migrating posterior lateral line primordium during early development in zebrafish. BMC Dev Biol. 2010; 10(1):120. https://doi.org/10.1186/1471-213X-10-120 PMID: 21144052
3. Dijkgraaf BYS. The functioning and significance of the lateral-line organs. Biol Rev Camb Philos Soc. 1963 Feb; 38:51–105. https://doi.org/10.1111/j.1469-185x.1963.tb00654.x PMID: 14027866
4. Metcalfe WK, Kimmel CB, Schabtach E. Anatomy of the posterior lateral line system in young larvae of the zebrafish. J Comp Neurol. 1985 Mar 15; 233(3):377–89. https://doi.org/10.1002/cne.902330307 PMID: 3980776
5. Haas P, Gilmour D. Chemokine Signaling Mediates Self-Organizing Tissue Migration in the Zebrafish Lateral Line. Dev Cell. 2006 May; 10(5):673–80. https://doi.org/10.1016/j.devcel.2006.02.019 PMID: 16878780
6. Dambly-Chaudière C, Cubedo N, Ghysen A. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. BMC Dev Biol. 2007; 7(1):23. https://doi.org/10.1186/1471-213X-7-23 PMID: 17394634
7. Valentin G, Haas P, Gilmour D. The Chemokine SDF1a Coordinates Tissue Migration through the Spatially Restricted Activation of Cxcr7 and Cxcr4b. Curr Biol. 2007 Jun; 17(12):1026–31. https://doi.org/10.1016/j.cub.2007.05.020 PMID: 17570670
8. Xu H, Ye D, Behra M, Burgess S, Chen S, Lin F. GBeta1 controls collective cell migration by regulating the protrusive activity of leader cells in the posterior lateral line primordium. Dev Biol. 2014; 385(2):316–27. https://doi.org/10.1016/j.ydbio.2013.10.027 PMID: 24201188

9. Venkiteswaran G, Lewellis SW, Wang J, Reynolds E, Nicholson C, Knaut H. Generation and Dynamics of an Endogenous, Self-Generated Signaling Gradient across a Migrating Tissue. Cell. 2013 Oct; 155(3):674–87. https://doi.org/10.1016/j.cell.2013.09.046 PMID: 24119842

10. Donà E, Barry JD, Valentín G, Quirín C, Khmelinskii A, Kunze A, et al. Directional tissue migration through a self-generated chemokine gradient. Nature. 2013 Nov 25; 503(7475):285–9. https://doi.org/10.1038/nature12635 PMID: 24067609

11. Stephens B, Handel TM. Chemokine Receptor Oligomerization and Allostery. In: Progress in molecular biology and translational science. Prog Mol Biol Transl Sci. 2013. p. 375–420. https://doi.org/10.1016/B978-0-12-394587-7.00009-9 PMID: 23415099

12. Thelen M, Legler DF. Membrane lipid environment: Potential modulation of chemokine receptor function. Cytokine. 2018 Sep; 109:72–5. https://doi.org/10.1016/j.cyto.2018.02.011 PMID: 29433906

13. Tarrant JM, Robb L, van Spriel AB, Wright MD. Tetraspanins: molecular organisers of the leukocyte surface. Trends Immunol. 2003 Nov; 24(11):610–7. https://doi.org/10.1016/j.it.2003.09.011 PMID: 14596886

14. Yañez-Mó M, Barreiro O, Gordon-Alonso M, Sala-Valdés M, Sánchez-Madrid F. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol. 2009 Sep; 19(9):434–46. https://doi.org/10.1016/j.tcb.2009.06.004 PMID: 19708882

15. Ovalle S, Gutiérrez-López MD, Olmo N, Turnay J, Lizarbe MA, Majano P, et al. The tetraspanin CD9 inhibits the proliferation and tumorigenicity of human colon carcinoma cells. Int J Cancer. 2007 Nov 15; 121(10):2140–52. https://doi.org/10.1002/ijc.22902 PMID: 17582603

16. Leung KT, Chan KYY, Ng PC, Lau TK, Chiu WM, Tsang KS, et al. The tetraspanin CD9 regulates migration, adhesion, and homing of human cord blood CD34+ hematopoietic stem and progenitor cells. Blood. 2011 Feb 10; 117(6):1840–50. https://doi.org/10.1182/blood-2010-04-281329 PMID: 21063023

17. Brzoska E, Kowalski K, Markowska-Zagrajek A, Kowalewska M, Archacki R, Plaskota I, et al. Sdf-1 (CXCL12) induces CD9 expression in stem cells engaged in muscle regeneration. Stem Cell Res Ther. 2015 Mar 24; 6(1):46. https://doi.org/10.1186/s13287-015-0041-1 PMID: 25890097

18. Arnaud MP, Vallée A, Robert G, Bonneau J, Leroy C, Varin-Blank N, et al. CD9, a key actor in the dissemination of lymphoblastic leukemia, modulating CXCR4-mediated migration via RAC1 signaling. Blood. 2015 Oct 8; 126(15):1802–12. https://doi.org/10.1182/blood-2015-02-628560 PMID: 26320102

19. Brand MM, Granato M, Nüsslein-Volhard C, Dahm R. Zebrafish: A practical approach. Keeping and raising zebrafish. 2002; 7:39.

20. Trikić MZ, Monk P, Roehl H, Partridge LJ. Regulation of Zebrafish Hatching by Tetraspanin cd63. Kanellopoulos J, editor. PLoS One. 2011 May 19; 6(5):e19683. https://doi.org/10.1371/journal.pone.0019683 PMID: 21625559

21. David NB, Sapède D, Saint-Etienne L, Thissé C, Thissé B, Dambly-Chaudière C, et al. Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proc Natl Acad Sci U S A. 2002; 99(25):16297–302. https://doi.org/10.1073/pnas.252339399 PMID: 12444253

22. Thissé C, Thissé B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc. 2008 Jan 20; 3(1):59–69. https://doi.org/10.1038/nprot.2007.514 PMID: 18193022

23. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004 Mar 8; 32(5):1792–7. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

24. Knobeloch K-P, Wright MD, Ochsenbein AF, Liesenfeld O, Lohier J, Zinkernagel RM, et al. Targeted Inactivation of the Tetraspanin CD37 Impairs T-Cell-Dependent B-Cell Response under Suboptimal Costimulatory Conditions. Mol Cell Biol. 2000; 20(15):5363–9. https://doi.org/10.1128/MCB.20.15.5363-5369.2000 PMID: 10891477

25. Takeda Y, Tachibana I, Miyako K, Kobayashi M, Miyazaki T, Funakoshi T, et al. Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes. J Cell Biol. 2003 Jun 9; 161(5):945–56. https://doi.org/10.1083/jcb.200212031 PMID: 12796480

26. Takeda Y, He P, Tachibana I, Zhou B, Miyako K, Kaneko H, et al. Double Deficiency of Tetraspanins CD9 and CD81 Alters Cell Motility and Protease Production of Macrophages and Causes Chronic Obstructive Pulmonary Disease-like Phenotype in Mice. J Biol Chem. 2008 Sep; 283(38):26089–97. https://doi.org/10.1074/jbc.M801902200 PMID: 18662991

27. Wright MD, Geary SM, Fitter S, Gregory W, Lau L, Sheng K, et al. Characterization of Mice Lacking the Tetraspanin Superfamily Member CD151 Characterization of Mice Lacking the Tetraspanin...
Superfamily Member CD151. Mol Cell Biol. 2004; 24(13):5978–88. https://doi.org/10.1128/MCB.24.13.5978-5988.2004 PMID: 15199151

28. Hemler ME. Tetraspanin Proteins Mediate Cellular Penetration, Invasion, and Fusion Events and Define a Novel Type of Membrane Microdomain. Annu Rev Cell Dev Biol. 2003; 19(1):397–422.

29. Hemler ME. Targeting of tetraspanin proteins—potential benefits and strategies. Nat Rev Drug Discov. 2008 Sep; 7(9):747–58. https://doi.org/10.1038/nrd2659 PMID: 18758472

30. Hassuna N, Monk PN, Moseley GW, Partridge LJ. Strategies for Targeting Tetraspanin Proteins. BioDrugs. 2009 Dec; 23(6):341–59. https://doi.org/10.2165/11315650-00000000-00000 PMID: 19894777

31. Gartlan KH, Belz GT, Tarrant JM, Minigo G, Katsara M, Sheng K-C, et al. A Complementary Role for the Tetraspanins CD37 and Tscs6 in Cellular Immunity. J Immunol. 2010 Sep 15; 185(6):3158–66. https://doi.org/10.4049/jimmunol.0902867 PMID: 20709950

32. Meyer A, Van de Peer Y. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). BioEssays. 2005 Sep; 27(9):937–45. https://doi.org/10.1002/bies.20293 PMID: 16108068

33. Brunet FG, Crollius HR, Paris M, Aury J-M, Gibert P, Jaillon O, et al. Gene Loss and Evolutionary Rates Following Whole-Genome Duplication in Teleost Fishes. Mol Biol Evol. 2006 Sep 1; 23(9):1808–16. https://doi.org/10.1093/molbev/msl049 PMID: 16809621

34. García-España A, Chung P-J, Sarkar IN, Stiner E, Sun T-T, DeSalle R. Appearance of new tetraspanin genes during vertebrate evolution. Genomics. 2008 Apr; 91(4):326–34. https://doi.org/10.1016/j.ygeno.2007.12.005 PMID: 18291621

35. Huang S, Tian H, Chen Z, Yu T, Xu A. The evolution of vertebrate tetraspanins: gene loss, retention, and massive positive selection after whole genome duplications. BMC Evol Biol. 2010; 10(1):306.

36. Howe DG, Bradford YM, Conlin T, Eagle AE, Fashena D, Frazer K, et al. ZFIN, the Zebrafish Model Organism Database: increased support for mutants and transgenics. Nucleic Acids Res. 2012 Oct 15; 41(D1):D854–60. https://doi.org/10.1093/nar/gks938 PMID: 23074187

37. El-Brolosy MA, Kontarakis Z, Rossi A, Kuenne C, Günter S, Fukuda N, et al. Genetic compensation triggered by mutant mRNA degradation Europe PMC Funders Group. Nature. 2019; 569(7751):193–7. https://doi.org/10.1038/s41586-019-1064-z PMID: 30944477

38. Huang S, Yuan S, Dong M, Yu C, Shen Y, et al. The phylogenetic analysis of tetraspanins projects the evolution of cell–cell interactions from unicellular to multicellular organisms. Genomics. 2005 Dec; 86(6):674–84. https://doi.org/10.1016/j.ygeno.2005.08.004 PMID: 16242907

39. Kaji K, Oda S, Miyazaki S, Kudo A. Infertility of CD9-Deficient Mouse Eggs Is Reversed by Mouse CD9, Human CD9, or Mouse CD81; Polyadenylated mRNA Injection Developed for Molecular Analysis of Sperm–Egg Fusion. Dev Biol. 2002 Jul; 247(2):327–34. https://doi.org/10.1006/dbio.2002.0694 PMID: 12086470

40. Suárez H, Andreu Z, Mazzeo C, Toribio V, Pérez-Rivera AE, López-Martin S, et al. CD9 inhibition reveals a functional connection of extracellular vesicle secretion with mitophagy in melanoma cells. J Extracell Vesicles. 2021 May 12; (7). https://doi.org/10.1002/jexv.12082 PMID: 34012515

41. Lin Q, Low LWL, Lau A, Chua EWL, Matsuoka Y, Lian Y, et al. Tracking genome-editing and associated molecular perturbations by SWATH mass spectrometry. Sci Rep. 2019 Dec 23; 9(1):15240. https://doi.org/10.1038/s41598-019-1064-z PMID: 31645615

42. Minina S, Reichman-Fried M, Raz E. Control of Receptor Internalization, Signaling Level, and Precise Arrival at the Target in Guided Cell Migration. Curr Biol. 2007 Jul; 17(13):1164–72. https://doi.org/10.1016/j.cub.2007.05.073 PMID: 17600713

43. Matsuda M, Nogare DD, Somers K, Martin K, Wang C, Chitnis AB. Lef1 regulates Dusp6 to influence neuronast formation and spacing in the zebrafish posterior lateral line primordium. Development. 2013; 140(11):2387–97. https://doi.org/10.1242/dev.091348 PMID: 23637337

44. Valdivia LE, Young RM, Hawkins TA, Stickney HL, Cavodeassi F, Schwarz Q, et al. Lef1-dependent Wnt/-catenin signalling drives the proliferative engine that maintains tissue homeostasis during lateral line development. Development. 2011; 138(18):3931–41. https://doi.org/10.1242/dev.062695 PMID: 21862557

45. Yauch RL, Hemler ME. Specific interactions among transmembrane 4 superfamily protein (TM4SF) proteins and phosphoinositide 4-kinase. Biochem J. 2000; 351 Pt 3:629–37. PMID: 11042117

46. Thomas ED, Cruz IA, Hailey DW, Raible DW. There and back again: development and regeneration of the zebrafish lateral line system. Wiley Interdiscip Rev Dev Biol. 2015 Jan; 4(1):1–16. https://doi.org/10.1002/wdev.160 PMID: 25330982

47. Dalle Nogare D, Chitnis AB. A framework for understanding morphogenesis and migration of the zebrafish posterior Lateral Line primordium. Mech Dev. 2017; 148:69–78. https://doi.org/10.1016/j.mod.2017.04.005 PMID: 28460893
48. Lee JH, Bae JA, Lee JH, Seo Y-W, Kho DH, Sun EG, et al. Glycoprotein 90K, downregulated in advanced colorectal cancer tissues, interacts with CD9/CD82 and suppresses the Wnt/beta-catenin signal via ISGylation of beta-catenin. Gut. 2010 Jul 1; 59(7):907–17. https://doi.org/10.1136/gut.2009.194068 PMID: 20581239

49. Huang C, Liu D, Masuya D, Kameyama K, Nakashima T, Yokomise H, et al. MRP-1/CD9 gene transduction downregulates Wnt signal pathways. Oncogene. 2004 Sep 30; 23(45):7475–83. https://doi.org/10.1038/sj.onc.1208063 PMID: 15334057

50. Colak-Champollion T, Lan L, Jadhav AR, Yamaguchi N, Venkiteswaran G, Patel H, et al. Cadherin-Mediated Cell Coupling Coordinates Chemokine Sensing across Collectively Migrating Cells. Curr Biol. 2019 Aug; 29(15):2570–2579.e7. https://doi.org/10.1016/j.cub.2019.06.061 PMID: 31386838

51. Johnson JL, Winterwood N, DeMali KA, Stipp CS. Tetraspanin CD151 regulates RhoA activation and the dynamic stability of carcinoma cell-cell contacts. J Cell Sci. 2009 Jul 1; 122(13):2263–73. https://doi.org/10.1242/jcs.045997 PMID: 19509057

52. Mohan A, Nalini V, Mallikarjuna K, Jyotirmay B, Krishnakumar S. Expression of motility-related protein MRP1/CD9, N-cadherin, E-cadherin, a-catenin and b-catenin in retinoblastoma. Exp Eye Res. 2007; 84 (4):781–9. https://doi.org/10.1016/j.exer.2006.06.014 PMID: 17316610

53. Jiang X, Teng M, Ji R, Zhang D, Zhang Z, Lv Y, et al. CD9 regulates keratinocyte differentiation and motility by recruiting E-cadherin to the plasma membrane and activating the PI3K/Akt pathway. Biochim Biophys Acta—Mol Cell Res. 2020;1867(2):118574.

54. Dalle Nogare D, Chitnis AB. NetLogo agent-based models as tools for understanding the self-organization of cell fate, morphogenesis and collective migration of the zebrafish posterior Lateral Line primordium. Semin Cell Dev Biol. 2020; 100(December 2019):186–98. https://doi.org/10.1016/j.semcdb.2019.12.015 PMID: 31901312