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Expression of two uncharacterized protein coding genes in zebrafish lateral line system

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ABSTRACT The lateral line system is a mechanosensory organ of fish and amphibians that detects changes in water flow and is formed by the coordinated action of many signalling pathways. These signalling pathways can easily be targeted in zebrafish using pharmacological inhibitors to decipher their role in lateral line system development at cellular and molecular level. We have identified two uncharacterized proteins, whose mRNA are expressed in the lateral line system of zebrafish. One of these proteins, uncharacterized protein LOC564095 precursor, is conserved across vertebrates and its mRNA is expressed in posterior lateral line primordium (pLLP). The other uncharacterized protein, LOC100536887, is present only in the teleost fishes and its mRNA is expressed in neuromasts. We show that inhibition of retinoic acid (RA) signalling reduces the expression of both of these uncharacterized genes. It is reported that inhibition of RA signalling during gastrulation starting at 7 hours post fertilization (hpf) abrogates pLLP formation, and inhibition of RA signalling at 10 hpf delays the initiation of pLLP migration. Here, we show that inhibition of RA signalling before and during segmentation (9-16 hpf) results in delayed initiation and reduced speed of pLLP migration, as well as inhibition of posterior neuromasts formation.

KEY WORDS: *Lateral line system, posterior lateral line primordium, neuromasts, uncharacterized proteins*

Introduction

The lateral line is a mechanosensory system that is present in fishes and most amphibians in order to detect the pattern of water movement and to spot the location of prey and predators (Coombs and Van Netten, 2005). Its functional units are neuromasts (Kornblum *et al.*, 1990; Bleckmann and Zelick, 2009). The lateral line system in zebrafish is divided into two parts: the anterior lateral line (aLL), present around the head; and the posterior lateral line (pLL), which runs in the trunk and the tail. These are derived from anterior and posterior lateral line placodes, respectively (Andermann *et al.*, 2002; Ghysen and Dambly-Chaudiere, 2004). The pLL is generated by the deposition of neuromasts by a migrating primordium (pLLP) during development. The migratory pLLP consists of around 140 cells, which emerge behind the otic placode at around 20-22 hpf and begin to migrate towards the tip of the tail, which is completed by 42 hpf (Gompel *et al.*, 2001; Dalle Nogare and Chitnis, 2017). During this migration, they deposit cells as proto-neuromasts at regular intervals throughout the horizontal myoseptum to generate 5 neuromasts (L1-L5) in the trunk region and 2-3 terminal neuromasts at the tip of the tail (Gompel *et al.*, 2001). These neuromasts undergo a process of morphogenesis, become epithelized and mature into rosettes with sensory hairs at their center (Lecaudey *et al.*, 2008; Hava *et al.*, 2009). Approximately 20 cells of the original placode remain to form neurons of lateral line ganglion which later follow migratory path of pLLP and establish connections with sensory hair cells (Metcalfe, 1985; Chitnis *et al.*, 2012). The migratory pLLP has a dynamic structure that varies from the leading end to the trailing end. Leading cells are mesenchymal and flatter in appearance, while cells at the trailing end are epithelial and have columnar morphology called proto-neuromasts, which later develop into rosettes (Lecaudey *et al.*, 2008). More cells are added to the leading end of migratory pLLP by division of existing cells that in turn shifts the newly formed neuromasts more towards the trailing end (Nechiporuk and Raible, 2008; Chitnis *et al.*, 2012).

Abbreviations used in this paper: aLL, anterior lateral line; DEAB, N,N-diethylaminobenzaldehyde; DMSO, dimethyl sulfoxide; pLL, posterior lateral line; pLLP, posterior lateral line primordium; RA, Retinoic acid.

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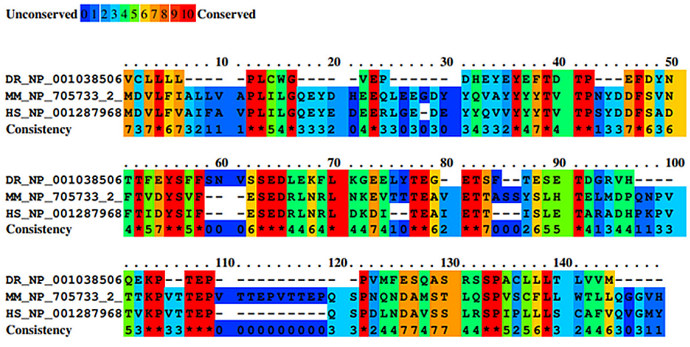
Several signalling pathways regulate the process of lateral line placode formation, primordium migration, proto-neuromast formation and maturation in zebrafish. Wnt, Fgf and Bmp signalling control the expansion of posterior lateral line placode (Nikaido *et al.*, 2017). Wnt-dependent Fgf signalling is responsible for proto-neuromast formation (Aman and Piotrowski, 2008). Fgf signalling also triggers proto-neuromast maturation, formation of sensory hair cells within the neuromasts, and induction of anterior lateral line placode (Nikaido *et al.*, 2017; Lecaudey *et al.*, 2008; Nechiporuk and Raible, 2008). Chemokine signals such as chemokine receptors (cxcr4 and cxcr7) and their ligand sdf1a regulate pLLP migration throughout the horizontal myoseptum (David *et al.*, 2002; Haas and Gilmour, 2006; Dambly-Chaudière *et al.*, 2007). Notch signalling regulates the development of sensory hair cell progenitors present in the neuromasts (Matsuda and Chitnis, 2010). Knockdown of *tgfβ1a* reduced neuromast numbers with increased inter-neuromast distance, thus highlighting the role of TGFβ signalling in pLL development (Xing *et al.*, 2015). It is reported that the RA signalling pathway regulates posterior lateral line placode formation during late gastrulation stage from 8 to 10 hpf (Sarrazin *et al.*, 2010). Inhibition of RA synthesis from 10 to 24 hpf delays initiation of pLLP migration (Nikaido *et al.*, 2017).

Whole-genome sequencing along with advancements of bioinformatics tools have identified many predicted open reading frames (ORFs) (Brent, 2005). When the proteins encoded by these ORF do not show significant homology with the proteins of known function, they are called uncharacterized, unknown, hypothetical, or predicted proteins (henceforth, uncharacterized) (Ijaq *et al.*, 2015). These uncharacterized genes comprise a significant fraction of the genome of most organisms. However, assigning functions to these predicted ORFs is challenging (Hawkins and Kihara, 2007; Ijaq *et al.*, 2015). Here, we have identified two uncharacterized proteins, and show that the mRNA of "uncharacterized protein LOC564095 precursor" and "uncharacterized protein LOC100536887" are expressed in zebrafish lateral line system. We show that RA signalling regulates their expression, and hypothesize that the effect of RA on pLLP migration and neuromast development in zebrafish embryos may be partly regulated through these proteins.

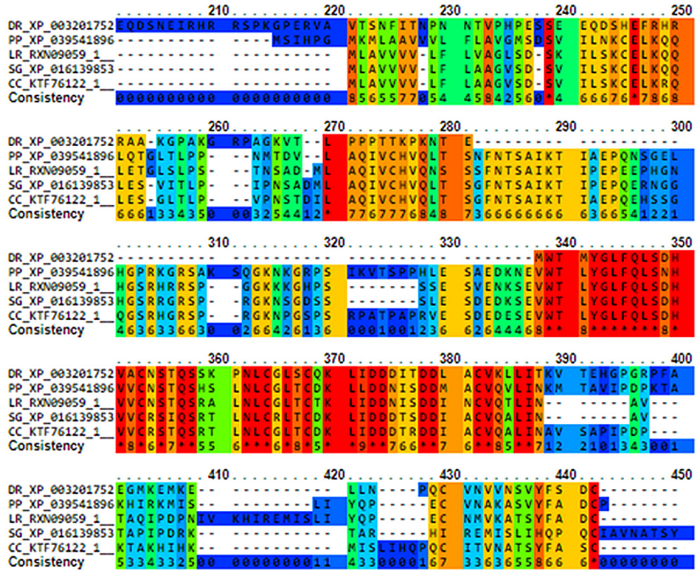
A



B

	DR NP_001038506.1	MM NP_705733.2	HS NP_001287968.1
DR NP_001038506.1	100.00	32.35	35.71
MM NP_705733.2	32.35	100.00	63.85
HS NP_001287968.1	35.71	63.85	100.00

C



D

	DR XP_003201752.1	PP XP_039541896.1	LR RXN09059.1	SG XP_016139853.1	CC KTF76122.1
DR XP_003201752.1	100.00	54.90	52.11	53.16	51.53
PP XP_039541896.1	54.90	100.00	71.73	72.25	71.72
LR RXN09059.1	52.11	71.73	100.00	76.04	73.44
SG XP_016139853.1	53.16	72.25	76.04	100.00	85.42
CC KTF76122.1	51.53	71.72	73.44	85.42	100.00

Fig. 1. Homologs of uncharacterized protein LOC564095 precursor and uncharacterized protein LOC100536887 in other species. (A,B) *The DUF4634 domain was taken for BLASTP search to identify the proteins containing this domain in other species. Multiple sequence alignment and percentage identity matrix of DUF4634 domain present in uncharacterized protein LOC564095 precursor (NP_001038506.1) of zebrafish with the domain from homologous proteins in human (NP_001287968.1) and mice (NP_705733.2).* **(C,D)** *Multiple sequence alignment and percentage identity matrix of uncharacterized protein LOC100536887 (XP_003201752.1) with its homologs in other species. LOC100536887 homologs are present only in the class Teleostei. Sequences were aligned using PRALINE, percentage identity matrix were generated using MUSCLE. DR,* Danio rerio*; MM,* Mus musculus*; HS,* Homo sapiens*; PP,* Pimephales promelas*; LR,* Labeo rohita*; SG,* Sinocyclocheilus graham*; CC,* Cyprinus carpio.

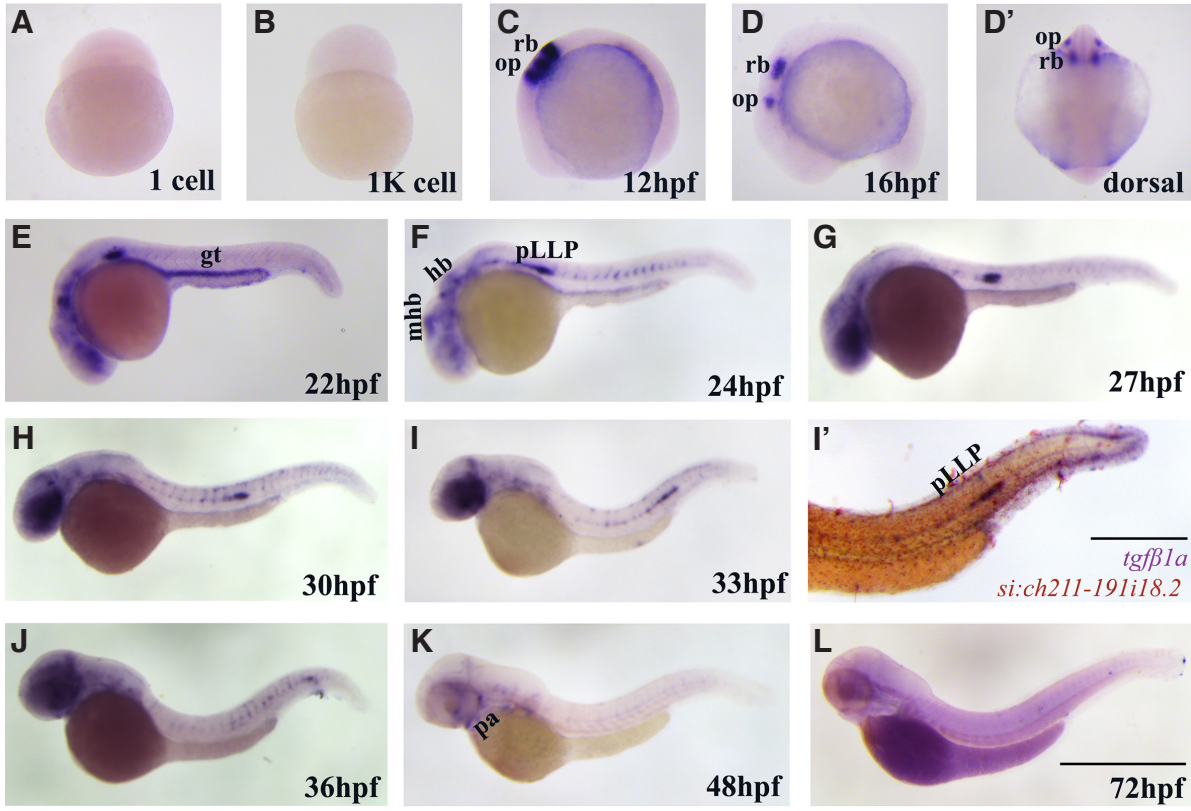


Fig. 2. Expression pattern of *si:ch211-191i18.2* mRNA at different developmental stages of zebrafish. (A-B) *1 cell and 1K cell stage embryos do not show any detectable expression.* **(C,D,D')** *12 and 16 hpf embryos showing expression in otic placode (op) and rhombomeres (rb).* **(E-H)** *22 hpf, 24 hpf, 27 hpf and 30 hpf embryos showing expression in gut (gt), midbrain-hindbrain boundary (mhb), hindbrain (hb) and migratory posterior lateral line primordium (pLLP).* **(I)** si:ch211-191i18.2 *expression at 33 hpf* **(I')** *Co-expression of* si:ch211-191i18.2 *(magenta) and* tgfβ1a *(purple) in pLLP at 33 hpf* **(J)** *36 hpf embryo showing expression in migrating pLLP* **(K)** *48 hpf embryos showing expression in pharyngeal arches (pa).* **(L)** *Expression is not detectable in 72 hpf embryos. Scale bar 500 μm for all images except I' for which the scale bar is 250 μm.*

Results

Uncharacterized protein LOC564095 precursor is conserved and uncharacterized protein LOC100536887 is a lineage-specific protein

Zebrafish uncharacterized protein LOC564095 precursor (NP_001038506.1) is a 124 amino acid long protein with a DUF4634 (Domain of Unknown Function 4634) domain. The DUF4634 domain comprises 83-98 percent of the total protein sequence where it is present. Multiple sequence analysis of DUF4634 domain, present in uncharacterized protein LOC564095 precursor with its homolog in mice (NP_705733.2) and humans (NP_001287968.1), showed 32.35% and 35.71% sequence identity respectively (Fig. 1 A,B). The identity between mice and humans was 63.85%.

Zebrafish uncharacterized protein LOC100536887 (XP_003201752.1) is a 367 amino acid long protein and has a DUF4106 domain, a lysozyme-like domain and a calcium-binding site. The BLAST analysis of this protein revealed that it is conserved among bony fishes belonging to the class Teleostei, suggesting that it is a lineage-specific protein (Fig. 1 C,D) (Lespinet, 2002). The DUF4106 domain is also present in Hypothetical Protein TVAG_358080 of protozoan parasite *Trichomonas vaginalis*, which does not contain the lysozyme-like domain, and calcium-binding site present in uncharacterized protein LOC100536887.

The mRNA of uncharacterized protein LOC564095 precursor is expressed in pLLP

Zebrafish uncharacterized protein LOC564095 precursor (NP_001038506.1) is encoded by *si:ch211-191i18.2* gene. It has two predicted transcripts, ENSDART00000150919.3 and ENSDART00000151034.2. We carried out whole-mount *in situ* hybridization (WISH) at different developmental stages of zebrafish to find out the temporal and spatial expression pattern of *si:ch211-191i18.2* mRNA. The antisense probe was designed to recognize both the splice variants. Its mRNA expression was not visible in the early embryonic stages (Fig. 2 A,B). At 12 and 16 hpf, mRNA expression was seen in the otic placode and rhombomeres (Fig. 2 C,D,D'). In 22 hpf embryos, expression was observed in the hindbrain, midbrain-hindbrain boundary, gut, and migratory pLLP, and was also seen in the later stages of development (Fig. 2E). The expression domain of *si:ch211-191i18.2* gene in migratory primordium continued to move behind the otic placode towards the tip of the tail as the development progressed (Fig. 2 E-J). Double *in situ* hybridization with *tgfβ1a* (a lateral line primordium marker) (Xing *et al.*, 2015) confirmed *si:ch211-191i18.2* expression in the pLLP (Fig. 2I'). In 48 hpf embryos, expression was observed in the pharyngeal arches (Fig. 2K), but no detectable expression was observed in the 72 hpf embryos (Fig. 2L).

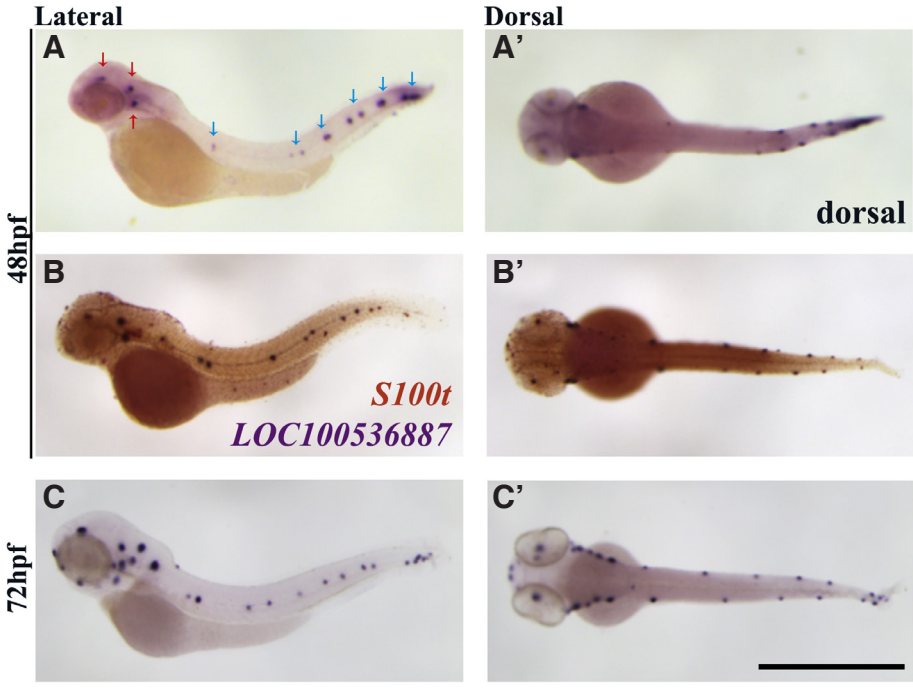


Fig. 3. Expression pattern of *LOC100536887* at different developmental stages of zebrafish embryos. (A,A') *48 hpf embryos showing expression in anterior (red arrow) and posterior (blue arrow) neuromasts.* **(B,B')** *Co-expression of* LOC100536887 *(purple) and* S100t *(magenta) in neuromasts at 48 hpf embryo.* **(C,C')** *72 hpf embryos showing expression in anterior and posterior neuromasts. Scale bar 500 μm.*

The mRNA of uncharacterized protein LOC100536887 is expressed in lateral line neuromasts

Uncharacterized protein LOC100536887 (XP_003201752.1) is encoded by *LOC100536887* gene. It has one known transcript XM_003201704.5. An antisense probe was designed to check the expression pattern of *LOC100536887* gene at different developmental stages of zebrafish embryos. Its expression was not detectable in embryos from 1 cell to 36 hpf (data not shown). At 48 hpf, its expression was observed both in anterior and posterior lateral line neuromasts, and was also seen in 72 hpf embryos (Fig. 3A and C). Double *in situ* hybridization with *S100t* (a neuromasts marker) (Kraemer *et al.*, 2008) confirmed the location of *LOC100536887* in neuromasts (Fig. 3B).

Inhibition of Retinoic acid signalling reduces the expression of si:ch211-191i18.2 *and* LOC100536887

RA signalling is required during late gastrulation for posterior lateral line development (Sarrazin *et al.*, 2010; Nikaido *et al.*, 2017). We checked whether RA has any effect on *si:ch211-191i18.2* (codes uncharacterized protein LOC564095 precursor) and *LOC100536887* (codes uncharacterized protein LOC100536887) expression. We treated the embryos with either 10μM N, N-diethylaminobenzaldehyde (DEAB), an inhibitor of retinaldehyde dehydrogenase; 1μM citral (that inhibits the oxidation of retinol to RA); or vehicle control dimethyl sulfoxide (DMSO). Early inhibition of RA from 9 to 16 hpf significantly reduced the expression of *si:ch211-191i18.2* gene at 24 and 36 hpf (Fig. 4A). It is a possible that the decreased expression of *si:ch211-191i18.2* may be due to delayed development of lateral line system upon RA inhibition. To rule out this possibility, we treated the embryos with DEAB or citral from 24 hpf at which point the primordium migration has already started. The expression of *si:ch211-191i18.2* was checked at 36 hpf after treating the embryos with RA inhibitors from 24 to 36 hpf. The RT PCR analysis showed that with 20μM DEAB and 2μM citral, there was a significant reduction of *si:ch211-191i18.2* expression at 36 hpf (Fig. 4B).

As *LOC100536887* gene is expressed 48 hpf onwards (Fig. 3), its expression upon RA signalling inhibition was checked at 48 hpf and 72 hpf. Early inhibition of RA between 9 to 16 hpf reduced the expression of *LOC100536887* in *D*EAB or citral treated embryos (Fig. 4C). The expression of *LOC100536887* was checked further by treating the embryos with 20μM DEAB and 2μM citral from 24 to 48 hpf. The reduced expression of *LOC100536887* transcript *w*as found at 48 hpf upon RA signalling inhibition (Fig. 4D).

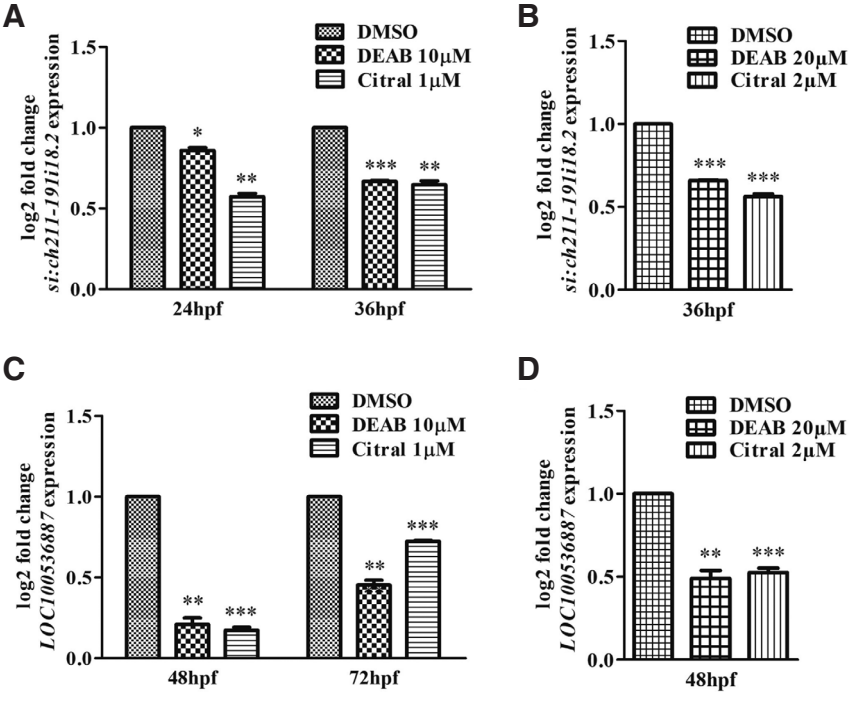


Fig. 4. Effect of DEAB and citral on *si:ch211-191i18.2* and *LOC100536887* gene expression. *RT-PCR analysis of* si:ch211-191i18.2 *transcript of DEAB, citral or DMSO treated embryos* **(A)** *at 24 and 36 hpf, drug treatment time 9 to 16 hpf* **(B)** *at 36 hpf, drug treatment time 24 to 36 hpf. RT-PCR analysis of* LOC100536887 *transcript of DEAB, citral or DMSO treated embryos* **(C)** *at 48 and 72 hpf, drug treatment time 9 to 16 hpf* **(D)** *at 48 hpf, drug treatment time 24 to 48 hpf. n=4, mean ±SEM, paired t test, two tailed; *p-value <0.05, **p-value <0.01 and ***p-value <0.001.*

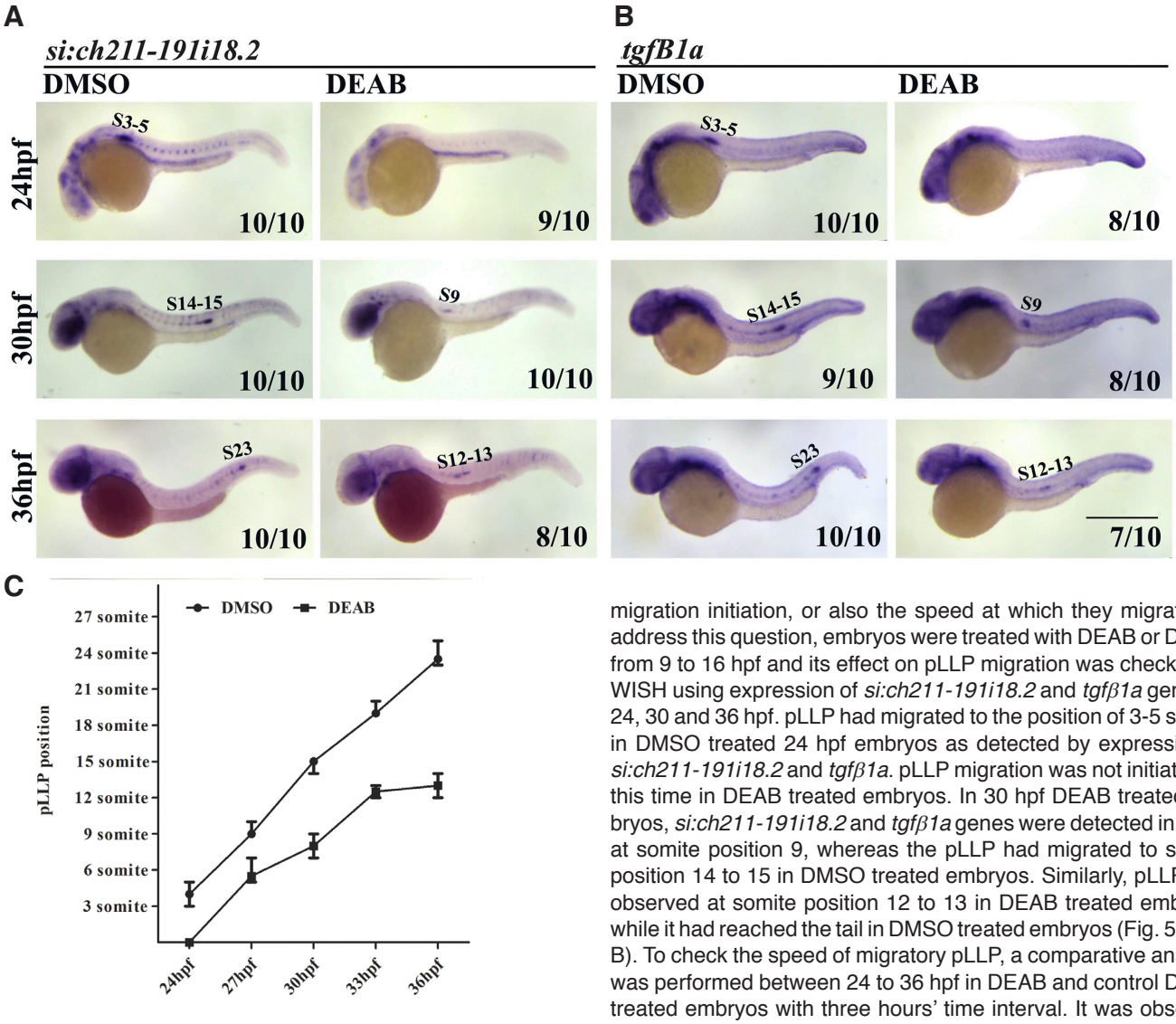


Fig. 5. Effect of DEAB on lateral line primordium migration. Initiation of pLLP migration was delayed and the speed of migration was slowed-down in DEAB treated embryos compared with DMSO treated control embryos. *Observations were done at 24 hpf, 30 hpf and 36 hpf using* **(A)** si:ch211-191i18.2 *and* **(B)** tgfβ1a *transcripts as pLLP markers. "S" represents the somite position where pLLP had reached. Scale bar 500 μm. Ratios at right corner of each image indicate number of embryos showing the pattern of expression.* **(C)** *The speed of pLLP migration as measured by its position with respect to somite number at mentioned time points (hpf) in DEAB and DMSO treated embryos between 24 to 36 hpf (n=6).*

Inhibition of retinoic acid signalling results in delayed initiation and reduced speed of pLLP migration

It is reported that inhibition of RA signalling by DEAB treatment from 7 to 24 hpf completely inhibits pLLP formation. DEAB treatment from 10 to 24 hpf does not alter pLLP formation but delays primordium migration, and treatment from 14 to 24 hpf had no effect on pLLP formation or migration (Nikaido *et al.*, 2017). Two uncharacterized genes reported here (*si:ch211-191i18.2* and *LOC100536887*) are expressed in pLLP and neuromast respectively. Both are down regulated upon RA signalling inhibition. We wanted to know whether inhibition of RA signalling affects only pLLP migration initiation, or also the speed at which they migrate. To address this question, embryos were treated with DEAB or DMSO from 9 to 16 hpf and its effect on pLLP migration was checked by WISH using expression of *si:ch211-191i18.2* and *tgfβ1a* genes at 24, 30 and 36 hpf. pLLP had migrated to the position of 3-5 somite in DMSO treated 24 hpf embryos as detected by expression of *si:ch211-191i18.2* and *tgfβ1a*. pLLP migration was not initiated by this time in DEAB treated embryos. In 30 hpf DEAB treated embryos, *si:ch211-191i18.2* and *tgfβ1a* genes were detected in pLLP at somite position 9, whereas the pLLP had migrated to somite position 14 to 15 in DMSO treated embryos. Similarly, pLLP was observed at somite position 12 to 13 in DEAB treated embryos, while it had reached the tail in DMSO treated embryos (Fig. 5A and B). To check the speed of migratory pLLP, a comparative analysis was performed between 24 to 36 hpf in DEAB and control DMSO treated embryos with three hours' time interval. It was observed that in DMSO treated embryos, the migration of pLLP is almost uniform, with an average speed of 1.6 somite length per hour. The pLLP migration was slow in DEAB treated embryos and moved at an average speed of 1 somite per hour. Moreover, the speed of pLLP migration in DEAB treated embryos was not uniform, as no change in pLLP position was observed between 33 to 36 hpf (Fig. 5C). These results suggest that RA inhibition not only abrogates or delays initiation of pLLP migration as reported earlier (Nikaido *et al.*, 2017), but also affects the speed of primordium migration in zebrafish embryos.

Inhibition of retinoic acid signalling affects neuromast deposition throughout the body

The primary posterior lateral line system is fully developed by 3 dpf (Nuñez *et al.*, 2009). It is reported that treatment with DEAB between 8 to 10 hpf reduced the number of total neuromasts at 4 dpf (Sarrazin *et al.*, 2010). As we observed earlier, RA signalling inhibition during late gastrulation and early segmentation stages reduced the speed of migratory primordium; we further checked how it affects the number of deposited neuromasts in late stages of development. We treated zebrafish embryos from 9-16 hpf with

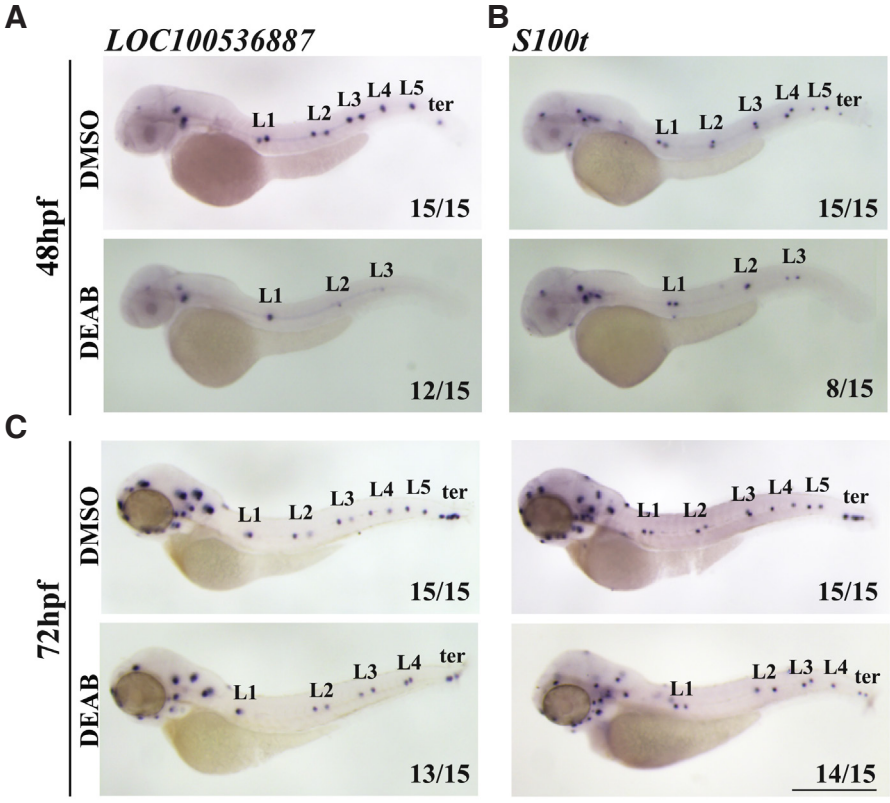


Fig. 6. Effect of DEAB on neuromast formation. (A) *DEAB treated embryos showing reduced number of neuromasts compared with DMSO treated control embryos at 48 hpf. L1-L5 posterior neuromasts and terminal (ter) neuromasts were formed in control embryos whereas only L1-L3 were formed in DEAB treated embryos. Observations were done using* LOC100536887 *and* S100t *transcripts as neuromasts markers* **(B)** *Posterior neuromasts number were reduced in DEAB treated embryos at 72 hpf. Ratios at right corner of each image indicates number of embryos showing representative pattern. Scale bar 500 μm.*

DEAB and fixed these at 48 and 72 hpf. *LOC100536887* was used along with known neuromast marker *S100t* (Kraemer *et al*., 2008) to probe the neuromast deposition upon RA signalling inhibition. We found that DEAB treated embryos showed a reduced number of neuromasts at 48 hpf. Only three neuromasts (L1-L3) were present in the trunk region in DEAB treated embryos, whereas the neuromast formation in trunk (L1-L5) and tail was normal in the vehicle (DMSO) control embryos (Fig. 6A). Four neuromasts (L1-L4), along with terminal neuromasts (ter), were observed in DEAB treated 72 hpf embryos, whereas in vehicle control there were five (L1-L5), along with terminal neuromasts (Fig. 6B).

Discussion

In this study, we report identification of two genes that are annotated as uncharacterized in non-redundant databases because these genes or their homologs in other species have no known function. The expression pattern of a gene provides valuable clues to predicting its function. We have cloned these two genes and describe their mRNA expression pattern during zebrafish embryogenesis. We show that mRNA of uncharacterized protein LOC564095 precursor is expressed in migratory pLLP, and uncharacterized protein LOC100536887 is expressed in both anterior and posterior neuromasts. Thus, these genes may be involved in the development and function of zebrafish lateral line system. We further show that inhibition of RA synthesis by DEAB or citral treatment reduces their expression, suggesting that the expression of these genes may be regulated by RA signalling pathway. It is claimed that RA plays a major role in inducing posterior placode formation and primordium migration initiation during late gastrulation (Sarrazin *et al*., 2010; Nikaido *et al*., 2017). We targeted RA signalling pathway to check its effect on speed of primordium migration and neuromasts deposition using these two uncharacterized genes along with *tgfβ1a* and *S100t* as lateral line markers. We treated the embryos with DEAB at 9 hpf before somitogenesis begins, and removed it at 16 hpf prior to primordium migration. A late initiation of primordium migration was observed in DEAB treated embryos. The migratory speed of pLLP was also affected in DEAB treated embryos, in which it was slow and irregular compared with DMSO vehicle control. The expression domain of migratory primordium was reduced in DEAB treated embryos as observed by the expression of both *si:ch211-191i18.2* and *tgfβ1a* gene (Fig. 5), which may indicate presence of smaller number of cells in the migratory primordium. There is a need to investigate whether inhibition of RA signalling reduces the number of migratory cells originating from the posterior lateral line placode. 60% of the length of migratory primordium is under the control of the Wnt signalling pathway (Dalle Nogare and Chitnis, 2017). As the primordium proliferates, the length of the leading Wnt system is reduced, gradually decreasing access of trailing end cells to Wnt dependent migratory factor. When access to this factor falls below a threshold level, the cells at the trailing end come under the influence of Fgf signalling pathway, halting migration and deposits as protoneuromasts. The rate at which primordium proliferates and the Wnt system shrinks determines the speed of migratory primordium (Aman *et al*., 2011; Valdivia *et al*., 2011; Dalle Nogare and Chitnis, 2017). We hypothesize that the reduced speed of pLLP migration in DEAB treated embryos could be a low rate of proliferation. The proliferation of migratory primordium regulate the process of neuromast deposition (Nechiporuk and Raible, 2008). The reduced number of neuromasts seen at 48 hpf in DEAB treated embryos may also because of reduced cell proliferation, a hypothesis that requires further investigation.

Materials and Methods

Bioinformatics and sequence analysis

The NCBI's Conserved Domain Database was used to identify the domains present in uncharacterized protein LOC564095 precursor and uncharacterized protein LOC100536887. NCBI protein-BLAST was used to identify the homologous proteins present in other vertebrates. The PRA-

LINE was used to analyse conserved amino acid residues. PRALINE uses homology-extended multiple alignment to enrich the information of each sequence present in the set. The output is visualized by means of different colour schemes to provide information about the position conservation of each residue (Simossis and Heringa, 2005). The MUSCLE multiple sequence alignment server, which is known for its speed and accuracy, was used to generate percentage identity matrix (Edgar, 2004).

Zebrafish maintenance

Tübingen, Albino and AB strains were used in all experiments. The animal work was approved by the Institutional Animal Ethics Committee. Embryos were raised in E3 media at 28.5°C. Embryos were treated with 0.003% of 1-Phenyl 2 thiourea (PTU) in E3 media at 20 hpf to inhibit pigmentation and fixed at different developmental stages in 4% paraformaldehyde (PFA).

Gene cloning

Zebrafish *si:ch211-191i18.2*, *LOC100536887, tgfβ1a* and *S100t* genes were amplified by PCR using cDNA prepared from 24 hpf or 48 hpf embryos. Total RNA was extracted using TRIzol reagent and cDNA was prepared using SuperScript® III (Thermo Fisher Scientific). The primers used for amplifying the genes were designed using http://primer3.ut.ee/ and are as follows: (*si:ch211-191i18.2* forward 5'-AGCAGATGGACAGATAACCTGAA-3' and reverse 5'-CTGAATTCATACCAGCTATCGTG-3'; *LOC100536887* forward 5'-ACCCTCTCAGGGGAACAGAT-3' and reverse 5'-CTGAGCTGGAACAAGCCGTA-3'; *tgfβ1a* forward 5'-CCGAGATGAAGCGCAGTATTC-3' and reverse 5'-GTCAAGGATTGCGGGTACACA-3'; and *S100t* forward 5'-CCAGCAGTCATCTCACCTCG-3' and reverse 5'- AGACCATCGTACAAACTCACATCT-3'). Amplified products were cloned in PCR-Blunt II-TOPO vector (Invitrogen) and verified by sequencing.

WISH Probe synthesis

The above clones were used for probes synthesis. *si:ch211-191i18.2*, *LOC10053688*, *tgfβ1a* and *S100t* plasmids were linearized with *Xho*I and transcribed by SP6 RNA polymerase to synthesize anti-sense probe. Digoxygenin labelling mix (Roche 11277073910) or Fluorescein labelling mix (Roche 11685619910) were used to synthesize labelled RNA probe.

Whole mount* in situ *hybridization

WISH was performed on zebrafish embryos as described earlier (Thisse and Thisse, 2008). Briefly, embryos fixed in 4 % PFA were dehydrated through a methanol series and stored at -20°C in 100% methanol. The embryos were rehydrated in PBST (1X PBS with 0.1% Tween-20), permeabilized by proteinase K treatment (10μg/ml in 1XPBST) and refixed with 4% PFA. These were kept in hybridization buffer (50% deionized formamide, 5X SSC, 50μg/ml heparin, 0.5mg/ml torula RNA, 9.2mM citric acid and 0.1% Tween-20) for 2-5 hours at 65°C. Later, the buffer was exchanged with hybridization buffer containing the probe. The next day, embryos were washed with formamide-SSC buffer and transferred into MABT (1X MAB with 0.1% Tween-20) buffer. Embryos were kept in 10% FBS (foetal bovine serum) and 2 % blocking reagent (Roche 1109617600) in MABT for 2 hours at room temperature (RT) for blocking and then incubated with anti-digoxigenin F_{AB} fragment coupled to alkaline phosphate (AP) (Roche 11093274910) or anti-fluorescein F_{AB} fragment coupled to AP (Roche 11426338910) diluted in blocking buffer for overnight at 4°C. On the 3rd day, embryos were washed 8 times with MABT, twice with pH9 buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2 and 0.1% Tween-20) at RT and transferred into BM purple (Roche 11442074001) in dark until colour developed.

Double* in situ *hybridization

For Double *in situ* hybridization, embryos were incubated in hybridization buffer containing both digoxygenin and fluorescein labelled probes targeting two different mRNA. On the 4th or 5th day after the detection of first transcript with BM purple, embryos were washed with PBST followed by AP inactivation by treating them with methanol. Later, they were incubated in blocking buffer containing diluted antibody corresponding to the second probe. The next day, embryos were washed with MABT followed by pH 9 buffer and incubated in dark with INT-BCIP (Roche 11681460001) for the development of second colour. The stained embryos were washed with PBST and transferred into 100% glycerol for imaging. Images were taken using Leica MZ16 stereo microscope.

Drug treatment

10mM stock solution of DEAB was prepared in DMSO, which was diluted in E3 media to a working concentration of 10-20μM. 1mM stock solution of citral was prepared and diluted in E3 media to a working concentration of 1-2μM. Embryos were treated with DEAB, citral or DMSO (vehicle control) in dark at 28.5°C. Embryos were washed three times with E3 media after treatment, and fixed at different stages of development as mentioned in results section.

RT PCR

Total RNA was extracted from pools of 10 embryos at different developmental stages of DMSO, DEAB and citral treated embryos using Direct-zol RNA miniprep kit (Zymo research). cDNA was synthesized using superscript IV (Invitrogen). The following reaction condition was set: 95°C, 5min; (95°C, 15sec; 60°C, 30sec, 72°C, 30sec) X 40 cycles; 95°C, 15min; 60°C, 1min; 95°C, 15min. Analysis was carried out in quadruplicates using *zEF1α* for normalization. For *si:ch211-191i18.2* 5'-TGCAGTCATTCAGGGGCTCA-3' forward and 5'-CACACTGATATCATCACACA-3' reverse, for *LOC100536887* 5'- GTGTGAGTAATAGTGCCACTG-3' forward and 5'- CCAAAATGTCAGTCGCATTC-3' reverse and for *zEf1a* 5'-ATCACCAAGGAAGTCAGCG-3' forward and 5'-ATCTTCCATCCCTTGAACCAG-3' reverse primers were used.

Author contribution

RKS conceived, supervised and provided resources for the project. SF carried out experiments and wrote the manuscript. RK and AD carried out bioinformatics analysis that identified uncharacterized proteins, including two reported in this manuscript. All authors read and approved the manuscript.

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