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Dpysl2 (CRMP2) is required for the migration of facial branchiomotor neurons in the developing zebrafish embryo

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ABSTRACT Dihydropyrimidinase-like family proteins (Dpysls) are relevant in several processes during nervous system development; among others, they are involved in axonal growth and cell migration. Dpysl2 (CRMP2) is the most studied member of this family; however, its role *in vivo* is still being investigated. Our previous studies in zebrafish showed the requirement of Dpysl2 for the proper positioning of caudal primary motor neurons and Rohon-Beard neurons in the spinal cord. In the present study, we show that Dpysl2 is necessary for the proper migration of facial branchiomotor neurons during early development in zebrafish. We generated a *dpysl2* knock-out (KO) zebrafish mutant line and used different types of antisense morpholino oligonucleotides (AMO) to analyze the role of Dpysl2 in this process. Both *dpysl2* KO mutants and morphants exhibited abnormalities in the migration of these neurons from rhombomers (r) 4 and 5 to 6 and 7. The facial branchiomotor neurons that were expected to be at r6 were still located at r4 and r5 hours after the migration process should have been completed. In addition, mutant phenotypes were rescued by injecting *dpysl2* mRNA into the KO embryos. These results indicate that Dpysl2 is involved in the proper migration of facial branchiomotor neurons in developing zebrafish embryos.

KEY WORDS: *facial branchiomotor neuron, cell migration, Dpysl2*

Introduction

Dihydropyrimidinase-like family proteins (Dpysls), also known as Collapsin response mediator proteins (CRMPs), are evolutionarily conserved cytosolic phosphoproteins expressed in several regions throughout the central and peripheral nervous systems during development (Wang and Strittmatter, 1996). They are known for being involved in axon growth, guidance and regeneration, neuronal polarity, apoptosis and cell migration in the nervous system (Schweitzer *et al.,* 2005).

The most studied Dpysl family member is Dpysl2 (CRMP2), a protein that was originally identified as a mediator of semaphorin signaling and known for being involved in the collapse of the axon growth cones (Goshima *et al.,* 1995). The features of zebrafish such as the transparency of the embryos and their rapid development (Kimmel *et al.,* 1995) together with the high homology with the human DPYSL2 (Schweitzer *et al.,* 2005) make it suitable to study this gene's functions. In zebrafish, Dpysl2 is clearly expressed in different regions of the central nervous system throughout the development: by 16 hours post fertilization (hpf) its expression can be observed in areas such as the telencephalon, the trigeminal ganglion, clusters in the hindbrain and in the dorsally located Rohon-Beard neurons (Schweitzer *et al.,* 2005); *dpysl2* expression increases during the following hours of development and it is detected in more regions such as the retinal ganglion by 48 hpf (Christie *et al.,* 2006). *Dpysl2* expression can be observed in the brain at least up to 97 hpf (Christie *et al.,* 2006). Previous studies in zebrafish have shown the requirement of Dpysl2 for the proper positioning of neurons in the spinal cord (Tanaka *et al.,* 2012; Morimura *et al.,* 2013) and also for proper retinal axon growth (Liu *et al.,* 2018). However, its functions need to be further analyzed in brain development. Dpysl2 is involved in a variety of processes and it has been detected in many regions of the developing brain (Schweitzer *et al.,* 2005; Christie *et al.,* 2006); therefore, it could be an important piece for the proper establishment of the neural circuit in the brain.

In this study, we focused on the role of Dpysl2 in the development of cranial motor neurons.

Abbreviations used in this paper: AMO, antisense morpholino oligonucleotide; CRMP, collapsin response mediator protein; Dpysl, dihydropyrimidinase-like family protein; FBM, facial branchiomotor; KD, knock-down; KO, knock-out; r, rhombomer.

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We used the CRISPR/Cas9 system to generate a *dpysl2* knock-out (KO) line to study Dpysl2, more specifically to study its involvement in the migration of facial branchiomotor (FBM) neurons during early stages of development, a process previously described by Higashijima *et al.,* (2000). In addition, we used Antisense Morpholino Oligonucleotides (AMO) to knock down *dpysl2* and corroborate our findings. We used two types of AMO to block either the translation or splicing of *dpysl2* mRNA.

Higashijima *et al.,* (2000) found that the facial branchiomotor neurons migrate caudally during the development; at 21 hpf most of these neurons are located at the region corresponding to the rhombomers (r) 4 and 5 and they start migrating caudally until they reach r6 and r7. Most of them have reached this location by 36 hpf. We observed abnormalities in the location of these neurons in both *dpysl2* KO mutants and morphants. Most of facial branchiomotor neurons were expected to be at r6 region, but were found between r4 and r5 at 50 hpf, indicating a role of Dpysl2 in this migration process.

Results

Dpysl2 knock-out affects the migration of the facial branchiomotor neurons in early stages of nervous system development in zebrafish

To generate a *dpysl2* KO line, a fish carrying mutations induced by CRISPR/Cas9 injection was crossed with WT fish. In the F1, we identified a specific mutation consisting on a 1bp insertion (G->CA) at the target site, 3bp upstream to the PAM site (Fig. 1B). This mutation led to a frame shift mutation and a premature stop codon as consequence. Due to the premature stop codon, the length of Dpysl2 protein was shortened from 573 to 71 amino acids. The fish carrying this specific mutation were crossed with *Tg(isl1:GFP)rw0* (Fig. 1A) to enable direct visualization of the cranial motor neurons in their offspring. The transgenic line *Tg(isl1:GFP)rw0* expresses Green Fluorescent Protein (GFP) in the cranial motor neurons; therefore is a very useful line to study genes related to the motor neuron development (Higashijima *et al.,* 2000).

Dpysl2 KO (*dpysl2*$^{-/-}$) embryos expressing GFP in the cranial motor neurons were fixed at 50 hpf for dorsal observation of the facial branchiomotor neurons migration, at this time most of these neurons are expected to be located at r6. We observed the localization of the facial branchiomotor neurons *in vivo* at 28hpf and the general morphology of some embryos before fixation. We found no differences between *dpysl2*$^{+/+}$ and *dpysl2*$^{-/-}$ embryos in the positioning of the facial branchiomotor neurons, nor abnormalities in their morphology (data not shown). Defects were observed in the location of the facial branchiomotor neurons at 50 hpf in the 77.4% of the observed KO mutants (n= 31). In most of the embryos, a great number of neurons failed to migrate caudally and more neurons than expected were located between r4 and r5 (Fig. 2B). In the typical distribution of the facial branchiomotor neurons at this time point, there is a much larger number of them at r6 than between r4 and r5; however, in 48.4% of the cases we observed more cells between r4 and r5 than at r6 and in a 29% the amount of cells in these two regions was nearly the same. When we examined the siblings of the KO mutant embryos that did not carry the mutation (*dpysl2*$^{+/+}$) at 50 hpf we also found some abnormalities; however, it only affected the 36.4% of them (n=22). Most of them expressed WT-like patterns in the distribution of the facial branchiomotor neurons (Fig. 2A). These observations suggest a role of Dpysl2 in the migration of the facial branchiomotor neurons during development.

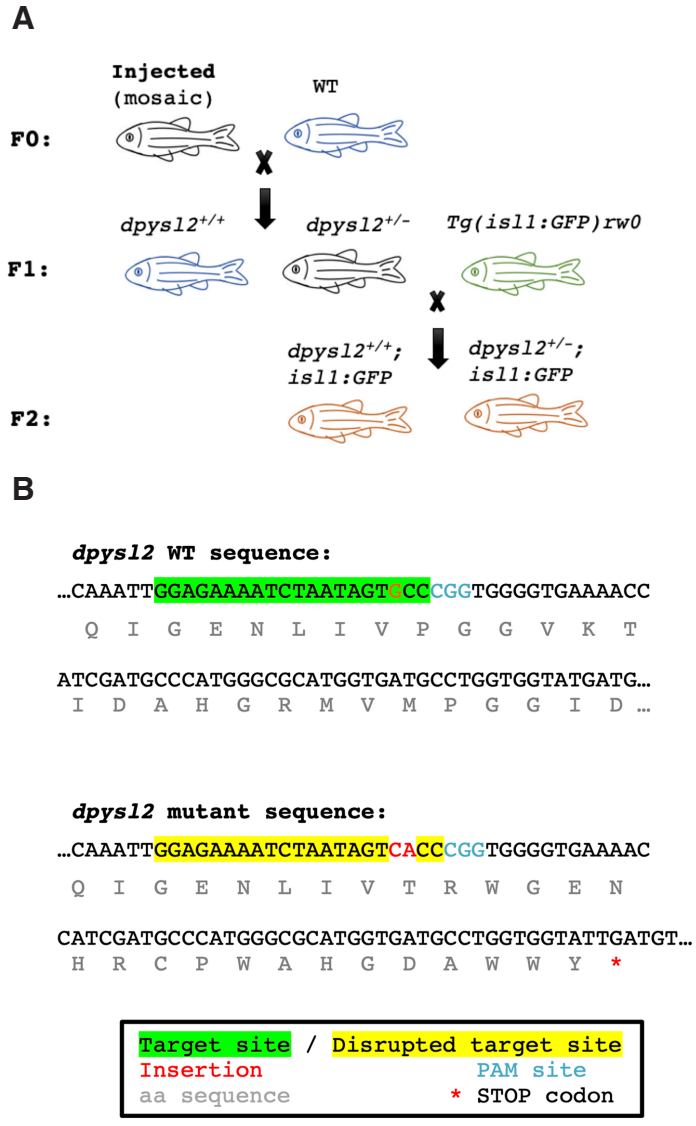

Fig. 1. Establishment of a *dpysl2* knock-out (KO) line. (A) *Scheme of the crossings performed to obtain the* dpysl2 *KO line. The fish carrying mutations induced by CRISPR/Cas9 injection was crossed with a WT fish. Posteriorly, the heterozygote descendants carrying the same specific mutation were crossed with* Tg(isl1:GFP)rw0 *to facilitate the observation of the cranial motor neurons in the following generations.* **(B)** *Fragments of WT and mutant* dpysl2 *sequence identified in the F1. 1bp insertion (G->CA) occurred 3bp upstream to the PAM site, leading to a frame shift mutation and its consequent premature stop codon. As a result, the amino acid length of Dpysl2 protein was shortened from 573 to 71 in this mutant line. The target site is indicated in green, the disrupted target site in yellow; red indicates the insertion mutation that occurred and the orange "G" points the corresponding site in the WT sequence; PAM sequence is marked in blue; block letters in grey indicate the amino acid sequence and the red star mark points out the location of the premature stop codon. These fragments correspond to exon 3 of* dpysl2.

Dpysl2 mRNA injection into dpysl2 KO embryos can rescue the mutant phenotype

To prove that the *dpysl2* loss-of-function was the cause of the abnormalities found in mutant embryos, we injected *dpysl2* mRNA at different concentrations (20, 50 and 100 ng/ml) into 1 to 2 cell stage *dpysl2*$^{-/-}$ embryos. *Dpysl2* mRNA injected and uninjected mutants were fixed at 50 hpf to observe dorsally the migration process of the facial branchiomotor neurons.

Among the embryos injected with 100 ng/ml of *dpysl2* mRNA (n= 23), 56.5% expressed a WT-like pattern; most of facial neurons were located at r6 by 50 hpf (Fig. 2C). After injecting lower concentrations of *dpysl2* mRNA the percentage of rescued embryos was also lower: 36.4% of the mutant embryos injected with 50 ng/ml of *dpysl2* mRNA (n=11) and 31.3% of the ones injected with 20 ng/ml (n= 16) exhibited WT-like patterns, indicating a dose dependency. These results support a role of Dpysl2 in the migration of the facial branchiomotor neurons.

No effects were observed when *dpysl2* mRNA was injected into WT embryos.

To confirm the relationship between the mutant phenotypes and the lack of *dpysl2*, we compared the frequency of embryos with abnormalities in the migration of the facial branchiomotor neurons and WT-like embryos observed in mutants (*dpysl2*$^{-/-}$), *dpysl2*$^{+/+}$ and *dpysl2* mRNA injected *dpysl2*$^{-/-}$ mutants (Fig. 2D). For each group, we calculated the frequency of phenotypes that were WT-like, slightly affected or severely affected and tested whether the differences in these frequencies were significant using a chi-square test. The results of statistical analysis support that the increase in the rate of severe abnormalities observed in *dpysl2*$^{-/-}$ embryos is related to the mutation that we generated.

Dpysl2 knock-down causes abnormalities in the migration of the facial branchiomotor neurons

There is evidence of the low correlation between phenotypes induced by a knock-down and the ones that result from a knock-out (Kok *et al.,* 2015), thus, the benefits of using knock-down techniques such as morpholino oligonucleotides has been questioned. However, previous research also showed that one of the causes of these discrepancies is the activation of a compensatory machinery; the knock-out of some genes leads to the upregulation of other genes, but this compensation does not take place after a knock-down (Rossi *et al.,* 2015). Therefore, a knock-down study can be a good complement to the knock-out one. We observed an incomplete penetrance in the phenotype of *dpysl2*$^{-/-}$ mutant embryos that could be due to the activation of compensatory systems; therefore, to confirm the role of Dpysl2 in the migration process of the facial branchiomotor neurons, we also evaluated the effects of a *dpysl2* knock-down. We injected different types of AMOs targeting *dpysl2* into 1 to 2 cell stage embryos obtained by crossing WT and *Tg(isl1:GFP)rw0* line zebrafish. We used either a translation blocking or a splicing blocking AMO to knock-down *dpysl2* and a control AMO to confirm that the effects were due to the knock-down of *dpysl2*, not to the AMO injection itself. Another typical reason for the common discrepancies between knock-down and knock-out phenotypes can be the presence of maternal WT mRNA in the mutants (Zimmer *et al.,* 2019). Translation blocking AMOs inhibit both maternal and zygotic mRNA, while splicing blocking AMOs affect only the zygotic mRNA (Eimon, 2014); for this reason, using both types of AMO in our research could help

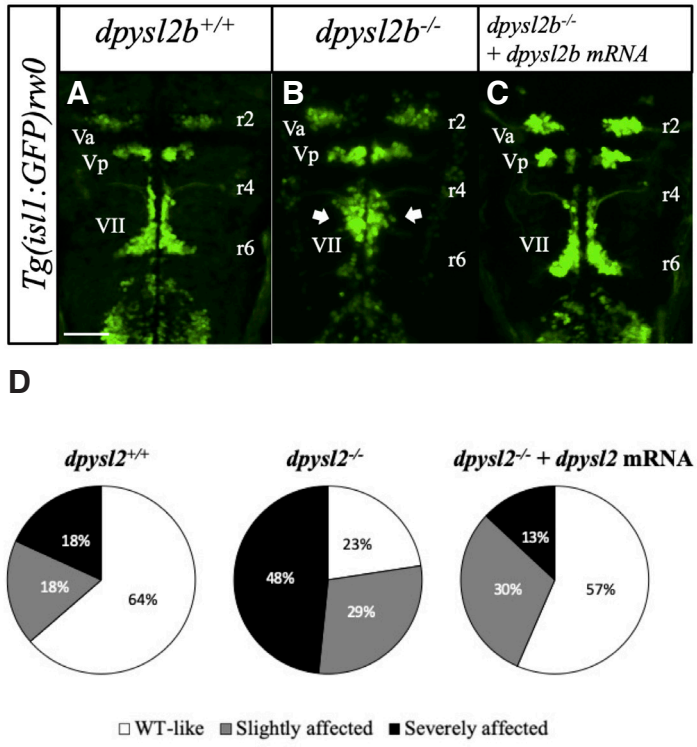

Fig. 2. *Dpysl2* knock-out (KO) affects the migration of facial branchiomotor (FBM) neurons. (A-C) *Representative images of the dorsal view of the cranial motor neurons at 50 hpf.* **(A)** *In* dpysl2$^{+/+}$ *embryo most facial branchiomotor neurons (VII) are located at r6.* **(B)** *The* dpysl2$^{-/-}$ *mutant exhibits clear abnormalities in the distribution of the facial branchiomotor neurons; most of them are still located between r4 and r5.* **(C)** dpysl2 *mRNA injected (100 ng/μl)* dpysl2$^{-/-}$ *embryo exhibits a WT-like pattern; most of facial branchiomotor neurons are located at r6. Abnormal regions are indicated with arrows. Va= Trigeminal anterior; Vp= Trigeminal posterior. Anterior is up. Scale bar, 50 μm.* **(D)** *Percentage of WT-like, slightly affected and severely affected phenotypes observed at 50 hpf in the facial branchiomotor neurons migration.* dpysl2$^{+/+}$ *(n= 22),* dpysl2$^{-/-}$ *(n= 31) and* dpysl2 *mRNA injected (100 ng/μl)* dpysl2$^{-/-}$ *(n= 23). (X^2 (4)= 13.598, p= 0.009< 0.05; indicates differences in the percentage of slightly affected severely affected and WT-like phenotypes between the groups. For the post-hoc analysis, a new P-value of 0.005 was established; this one determines that, although the percentage of slightly affected samples is similar, the number of severely affected and WT-like samples in the* dpysl2$^{-/-}$ *group differs significantly to the others with p < 0.005.*

to understand better the machinery regulating *dpysl2*. The three different types of AMOs were injected at the same concentration.

AMO injected and uninjected samples were fixed at 50 hpf as well. Embryos were observed dorsally to confirm whether the migration from r4 and r5 to r6 and r7 was completed successfully. Results showed clear abnormalities in 83.9% of the translation blocking injected embryos (n=31) and both slightly (9.3%) and severe (74.4%) defects in the splicing blocking AMO injected ones (n= 43). In the splicing blocking injected embryos, the characteristic structure of the facial nucleus was changed; neurons seemed to accumulate linearly between r4 and r6 instead of extending at r6 (Fig. 3D). In embryos injected with translation blocking AMO the effects were more severe; the structure of both facial and trigeminal nucleus was completely affected in most of cases (Fig. 3C). In contrast, in both uninjected and control AMO injected embryos, the migration

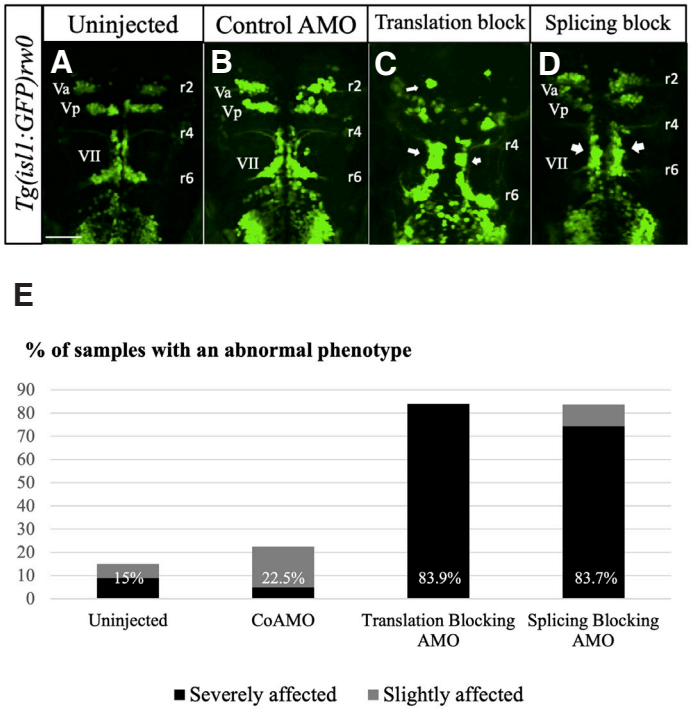

Fig. 3. Dpysl2 knock-down (KD) affects the migration of facial branchiomotor (FBM) neurons. *Representative images of the dorsal view of cranial motor neurons at 50 hpf and corresponding data.* **(A)** *Uninjected and* **(B)** *control AMO injected embryos.* **(C)** *Translation blocking AMO injected and* **(D)** *splicing blocking AMO injected embryos exhibit clear abnormalities in the distribution of facial branchiomotor neurons (VII). Abnormalities are indicated with arrows. Va, trigeminal anterior; Vp, trigeminal posterior. Anterior is up. Scale bar, 50 μm.* **(E)** *Percentage of embryos with an abnormal phenotype, slightly or severely affected, in the facial branchiomotor neurons migration at 50 hpf. 9% of uninjected samples (n= 33) were severely affected, 6% of them exhibited mild abnormalities; most of the control AMO injected embryos (17.5%) were slightly affected, while only 5% of them showed severe defects (n= 40); 83.9% of the translation blocking AMO injected (n= 31) and 74.4% of splicing blocking AMO injected (n= 43) had severe abnormalities; 9.3% of splicing blocking AMO injected samples were slightly affected by the morpholino injection.*

of the facial branchiomotor neurons was not really affected (Fig. 3A, B). 15% of the uninjected embryos (n= 33) exhibited abnormalities and 22.5% of the control AMO injected samples were affected to some degree (n= 40). These observations support the idea that Dpysl2 is involved in the facial branchiomotor neuron migration, at least during the early stages of the nervous system development.

Discussion

The role of Dpysl family proteins in axonal growth and cell migration in the nervous system has been studied in different species. Dpysl2 role has also been studied in zebrafish to understand better its functions *in vivo*. However, here we used different means to show for the first time the involvement of this protein in the migration of the cranial branchiomotor neurons in zebrafish. We generated a *dpysl2* KO line by using the CRISPR/Cas9 technology and also knocked it down using different types of morpholino oligonucleotides.

Our studies using both CRISPR/Cas9 system and AMOs show that Dpysl2 has a role in the migration of facial branchiomotor neurons during the development of zebrafish nervous system. During normal development, facial branchiomotor neurons migrate progressively from r4 and r5 to r6 and r7 and most of these neurons have reached their location in r6 and r7 by 36 hpf (Higashijima *et al.,* 2000). We waited until 50 hpf to observe the *dpysl2* KO mutant and *dpysl2* AMO injected embryos, a time when most of facial branchiomotor neurons should be already at the caudal region of the facial nucleus. However, even at this time, we observed abnormalities in the positioning of these neurons.

When analyzing the migration process of the facial branchiomotor neurons some differences between mutants and morphants were observed. Previous reports expressed that a great number of morphant phenotypes were not observed when analyzing the corresponding mutants (Kok *et al.,* 2015), but these discrepancies might be explained by different reasons. *Dpysl2* morphant phenotypes were more severe and constant than the mutant ones.

Dpysl2 KO mutants express variability in both the degree of expression of the phenotype and the pattern of the abnormalities observed. This variability could be dependent both on the level of penetrance of the mutation or on the degree of compensation by another genes. It has been reported previously that, in some cases, knocking out a gene will lead to the activation of a compensatory pathway and, as a result, other genes will be upregulated. The exact way this mechanism of adaptation to the cell environment works is not clear; however, it is not activated after an AMO induced knock-down and can be the cause of phenotypic differences between mutants and morphants (Rossi *et al.,* 2015). Dpysl family members are expressed in similar regions throughout the nervous system and some of them accomplish similar roles. Previous studies have shown similar effects after knocking down *dpysl2* (CRMP2) and *dpysl3* (CRMP4) (Tanaka *et al.,* 2012; Morimura *et al.,* 2013) therefore, it is possible that *dpysl3* or another *dpysl* family member is compensating the lack of *dpysl2* in the KO mutants.

The phenotype induced by the injection of splicing blocking AMO was similar in all cases; most of cells were accumulated between r4 and r6 changing the usual distribution of the facial branchiomotor neurons (Fig. 3D). When the translation blocking AMO was injected, the phenotype observed was more severe (Fig. 3C), probably due to the higher efficiency inhibiting the production of Dpysl2 protein. Translation blocking AMOs bind to the post-spliced mRNA (both maternal and zygotic mRNA) and block the progression of the ribosomal initiation complex; splicing blocking AMOs block the proper processing of pre-mRNA (Eimon, 2014), they do not affect the maternal mRNA. The presence of maternal mRNA could mitigate the effects of the knock-down and this could explain why the phenotype induced by the splicing blocking AMO is less severe than the induced by the translation blocking AMO. The lack of a morphant phenotype after control AMO injection supports the specificity of the effect of knocking down *dpysl2*; it is unlikely that the severe phenotype induced by the translation blocking AMO is due to an off-target effect. In the same way, the protein produced by the maternal WT mRNA could explain the WT-like phenotypes in the KO mutants generated by crossing heterozygous parents (Zimmer *et al.,* 2019).

The phenotypes observed in *dpysl2* KO mutants were more diverse than the morphant phenotypes; however, in both cases, we found abnormalities in the migration of the facial branchiomotor neurons. Therefore, our analysis let us assess that Dpysl2 has a role in the migration of the facial branchiomotor neurons during the

development of the zebrafish nervous system. This is consistent with previous researches that indicate a role of neuropilin1 (Nrp1) and the semaphorin Sema3A in the patterning of the facial nerve in mice (Schwarz *et al.,* 2004). As a regulator of the Sema3A signaling in different processes, it is understandable that Dpysl2 participates also in the patterning of the facial nerve.

During our studies, we also analyzed the descendants from heterozygous fish that did not carry our specific mutation and compared them with WT embryos. We identified them as *dpysl2*$^{+/+}$ to make a difference between these fish and the WT ones descending from WT parents. 63.6% of the *dpysl2*$^{+/+}$ embryos fixed at 50 hpf and observed dorsally (n= 22) exhibited WT-like patterns. In the remaining samples we found some abnormalities as we did in 20.0% of WT embryos (n= 35). Statistical analysis was performed between *dpysl2*$^{+/+}$ and WT groups. The results showed that the frequencies of WT-like and abnormal samples observed at 50 hpf in the *dpysl2*$^{+/+}$ embryos were not significantly different compared to the WT ones. On the other hand, frequencies were significantly different between *dpysl2*$^{+/+}$ and *dpysl2*$^{-/-}$ fish (Fig. 2D). In addition, we observed that *dpysl2* mRNA rescued the mutant phenotype in a dose-dependent manner. All these results suggest that the high percentage of abnormal samples in the *dpysl2*$^{-/-}$ is related to the loss-of-function of *dpysl2*.

In sum, although we observed some differences between the knock-out and knock-down phenotypes, both methods affect the same process, indicating that Dpysl2 has actually a role in the migration of the facial branchiomotor neurons during zebrafish development.

Materials and Methods

Animals

Zebrafish (Danio rerio) were maintained according to standard procedures (Westerfield, 2000). Zebrafish embryos were incubated at 28.5 °C as indicated by Kimmel *et al.,* (1995). To prevent pigmentation, 0.003% 1-phenyl-2-thiourea (PTU) was added to the fish water before 24 hours after the fertilization (Westerfield, 2000). RIKEN Wako (RW) wild type and *Tg(isl1:GFP)rw0* transgenic zebrafish strains were obtained from the Zebrafish National BioResource Center of Japan (https://shigen.nig.ac.jp/zebra/). All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Waseda University (2017-A029, 2018-A005 and 2019-A027).

Generation of a dpysl2 KO mutant line by CRISPR/Cas9

To generate a *dpysl2* KO by using CRISPR/Cas9 system, we selected our target sequence as 5'- GGAGAAAATCTAATAGTGCC - 3' and selected the oligonucleotides sequences, partial sequence of sgRNA, as follows. Oligo 1: 5'–TAGGAGAAAATCTAATAGTGCC - 3'; Oligo2: 5'- AAACGGCACTATTAGATTTTCT -3' (eurofins, Tokyo, Japan). These oligonucleotides were subcloned into DR247 plasmid, which was a gift from Keith Joung (Addgene plasmid # 42250; http://n2t.net/addgene:42250; RRID:Addgene_42250) (Hwang *et al.,* 2013) and the sequence was confirmed. pT3TS-nCas9n was a gift from Wenbiao Chen (Addgene plasmid # 46757; http://n2t.net/addgene:46757; RRID:Addgene_46757) (Jao *et al.,* 2013).

sgRNA and Cas9 mRNA were synthesized using the mMessage mMachine kit (Ambion, Austin, Texas, USA). Synthesized *dpysl2* sgRNA and Cas9 mRNA were co-injected into 1 to 2 cell stage zebrafish embryos. Each embryo was injected with about 0.5 nl of solution containing a mixture of about 300 ng/ml sgRNA, 900 ng/ml Cas9 mRNA, 250 ng/ml GFP mRNA and 0.05% Phenol Red (PR). GFP mRNA was used as indicator to confirm the success of the injection.

Genomic DNA (gDNA) was extracted from CRISPR/cas9 injected embryos as a first screening to confirm the success of the knock-out generation. The target region was amplified by PCR using the following primers: *dpysl2* forward, 5'- CCTCTAAACGTGGCCCCTGA -3'; *dpysl2* reverse, 5'- ATACCACCAGGCATCACCAT -3'. PCR products were cloned into a plasmid using the pGEM T-easy vector (Promega, Madison, Wisconsin, USA). Sequences were compared to the wild-type (WT) ones to identify the possibly induced mutations. The mutation carrier was crossed with WT fish to obtain the F1. After a specific mutation was identified in the F1, primers to identify other mutants with the same sequence were designed as follows. *dpysl2*_forward, 5'- TCGCGATGCCGGCTCAGAAT -3'; *dpysl2*_WT_reverse, 5'- GGTTTTCACCCCACCGGGCA -3'; *dpysl2*_Mutant_reverse, 5`-GTTTTCACCCCACCGGGTGA-3'. gDNA from the following generations was extracted either from the caudal fin of an adult fish or from the posterior half of an embryo's body and genotyped by PCR with these primers.

F1 fish were crossed with *Tg(isl1:GFP)rw0*, a transgenic line that expresses GFP in the cranial motor neurons, facilitating their direct visualization (Higashijima *et al.,* 2000).

AMO and mRNA injections

The sequences for the morpholinos (Gene Tools, Philomath, Oregon, USA) blocking the translation of *dpysl2* and for the control morpholino were used as described previously (Tanaka *et al.,* 2012; Morimura *et al.,* 2013). The sequence for the splicing blocking AMO of *dpysl2* was 5'-CACTCTGGAAACACAGATAAACACA- 3'. Approximately 1 nL of AMO (1 mg/ml) was injected into 1 to 2 cell stage embryos as described (Nasevicius and Ekker, 2000).

For rescue experiments, *dpysl2* mRNA was synthesized using the mMessage mMachine Kit (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). This mRNA was injected at different concentrations into 1 to 2 cell stage mutant embryos as specified in the results.

Microscopy and imaging

For fluorescent microscopy a FV1000 confocal laser scanning microscope (Olympus) with UNPlanFL 20x (NA= 0.50) and LUMPlanFLN 40x (NA= 0.80) water immersion objectives was used. The images were processed using Adobe Photoshop and Adobe Illustrator.

Data analysis

We used ImageJ to measure the area of facial branchiomotor neurons occupying the regions from r4 to r5 and from r5 to r6. The sample was considered WT-like when the r5-r6 area was considerably bigger than r4-r5 (2.5 times or more); slightly affected when the region from r5 to r6 was between 1.5 and 2.4 times bigger than r4 to r5; and severely affected if r5-r6 had similar or smaller size than r4-r5 (less than 1.4 times bigger).

IBM SPSS Statistics 25 was used for statistical analysis. Chi-squared test was performed to determine whether the differences in frequencies between groups were statistically significant. When we compared the frequencies of slightly affected, severely affected and WT-like phenotypes between three groups, a P-value< 0.05 indicated significant differences were present. A post-hoc analysis was performed after the chi-squared test (Beasley and Schumacker, 1995); the z-scores were analyzed to determine a new specific P-value and determine which group was significantly different compared to the others. The new P-value is indicated in the corresponding figure.

Conflict of interest

The authors declare no conflict of interest.

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