Role of Fmn2b in the development of lateral line system of zebrafish (*Danio rerio*)

Project Thesis

Submitted To

Biology Department, IISER Pune

In Partial Fulfilment of the Requirements for the Award of

5 Years BS-MS dual degree

By

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Under the guidance of

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Certificate:

This is to certify that this dissertation entitled "**Role of Fmn2b in the development of the lateral line system in zebrafish (*Danio rerio*)**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by **Ghorpade Smita Madhukar (20111012)** at IISER Pune under the supervision of Dr. Aurnab Ghose, Associate Professor, Biology Department" during the academic year 2015-2016.

Date: 28-03-16

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Declaration:

I hereby declare that the matter embodied in the report entitled “Role of Fmn2b in the development of the lateral line system in zebrafish (*Danio rerio*)” are the results of the investigations carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr. Aurnab Ghose and the same has not been submitted elsewhere for any other degree.

Date: 28-03-16 Ghorpade Smita Madhukar

Acknowledgements:

First and foremost, I would like to take this opportunity to express my deep sense of gratitude towards my respected guide, Dr Aurnab Ghose, for his unwavering support, sound guidance, inspiring direction and untiring encouragement that helped me to complete this work with enthusiasm and zeal. I would also like to thank Prof. N. K. Subhedar and Dr. L.S. Shashidhara for their invaluable guidance.

I am infinitely indebted to my lab-mates Dhriti and Ratnakar, for their much-needed assistance and valuable insights during my experiments. I must thank also my other labmates, especially Aditi, Devika, Abhishek R., Ninad, Bhavin, Abhishek S., Ketakee, Tanushree, Sampada, Lakshmi, Meenakshi for their help and support and for maintaining an air of friendliness in the lab, which is worth more appreciation than it usually receives.

Thanks are due also to Vijay for taking pains beyond his duty in order to make my work a little bit smoother.

This work would not have been possible without the constant support and encouragement I have been fortunate to receive for the past 5 years from all my teachers and friends at IISER Pune.

To my batch-mates in particular, I give my heartfelt thanks, for the great times and cherished memories.

Lastly, I thank most of all my parents for their constant support and encouragement, and for never losing faith in me.

I beg forgiveness in case I forgot to mention anyone.

Ghorpade Smita Madhukar

Abstract:

Formins are a family of proteins that are involved in nucleating and elongating actin filaments and are hence hypothesized to be involved in the development of nervous system. The project aims at studying the role of one member of the Formin family, Formin2b, in the development of lateral line system by studying the defects in development caused by the knockdown of the protein in zebrafish embryos. Behavior experiments showed balance defects and a defect in escape responses suggesting defects in the development of inner ear and/or mechanosensory lateral line of the embryos. Using antisense morpholinos to reduce Fmn2b levels in the embryos, it was found that there are defects in the development of lateral line in the embryos with Fmn2b knockdown. The number of neuromasts in the lateral line system and the interval between consecutive neuromasts was affected due to Fmn2b knockdown. This could be because of defects in actin bundling during the maturation and deposition of neuromasts.

Contents

Introduction:

Actin cytoskeletal dynamics plays a major role in many processes in the cell in both unicellular and multicellular organisms. It gives cells resistance to deformation and helps in maintaining shape. It also regulates cell motility and various other functions like force generation, signalling, intracellular transport etc. It also has a specialized role in function of cilia, flagella, filopodia and lamellipodia. Thus, the regulation of actin dynamics is also very important for the proper functioning of cells. Actin dynamics involves polymerization and depolymerisation of the actin filaments depending on the cell's requirements.

The polymerization of actin filaments is the process of nucleating a new actin filament or adding new actin subunits to the existing acting filament leading to its elongation. Depolymerization is the opposite process in which subunits are removed from the actin filament. The addition of actin subunits during polymerization is a kinetically unfavourable process. Also, initial nucleation is a rate limiting step in this process. Hence other agents, known as actin nucleators are required to facilitate this process.

Three major classes of actin nucleators are known so far (Chesarone and Goode, 2009):

1. Actin related protein (Arp) 2 and 3, also known as Arp 2/3 complex.
2. Formins
3. Tandem monomer binding nucleators: Includes Spire (Quinlan et. al., 2005), Cordon bleu or Cobl (Ahuja et. al., 2007) and Leiomodin or Lmod (Chereau et. al., 2008).

Arp 2/3 nucleates actin and remains attached to the actin filament even after nucleation. This allows it to aid the formation of branches at 70^{O} from the initial filament (Goode and Eck, 2007), leading to the formation of branched actin networks. Arp 2/3 is mainly responsible for actin nucleation in the lamellipodia.

Spire acts by forming a pre nucleation complex of 3-4 actin subunits to which more subunits are then added from the sides (Quinlan et. al., 2005). Cobl also functions by a similar mechanism.

Formins function as dimers and are mainly involved in nucleation and elongation of bundled actin filaments. Formins work by capturing two actin monomers and thus leading to their dimererization (Goode and Eck, 2007). Formins are activated by Rho GTPases.

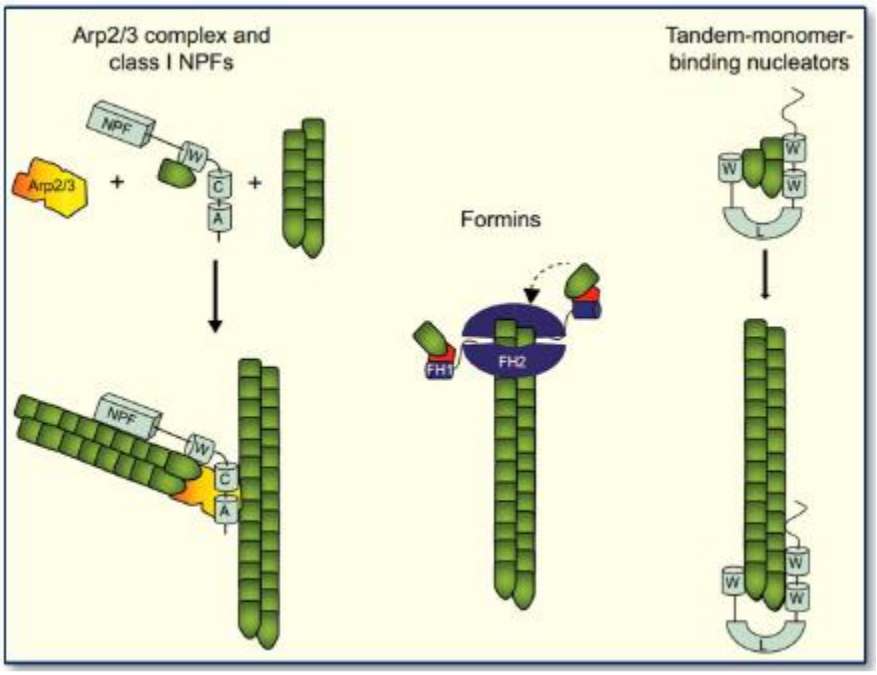


Fig. 1.1. ***Mechanism of action of different actin nucleators (Weston et. al., 2012)***

Multiple homologs of formins are found in eukaryotes. In mammals, for instance, the number of formin genes identified in 15 (Higgs and Peterson, 2005). There are three major regions of homology found in formins across species, including the FH1 and FH2 domains. Formins have been classified into following 7 classes depending upon the dissimilarities in the FH2 domains:

1. the original formins (Fmn1 and Fmn2)
2. the Diaphanous formins (Dia)
3. the “inverted” formins (INF)
4. the formin homology domain containing proteins (FHOD)
5. the disheveled-associated activators of morphogenesis (Daam)
6. Delphilin, and
7. the formin-related (or formin-like) proteins identified in leukocytes (FRL, FMNL)

Formin 2 is one of the members of the formin family of proteins. It was first discovered in murine and human systems. It shows a high degree of homology with the earlier known members Formin 1 and the drosophila homolog of formins, *cappuccino*. It had been shown to modulate a number of processes in mammals, including humans, like asymmetric cell division in mammalian oocyte maturation (Kwon and Lim, 2011), human adipogenesis (Peng and Liou, 2012) and vesicle transport (Schuh, 2011). In mice, it was shown to be highly expressed in the developing and mature central

nervous system (Leader et. al., 2000). Recent studies have also shown that mutations in Fmn2 lead to learning disability and cognitive deficits in humans. These finding made suggested that Fmn2 might play a role in axon guidance in the developing nervous system.

Earlier studies in the lab support this hypothesis that Fmn2 plays a role in axon guidance by modulating the cytoskeleton in the growth cone (Sahasrabudhe et. al., 2015). Fmn2 knockdown in neurons in vitro results in reduced number of filopodia in the growth cone and also a deficit in growth cone movement. Also, in vivo, it showed to affect the guidance of the spinal commissural neurons in chick embryos.

However, how these deficits in axon guidance shown in vitro translate into behaviour is yet unknown. As chick embryo is unsuitable for doing behaviour experiments, zebrafish was used for this study as it provides with many advantages like a comparatively simple nervous system and genetic accessibility. Zebrafish also provides other advantages such as easier observations due to transparency, faster development etc. Also, as the zebrafish embryonic development takes place outside of the mother, it allows for behavioural experiments in early stages of development. Zebrafish have two orthologs of Fmn2, Fmn2a and Fmn2b. We decided to study Fmn2b because it is more conserved across taxa as compared to Fmn2a.

Preliminary studies done in the lab have shown that Fmn2b is indeed expressed in the developing nervous system (Rajan Dasgupta, unpublished work). In situ hybridisation experiments revealed that Fmn2 is expressed in the developing brain, spinal cord and the retinal ganglion cell layer in the eyes in 48 hpf embryos.

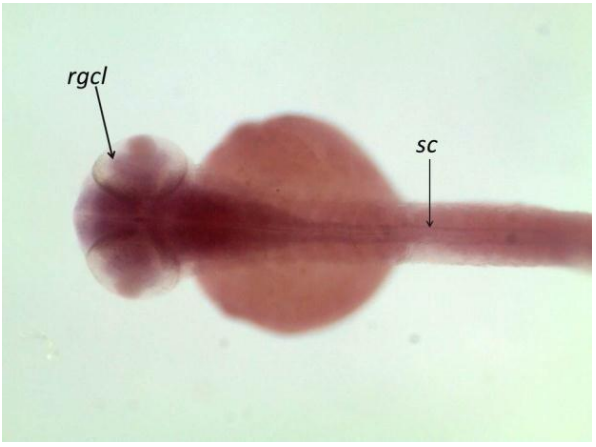


Fig.1.2. ***Expression of Fmn2b in 48hpf embryos visualized using whole mount RNA ISH (Rajan Dasgupta, 2012):***

Formin2b expression was seen in the brain, spinal cord and in the RGC layer of the eye.

Screening for various neural tracts also revealed that Fmn2b knockdown in the embryo affects the midline crossing of the spiral fiber neurons in the hindbrain of the 96 hpf embryos (Ratnakar Mishra, unpublished work). The Mauthner cell- spiral fiber neuron system has been shown to play a role in escape responses in zebrafish (Lorent et. al., 2001).

This led us to do behaviour assays to check whether Fmn2b knockdown leads to defect in escape response in the zebrafish larvae (Dhriti Nagar, unpublished work). These assays showed that the Fmn2b knockdown larvae showed a defect in C-start component of the escape response.

Along with defective escape response, these larvae also showed balance defect. As the inner ear is the primary organ responsible for maintaining balance, we hypothesized that Fmn2b knockdown could have led to defective development of the inner ear. As the inner ear and the sensory organs of the lateral line system, neuromasts are very similar in their cellular structure, there was a probability that Fmn2b knockdown also affected the neuromasts and the development of the lateral line. The lateral line system is more accessible and easier to visualize as compared to the inner ear and hence, we decided to check if the knockdown of Fmn2b has any effect on the development of the lateral line system.

Lateral line system:

The lateral line system is a system of sensory organs called neuromasts and the neurons innervating them. It is located along the lateral sides of the body of the fish, thus giving it its name. The lateral line is divided into the anterior and the posterior lateral line. The anterior lateral line includes the neuromasts present in the head region while the posterior lateral line includes neuromasts on the trunk and tail of the larva (Metcalfe et al., 1985; Gompel et al., 2001).The posterior lateral line consists of 8 to 9 neuromasts, 15-20 afferent neurons and 9 efferent neurons (Metcalfe et. al., 1985).

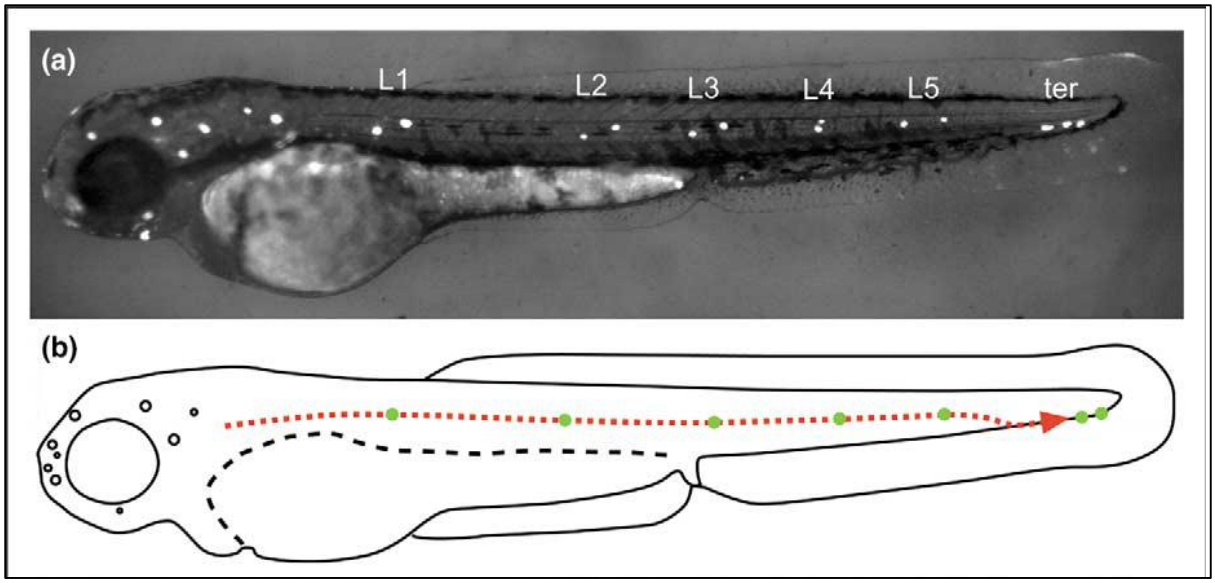


Fig. 1.3. ***Lateral line system of zebrafish (Ghysen and Dambly Chaudiere, 2004****):*
(A) The larva has been stained with Yo Pro in order to visualize the neuromasts
(B) Schematic of the lateral line system of the zebrafish larvae

Neuromasts are the sense organs that have hair cells at their center and support cells surrounding the hair cells. The hair cells are indistinguishable from the hair cells present in the vertebrate ear (Chitnis et. al., 2012).

The neuromasts form the mechanosensory organs of the lateral line deposited in a stereotyped pattern across the lateral sides of the larvae. They mediate 'touch at a distance' i.e. they detect the changes in flow of water a few centimeters from the fish's body. The neuromasts are thought to play a role in surface feeding, obstacle avoidance, subsurface detection of prey, predator etc (Chitnis et. al., 2012).

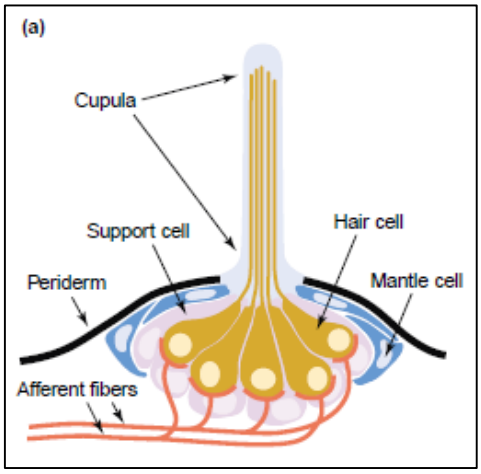


Fig.1.4. ***Structure of neuromast (Ghysen and Dambly Chaudiere, 2004):***
The schematic shows the hair cells with stereocilia, the support cells and the mantle cells

Development and migration of lateral line:

The development of the lateral line begins at around 20-24hpf from the sensory placode just caudal to the inner ear. The primary lateral line primordium (pLLP) migrates along the horizontal myoseptum and deposits neuromasts along its way. The migration is complete at 42hpf while the neuromasts are completely differentiated by 48hpf[3].

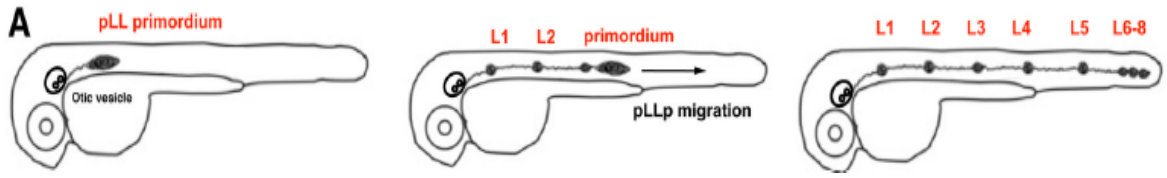


Fig. 1.5: ***Development of lateral line system in zebrafish (Chitnis et. al. 2012):***
Migration of the pLLP towards the tail of the larva. Neuromasts are deposited as the pLLP progresses forward.

As the pLLP migrates, it deposits neuromasts which are around 25-30 cells at an interval of 5-6 somites. The migrating primordium can be distinguished into 2 zones (Lecaudey et al., 2008):

1. The leading zone that consists of undifferentiated cells in the primordium. These leading cells navigate along the horizontal myoseptum and take the cells of the trailing edge with them as they progress. They form protrusions that scan the surrounding area and sense the guidance cues much like the growth cone in a navigating axon.
2. The trailing edge that consists of distinct rosettes of cells. These rosettes are protoneuromasts which are later deposited and go on to form mature neuromasts.

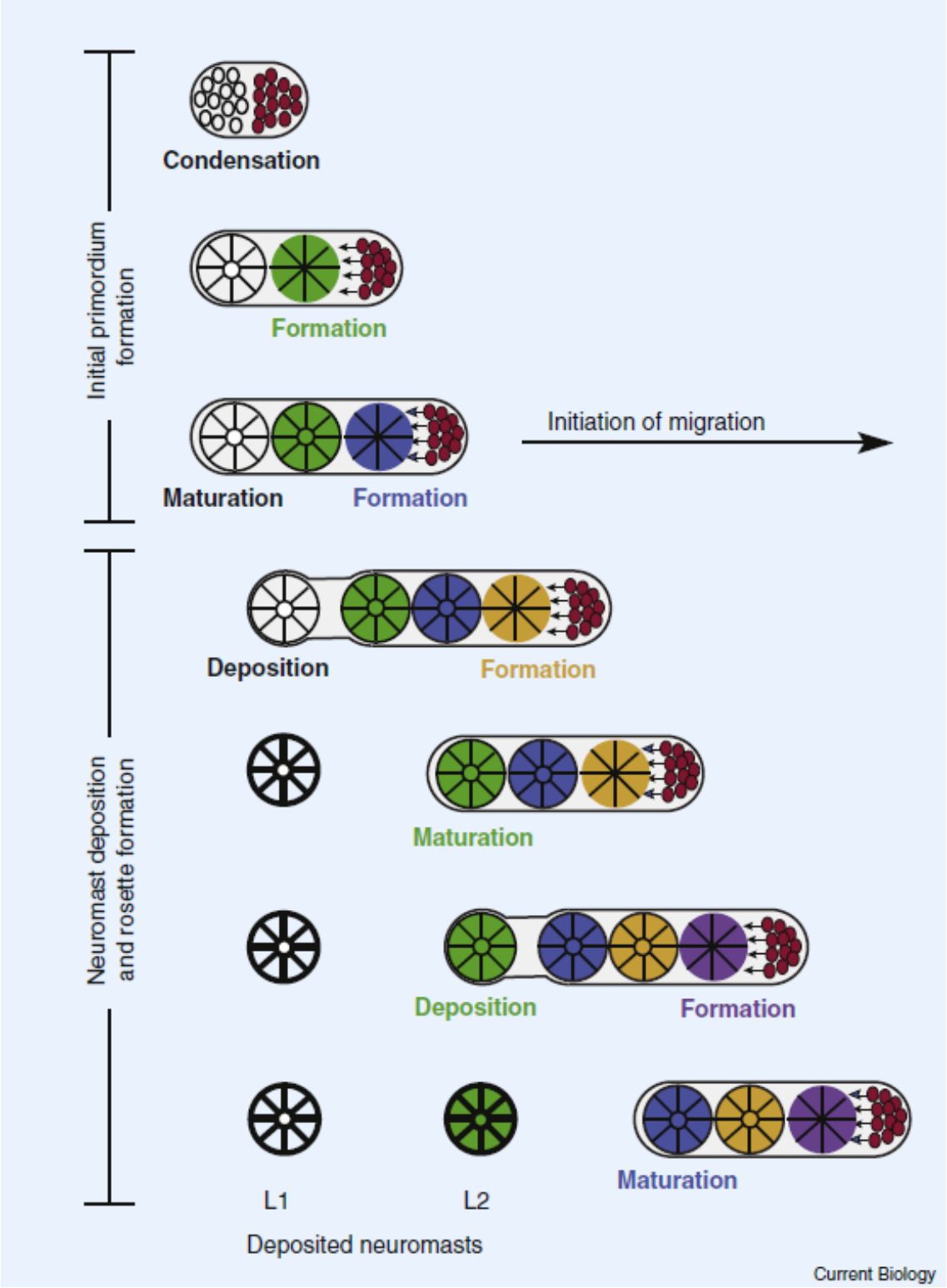


Fig. 1.6. ***Deposition of neuromasts (Ma and Raible, 2009):***
As the pLLP progrsses forward, protoneuromasts are formed at the trailing edge and are eventually deposited.

The leading edge cells have a mesenchymal morphology and are flat. However, as they mature, the cells become more columnar and gain an epithelial morphology (Lecaudey et al., 2008). Simultaneously, actin in the cells gets associated with the apical junctions leading to apical constriction (Hava et al., 2009). This leads to the formation of a more center oriented rosettes. The cell at the center gets specified to form the hair cell progenitor. All these changes together lead to the formation of proto neuromast.

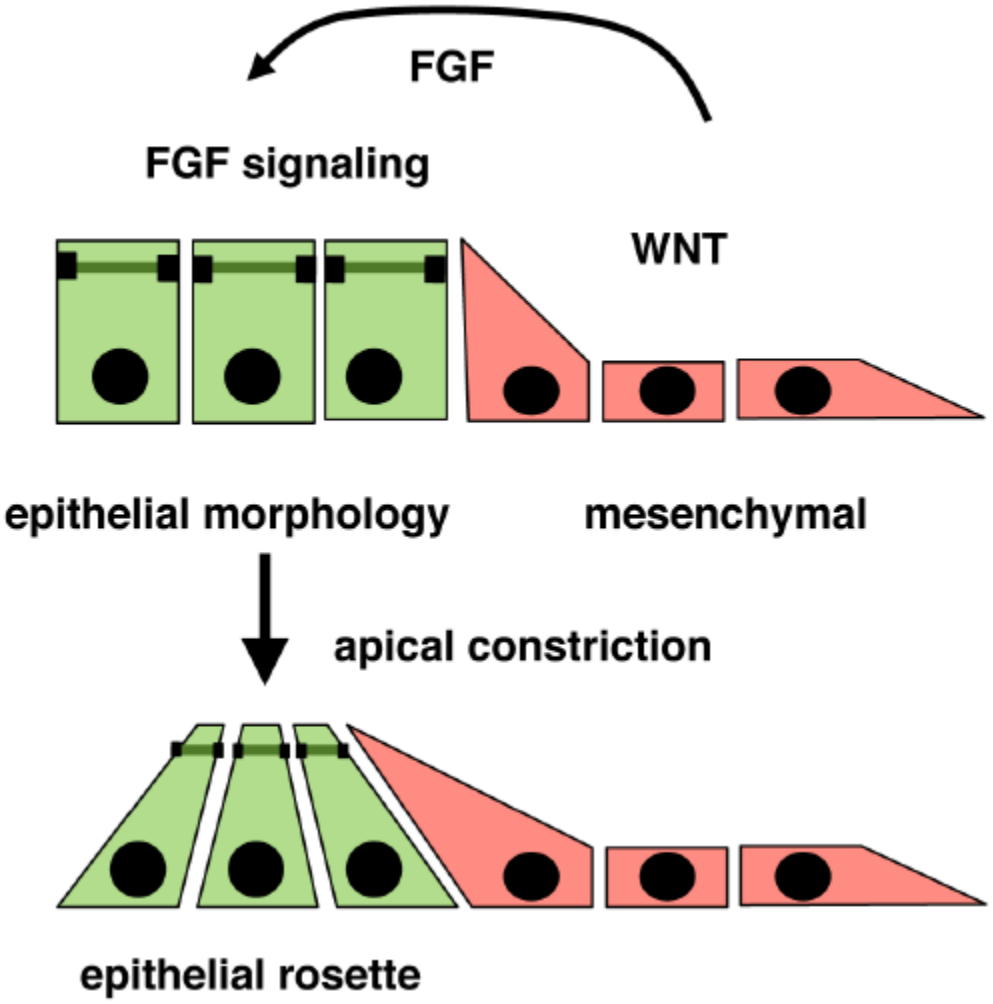


Fig. 1.7. ***Apical constriction of cells in trailing zone to form rosettes (Chitnis et. al., 2012):***

Actin associates with the apical junctions in order to cause the apical constriction of cells

Signaling plays a very important role in the migration of the primordium, maturation and deposition of the neuromasts. There is no extrinsic gradient of guidance cues present in order to give directionality to the pLLP migration. The migration is controlled

by the formation of a local dynamic gradient of chemokine Cxcl12a (Sdf1a) created the differential distribution of the guidance receptors Cxcr4b and Cxcr7b in the primordium. Cxcr4b is present all over the migrating primordium and is capable of binding Cxcl12a and inducing migration. Cxcl7b, however, is present only in the trailing zone of the primordium and internalizes Cxcl12a. Thus Cxcl12a is depleted in the vicinity of the trailing zone creating a local gradient of Cxcl12a and hence imparting directionality to the migration of the primordium (Burns et al., 2006; Boldajipour et al., 2008; Naumann et al., 2010).

The formation of proto neuromasts is initiated by the establishment of Wnt dependant FGF signaling in the trailing zone of the primordium. Wnt signaling is active in the leading zone of the primordium leading to the production of FGF3/10 in the leading edge which then diffuses and sets up FGF signaling in the trailing zone. There is no FGF signaling in the leading edge despite the production of the ligand due to the absence of the FGF receptor in the leading edge (Aman and Piotrowski, 2008).

Other signaling pathways like notch/delta and cadherins also play a major role in the maturation of a neuromast.

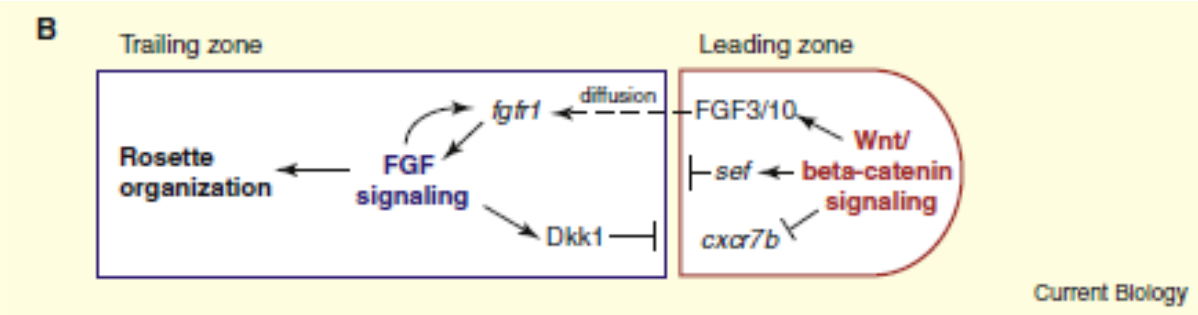


Fig. 1.8. ***Signalling pathways in the leading and trailing zone of the pLLP (Ma and Raible, 2009):***

Wnt dependant FGF signaling is established in the trailing zone of the primordium

Genesis of hypothesis and experimental design:

Behavioral experiments done in the lab (Dhriti Nagar, unpublished data), showed balance defects in the Formin2b knockdown larvae. The primary organ responsible for maintaining balance in zebrafish is the inner ear. The hair cells in the inner ear have stereocilia that play a role in this. The stereocilia are structurally similar to the filopodia in the growth cone of axons and are rich in actin cytoskeleton. In vitro studies in the lab have shown that formin2b knockdown affects the number of filopodia in a navigating axon. This suggested that Formin2b plays a role in development of the hair cells in the inner ear.

The lateral line system has a cellular structure similar to the inner ear and is more accessible as compared to the inner ear. The leading edge of the migrating primordium forms protrusions in order to sense the surrounding similar to a navigating axon. It has also been shown in the lab that the movement of axon towards its target is affected due to Fmn2b knockdown. Thus the similarity between the growth cone, inner ear and lateral line system combined with the observations from the lab led us to hypothesize that formin2b might play a role in the development on the lateral line system of zebrafish. The hypothesis was further strengthened by the fact that actin cytoskeletal dynamics play a major role in the formation of epithelial rosettes in the proto neuromast. As major role of formins is regulation of actin cytoskeleton, this hinted that formins might also play a role in specification of the neuromasts in the pLLP.

The experimental design involves knocking down of the Fmn2b gene using morpholino antisense oligonucleotide ‘knockdown’ technology and then labeling and looking at the development of lateral line. The larvae were grown for 4 days and then fixed. After fixation the neuromasts were labelled using DiI and then imaged.

Materials and methods:

Buffers and solutions:

1. 10X PBS (pH 7.4): 80g of NaCl, 2g Kcl, 14.4g Na2PO4, 2.4g KH2PO4 in 1litre H2O
2. 4% PFA: 4% w/v Paraformaldehyde is dissolved in suitable volume of 1X PBS by constant stirring at 65°C. pH is then adjusted to 7.2-7.4.
3. 60X E3 buffer: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM $MgSO_4$ and a few drops of methylene blue.
4. DiI Stock: 0.5 mg/ml stock of DiI was prepared in DMSO and stored at $4^{O}C$. Working solution was prepared by diluting the stock in 1X PBS.

Zebrafish husbandry: Zebrafish were stored in tanks with continuous circulation of water through them. The temperature was maintained at $28^{O}C$. For the current project, wild type Indian strain of zebrafish (*Danio rerio*) of both short and long fin variety was used. The night dark cycle of 14 hours of light and 10 hours of darkness was maintained in the fish room.

Breeding: The tank used for breeding the fishes is shown in figure 3.1. There was another tank with slits at its base placed inside a one litre tank so as to allow the eggs to pass through to the bottom of the tank. This was done in order to prevent the fishes from devouring the eggs.

Fig. 3.1. ***Breeding tank:*** *The image shows a breeding containing male fish (right) and female fish (left) with a separator placed in between them.*

One male and one female were placed in a breeding tank (separate from the system) on the night before and a separator was placed in between them so as to prevent mating during the night. The breeding tank was placed in such a way that it received adequate amount of light during the light cycle. After the dark cycle of 10 hours, the separator was removed just before the lights came on the next morning. The fishes were allowed to breed for around 30 minutes after which the eggs were collected from the breeding tank. The eggs were then either transferred to petri plates containing E3 medium directly or after morpholino injections (100 eggs per plate).

Feeding: The larvae were fed with synthetic larval food 4 times a day (10 a.m., 2 p.m., 6 p.m. and 9 p.m.) till 7dpf and artemia thereafter. Adult fishes were fed artemia in the morning and solid food in the evening. Along with normal feeding, the breeding pairs were again fed at night with solid food.

Morpholino based knockdown technology: Morpholinos are 25bp long oligonucleotides with a morpholine ring in place of a ribose or deoxyribose sugar and phosphorodiamidate linkages in place of anionic phosphates. This gives a neutral backbone to morpholino oligos and hence prevents it from being recognized by enzymes or signalling molecules. The morpholino oligos are used for translational blocking in zebrafish. This is accomplished by designing the oligos in such a way that they bind to the 5' UTR near the translational start site thereby preventing the assembly of ribosomes (Bill et. al., 2009).

As morpholinos are unrecognizable to enzymes, they are not degraded inside the cell. Instead, they bind to their target RNA and block its activity till the RNA is naturally degraded. The morpholino is then released once the target RNA has been destroyed.

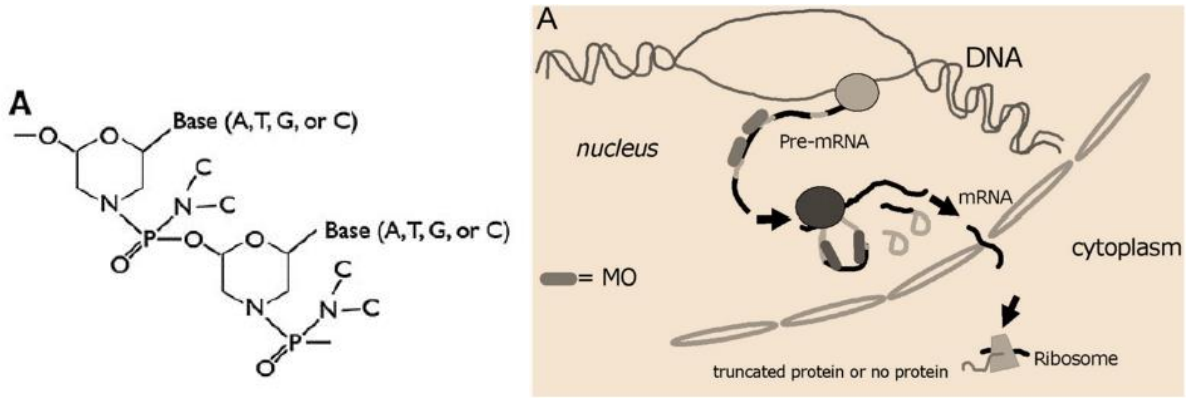


Fig 3.2. ***Mechanism of morpholino mediated knockdown (Bill et. al. 2009):***
(A) Structure of the morpholino oligonucleotide.
(B) Morpholino oligos inhibiting translation of the targeted protein by binding its mRNA.

Needle pulling: Microinjection needles were made from glass capillaries of 1mm diameter. Capillaries were pulled on a Model P-97 Flaming/ Brown micropipette puller (Sutter instruments Co.) to make the fine-tipped needles. The settings of the instrument are as follows:

Heat: 540 Pull: 30 Velocity: 120 Time: 200

Needle calibration: Microinjections were done using the model PLI-100 pico-injector microinjection apparatus from Harvard apparatus. In order to calibrate the needle, the morpholino was taken up in the needle by applying negative pressure and then, some of it was injected into a layer of mineral oil. The diameter of the drop was measured microscopically and the volume of the drop was calculated according to the instructions provided by the manufacturer of the apparatus. The applied pressure is then adjusted in order to get the desired injection volume.

Microinjection to embryos:

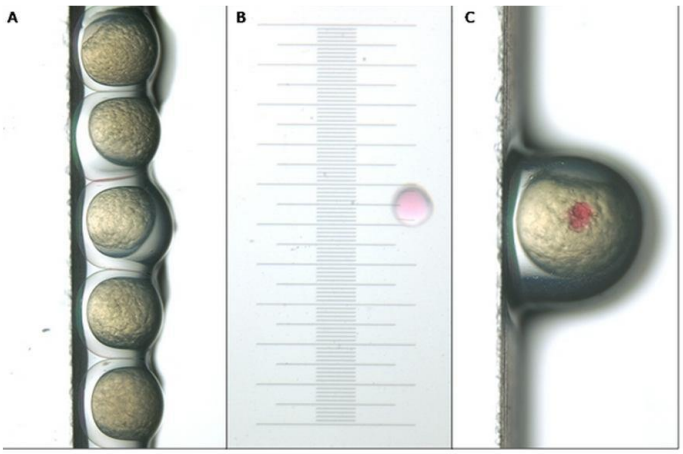


Fig. 3.3. ***Microinjection in zebrafish embryo at 1-2 cell stage:***

(A)The zebrafish embryos were lines up against a microscopic slide.
(B)The size of the morpholino dose was adjusted to a drop size of 150 micron, thus injecting 2 nl of morpholino in each egg.
(C)Morpholino was injected into the egg. (Jonathan et. al. 2009)

The eggs were collected after 15-20 minutes after the removal of the separator so as to ensure that they were still in one celled stage. The morpholinos were heated at 65^{O}C and snap cooled on ice prior to the injections. This was done in order to reduce the formation of secondary structures of the oligonucleotides. The morpholinos were then taken up in the needles and the needle was calibrated so as to inject 1.8-2 nl morpholino per injection. The eggs were aligned along the edge of a glass slide and the injections were done in the yolk of each egg while the embryo was still at 1-2 celled

stage (Fig. 3.3). The morpholinos used in this study were supplied by Gene Tools LLC and the sequences are as follows:

Standard Control: 5'- CCTCTTACCTCAGTTACAATTTATA-3'
Fmn2b_z_tb: 5'-ATGAGCGGCGGCGGTTTCAAGCCAT-3'

DiI staining: The larvae were injected with morpholino oligonucleotides at one or two celled stage and then allowed to grow. At 4dpf, they were fixed in 4% paraformaldehyde. Fixation was done at 4^{O}C for 24 hours. The larvae were then incubated in 3 µM DiI. Once in DiI, the larvae were kept on a shaker at room temperature for 30 minutes to ensure that the labelling was homogenous. After that, they were moved to 38^{O}C incubator and stored there for 22 hours. Once the incubation was complete, the larvae were rinsed in 1X phosphate buffered saline (PBS) thrice and then given 5-6 washes in 1X PBS for 20 minutes each. The larvae were then imaged under a stereomicroscope. Fluorescence imaging was done to visualize the DiI staining and bright field imaging was done to image somite boundaries.

Statistical analysis: Unpaired two-tailed student's t-test was used to analyse the difference between the experimental and control groups. The difference was considered significant if the p-value was less than 0.05. All analysis was done using Graphpad Prism 6.0 or Ms-Excel.

Results and discussion:

Morpholino microinjections:

The larvae were studied at 5dpf for any defect in morphology after morpholino injections. It was seen that the larvae injected with control morpholino had morphology similar to wild type (pictures not shown). However, the larvae injected with Fmn2b morpholino had defects in their morphology. The most common defect that was seen was the larvae had a twisted tail (Fig. 4.2). A few of the Fmn2b injected larvae had extreme defects like twisted, short bodies and cardiac edemas (Fig. 4.1). Such larvae were then excluded from further analysis.

As these extreme defects were not seen in the control morpholino injected larvae, it was confirmed that the defects are due to the knockdown of the Fmn2b protein and not because of any non-specific effect of the morpholino.

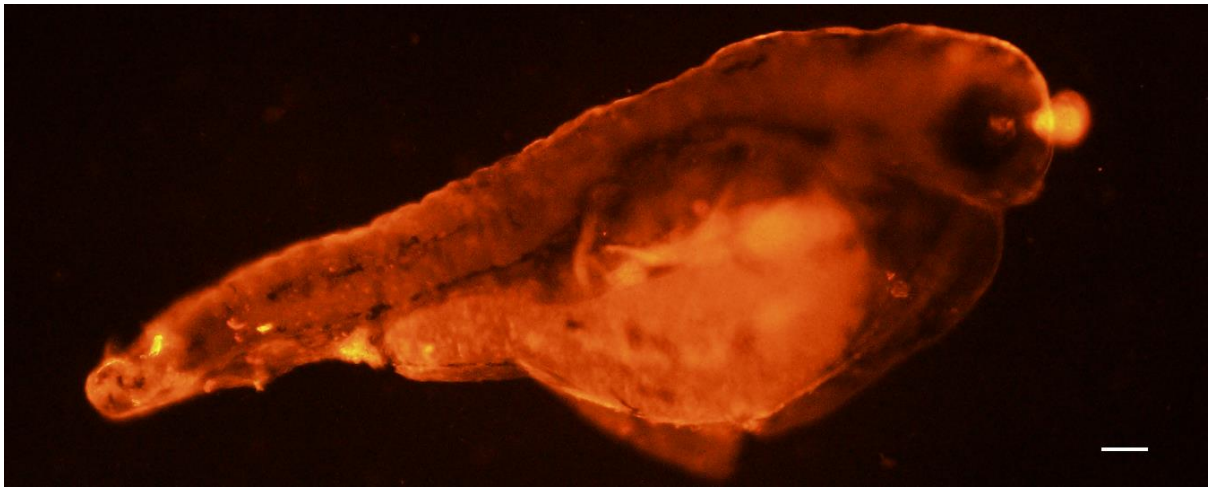


Fig 4.1. ***Fmn2b morpholino injected larva:*** *The larva shows extreme defects like short body and cardiac edema (Image taken under red fluorescent channel). Scale bar=200um.*

Defects in the lateral line system:

As mentioned earlier, the behavioral experiments done in our lab showed balance defects in formin 2b knockdown larvae and made us hypothesize that Fmn2b plays a role in the development of inner ear. Due to the similarity of the lateral line system and inner ear and due to its accessibility, we decided to test whether the lateral line system system had developmental defects due to Fmn2b knockdown. The protocol for labelling the lateral line using DiI was standardized. The protocol allowed us to visualize neuromasts, the sensory organs of the lateral line system using fluorescence

microscopy. Various aspects of the lateral line system, such as the number of neuromasts and the distance between them were analyzed in order to study the effect of the knockdown on the system.

1. Number of neuromasts:

The lateral line system has a stereotyped number of neuromasts. Also, as the neuromasts are deposited as a result of collective migration of cells similar to that of neuronal pathfinding, we analysed the number of primary neuromasts deposited in the control and Fmn2b knockdown larvae. Fig. 4.1(A) shows the control morpholino injected larva while (B) shows the Fmn2b morpholino treated larva.

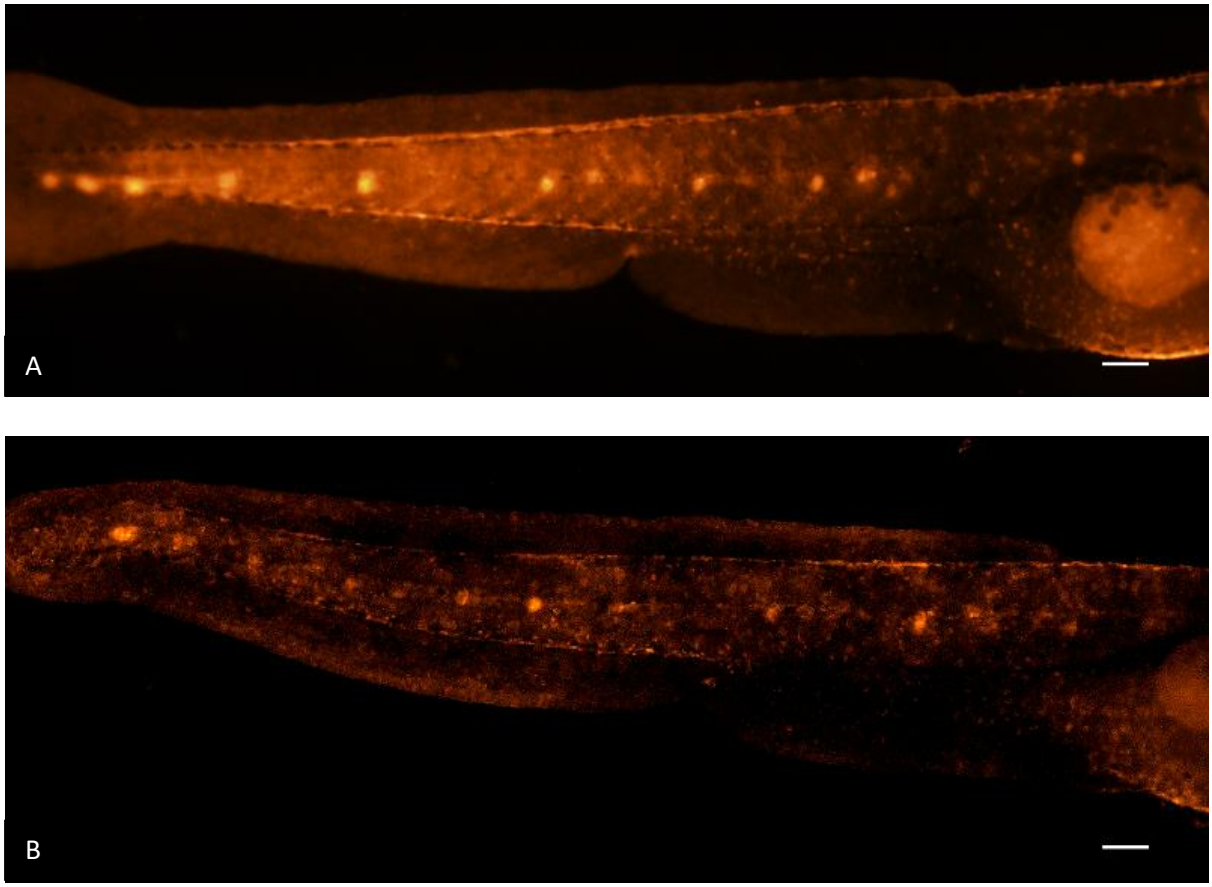


Fig.4.2: ***Control and Fmn2b knockdown larvae labelled with DiI:*** *The white arrows show the neuromasts.*
(A) ***Control morpholino injected larva:*** *5 neuromasts present at approximately equal interval.*
(B) ***Fmn2b morpholino injected larva:*** *Only three primary neuromasts are present and the distance between them is increased as compared to control morpholino injected larva.*
Scale bar= 200um

We found that indeed there is a significant difference in the number of neuromasts in the larvae in the two groups as shown in fig. 4.3. The number of neuromasts in the larvae with the knockdown was less as compared to those in the control group ($p < 0.0001$). The total number of larvae analysed was 67 (134 lateral lines) for control group and 46 (92 lateral lines) for experimental group.

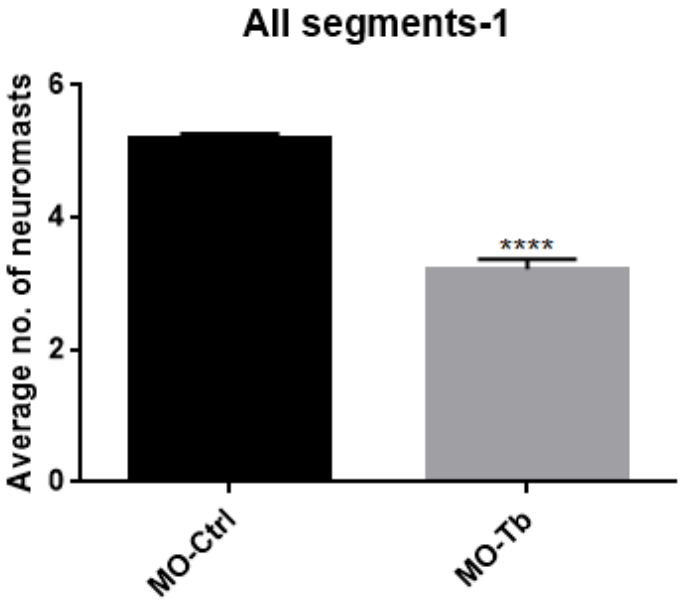


Fig. 4.3. ***Average number of neuromasts:*** *The number of neuromasts found along the entire lateral line system on each side of the larva (N=67 for MO-Ctrl and N=46 for MO-Tb; ****: p<0.0001).*

A reduction in the number of neuromasts suggested either a defect in the migration of the primordium or a defect in the deposition of neuromasts by the migrating primordium. If there was a defect in the migration of the primordium, the number of neuromasts will be more severely affected in the caudal regions as compared to the rostral regions. In order to check this possibility, the number of neuromasts was analysed by considering different segments (fig 4.4).

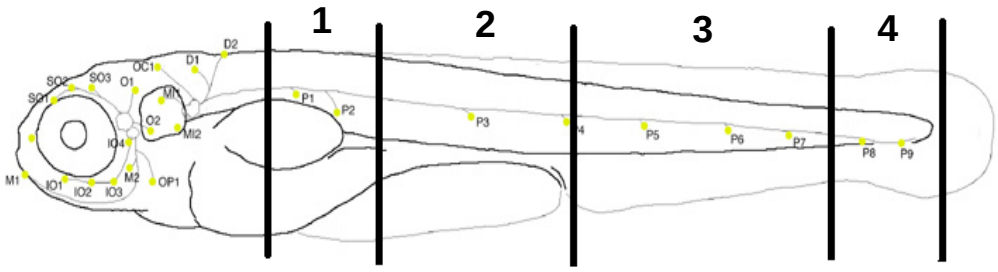


Fig 4.4. ***Segmentation of larva:*** *The larva was divided into 4 segments in order to analyse the number of neuromasts.*

Segment 4 was not considered for analysis as the number of neuromasts is not constant and the somite boundaries are not clearly visible in the tail.

It was seen that the number of neuromasts in all the segments was significantly affected (fig. 4.5). The number of larvae analysed was 40 (80 lateral lines) for control group and 43 (86 lateral lines) for the experimental group. This suggests that the defect is not in the migration of the primordium, but in the deposition of neuromasts. Also, this defect is uniform across the entire lateral line.

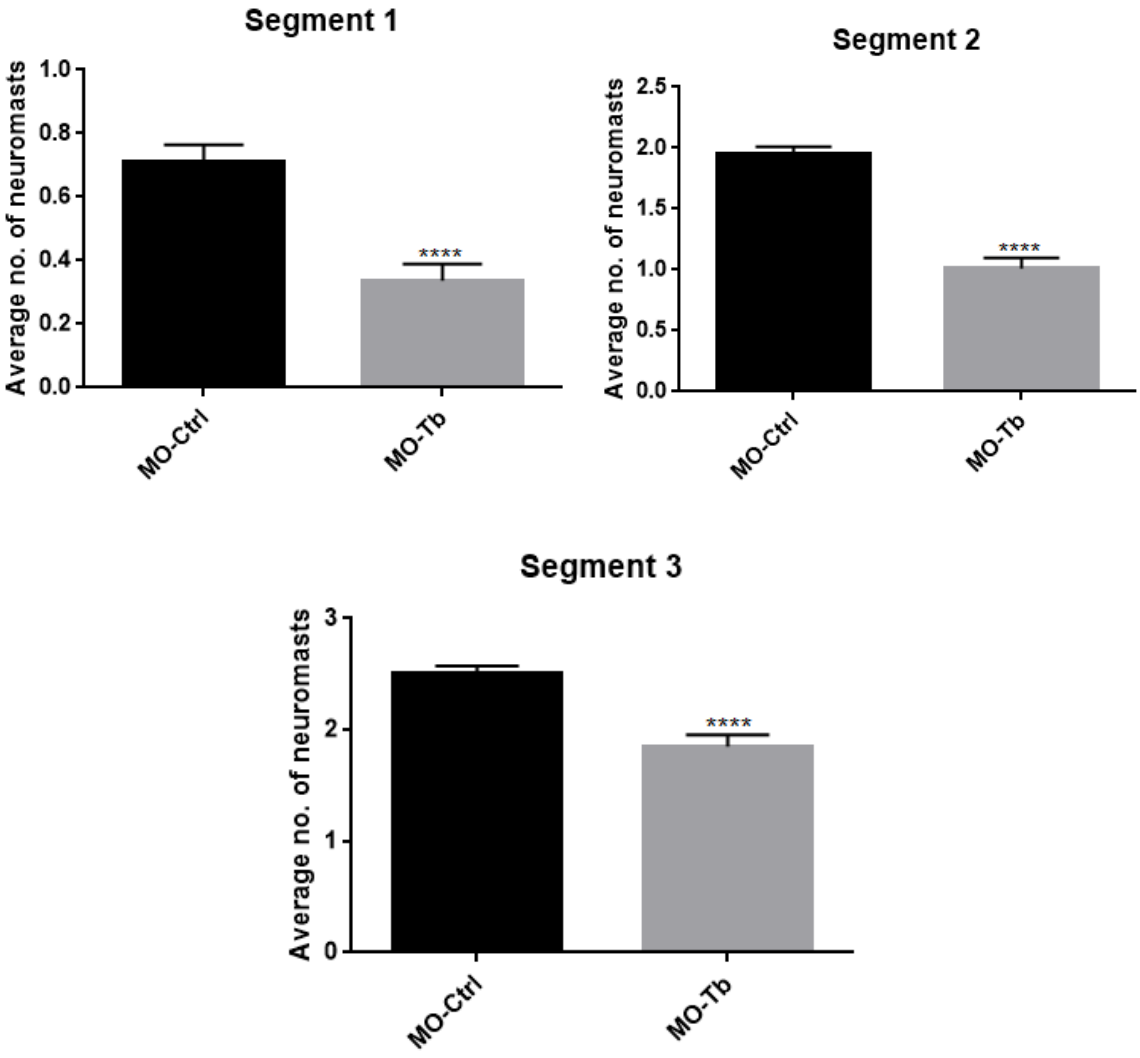


Fig. 4.5. ***Average number of neuromasts per segment:*** *The number of neuromasts found in different segments on each side of the larva (N=40 for MO-Ctrl and N=43 for MO-Tb; ****: p<0.0001).*

Similar results are seen when the number of larvae having a particular number of neuromasts is plotted. It is seen that whereas the number of neuromasts present in each segment is more or less constant in the control larvae, there is a lot of variability

seen in the Fmn2b knockdown larvae (fig. 4.6). Also, some of the larvae in the Fmn2b knockdown group have less than the expected number of neuromasts per segment.

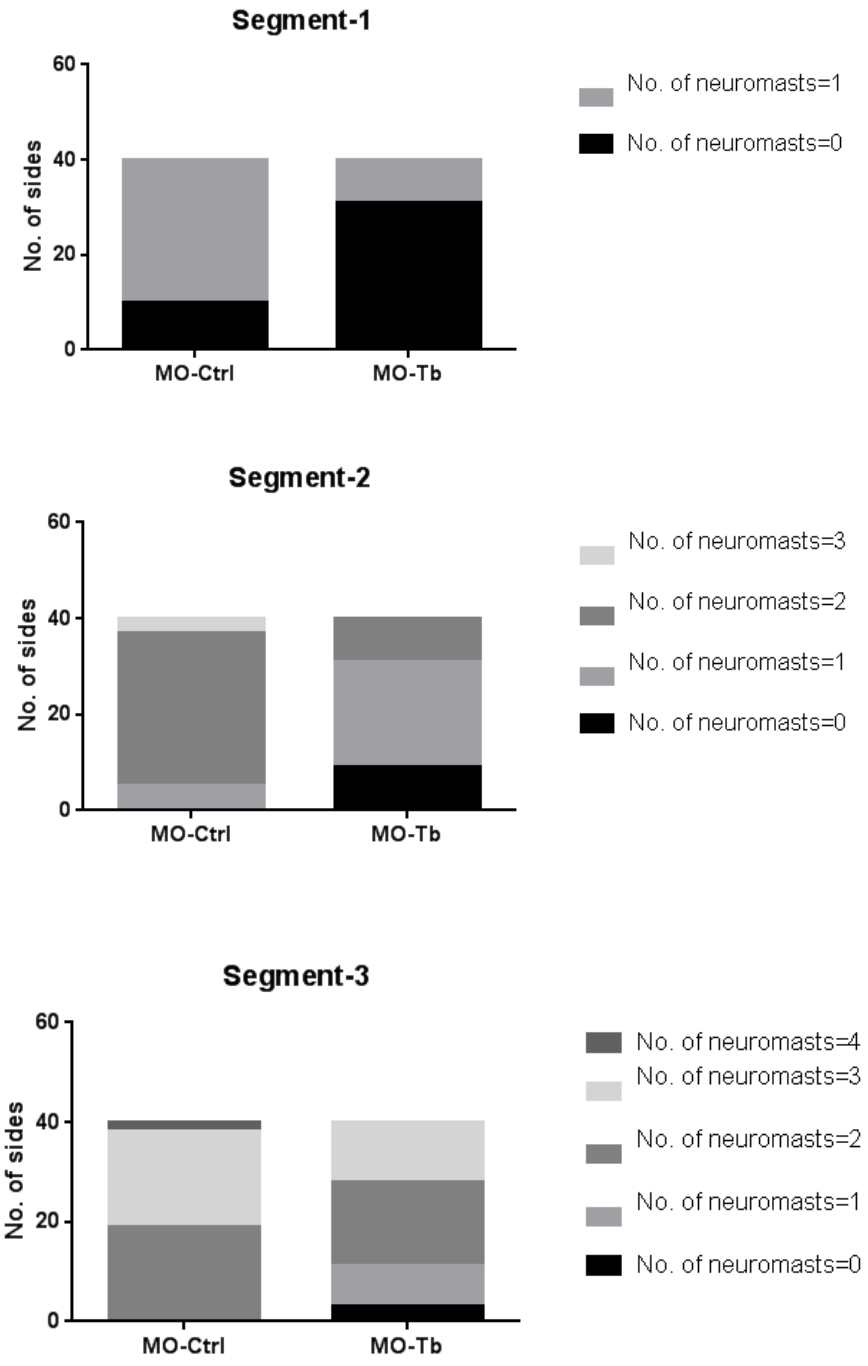


Fig. 4.6. ***Number of sides of larvae having a particular number of neuromasts:*** *It is seen that there is a lot of variability in the number of neuromasts found per segment in the MO-Tb group.*

2. Distance between neuromasts:

It has been shown earlier that there is a lot of variability in the position at which the neuromast is deposited, but the distance between the neuromasts remains more or less constant (Gompel et. al., 2001).

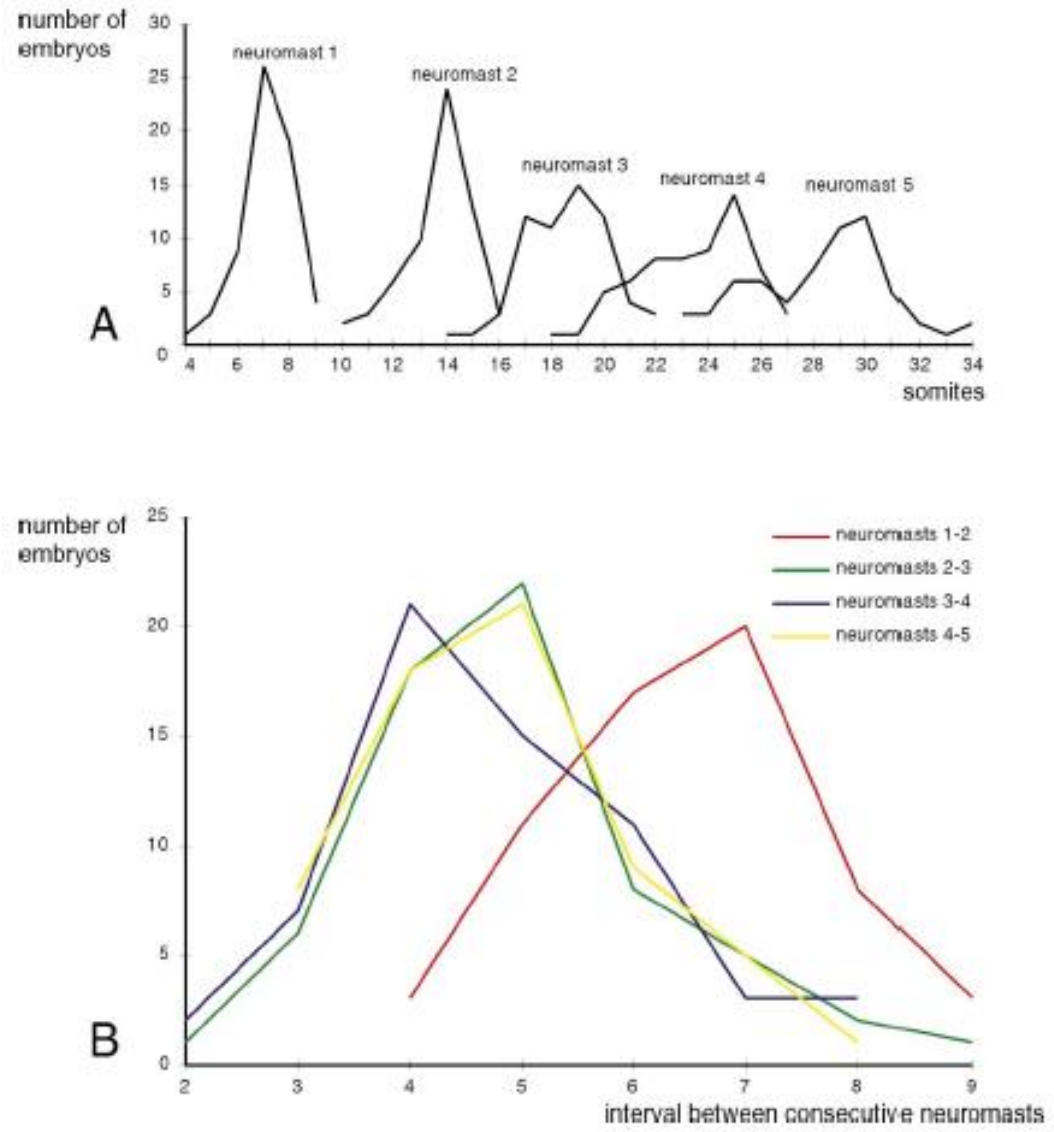


Fig. 4.7. ***Position and interval between neuromasts (Gompel et. al. 2011):***

(A) There is variability in the absolute position where the neuromast is deposited. This variability increses towards the caudal end.

(B) The interval between the neuromasts is constant irrespective of the position of neuromast.

This suggests that the deposition of a new neuromast depends not on the absolute position but on either physical or temporal distance from the previous neuromast. This could also mean that the number of neuromasts can vary depending on the distance between the neuromasts; if the distance is less, there can be more number of neuromasts while if the distance is more, the number of neuromasts will be less. Hence, we decided to check if this regulation of distance between the neuromasts is affected in Fmn2b knockdown larvae.

It is seen that there is a significant increase in the distance between the neuromasts in the Fmn2b knockdown larvae as compared to the control larvae (fig. 4.8). This increase in distance between the neuromasts could be the reason why the number of neuromasts is reduced in the Fmn2b knockdown larvae.

The number of somites between the neuromasts is used as a measure of distance between them. Zebrafish have around 30 somites at the completion of somitogenensis irrespective of the length of the embryo. Hence, when number of somites is used as a measure of distance, the effect of length on the positioning of the neuromasts is already taken into account.

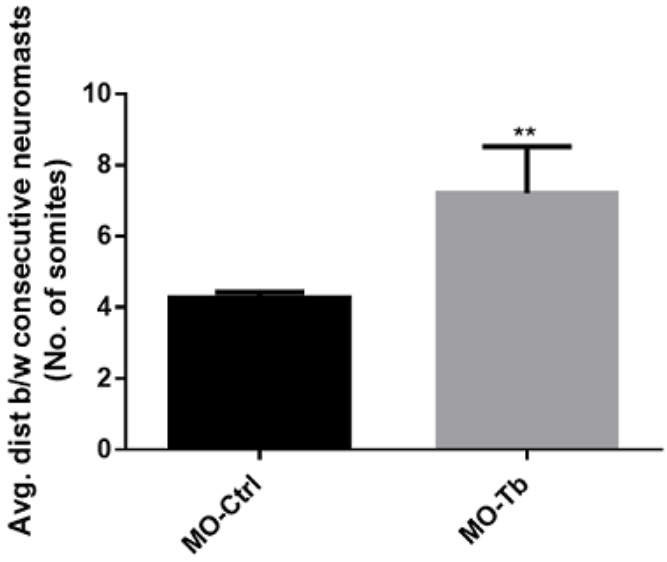
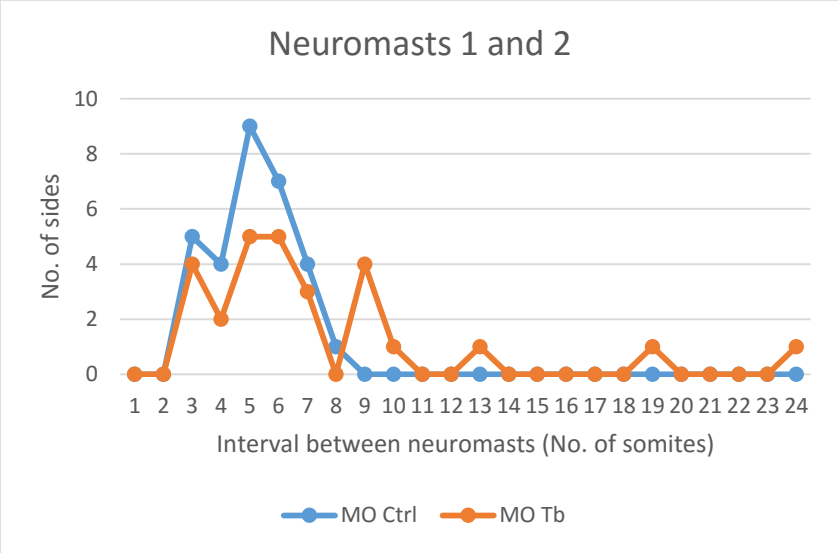
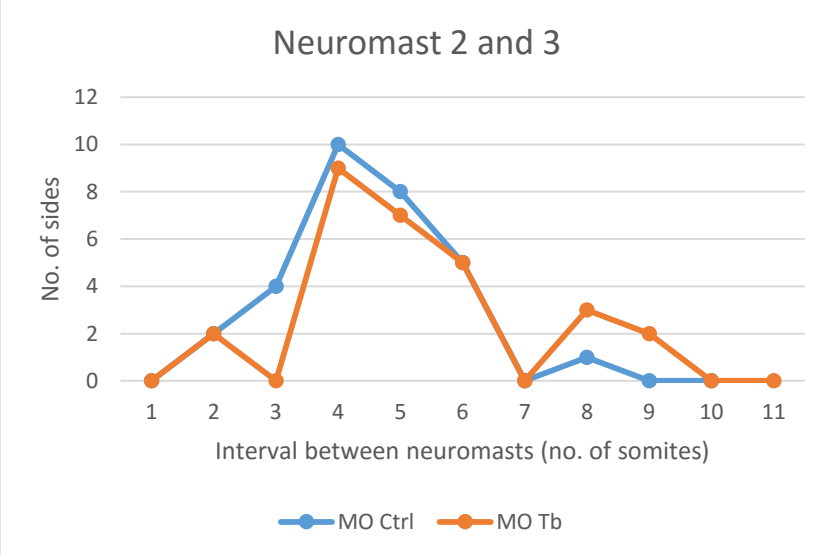


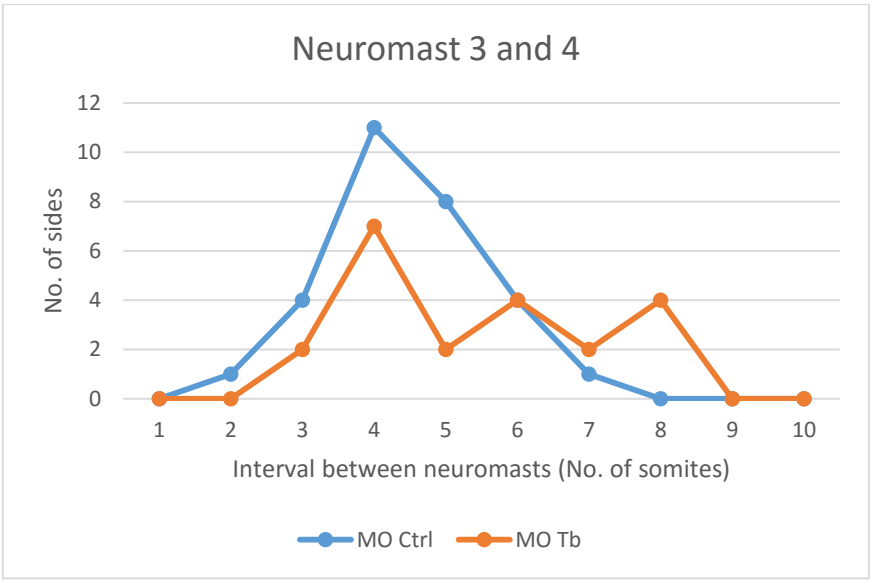
Fig. 4.8. ***Average distance between the neuromasts:***
*The distance between consecutive neuromasts is significantly increased in MO-Tb group. (N=20 for each group; **: p<0.001)*

When the distribution of the distance between consecutive neuromasts is plotted (fig. 4.9), it is seen that the distance between the neuromasts in all the segments in the Fmn2b knockdown group is more random as compared to the control group. A majority of larvae in the control group have an interval of 4-6 somites in between them. However, the Fmn2b knockdown larvae show a much wider distribution in terms of number of somites between consecutive neuromasts.

Even though the number of larvae analysed for each group is the same (N=15), as any of the Fmn2b knockdown larvae do not have 4-5 neuromasts, the number falls in the graph showing the interval between neuromasts 4 and 5.







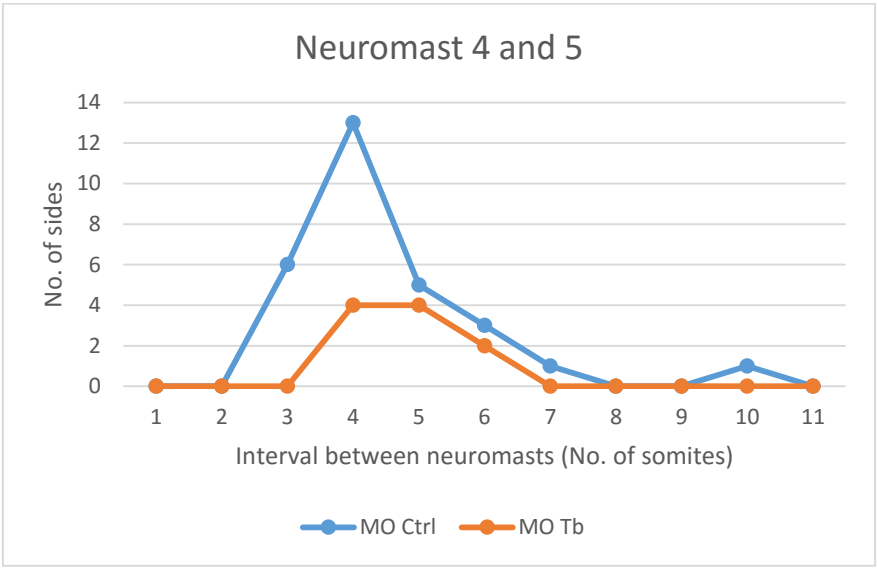


Fig. 4.9. ***Intervals between consecutive neuromasts:***
The distribution of the distances between consecutive neuromasts is much wider in the MO-Tb larvae as compared to the MO-Ctrl larvae.

These results show that the regulation of distance between the neuromasts is lost in Fmn2b knockdown larvae causing deposition of fewer neuromasts at abnormal intervals. However, as the tail neuromasts are seen even in the Fmn2b knockdown larvae, this further testifies that the defects are due to faulty deposition of the neuromasts and not due to problems in migration of the primordium.

One possible mechanism for the defective deposition of neuromasts could be that due to absence of Fmn2b in the cells affects the actin assembly in cells, and causing problems in the apical constriction of cells in the rosette. This prevents/ delays the

formation of protoneuromast and hence affects the deposition of the neuromasts. Further studies need to be done in order to confirm this hypothesis.

As lateral line is one of the organs involved in the maintenance of balance in zebrafish, the defects in the lateral line characterized here can explain the balance defects seen the behaviour experiments. However, as the primary organ responsible for balance is the inner ear, experiments still have to be done to check for defects due to Fmn2b knockdown in the inner ear.

Conclusions and future work:

The behaviour experiments on Fmn2b knockdown zebrafish suggest that Fmn2b might play a role in the development of the inner ear. Given the similarity between inner ear and lateral line and other evidences like role of actin in deposition of neuromasts, similarity between the migrating primordium and growth cone led us to study the effect of Fmn2b knockdown on the lateral line system of the zebrafish. I have tried to elucidate the role of Fmn2b in the deposition of neuromasts during the lateral line primordium migration. It was seen that the number of neuromasts in the Fmn2b larvae is reduced as compared to the controls. Also, the distance between the neuromasts is increased suggesting a defect in deposition of neuromasts. However, the exact mechanism of how Fmn2b plays a role in deposition of neuromasts is still unclear.

To illustrate the mechanism by which Fmn2b functions in development of the lateral line, the first step would be to analyse the extent of knockdown of protein by the morpholino. This can be done by western blots. However, as there is no commercially available antibody against Fmn2b, western blots cannot be done. Other methods like using sham controls and rescue experiments need to be done in order to confirm that the phenotype seen is due to Fmn2b knockdown by the morpholino. Other methods of knockdown, for example, CRISPR-Cas based knockdown can also be used. As CRISPR inhibits transcription, the extent of knockdown could then be studied using Q-PCR.

In order to get more insight about how the deposition of neuromasts is affected, live imaging can be done using transgenic CldnB::lynGFP larvae which express GFP in the neuromasts. Other aspects like the size and structure of neuromasts can also be analysed using this transgenic line.

It has been shown earlier (Gompel et. al., 2001), the deposition of neuromasts is dependent on the spacing between the neuromasts and not on their absolute position. This means that the reduced number of neuromasts in the Fmn2b knockdown larvae could be because of the loss of regulation of spacing between the neuromasts. The loss in regulation of spacing could be because of defects in rosette formation due to improper actin assembly as discussed earlier. Experiments need to be done to check

whether there indeed is a defect in actin assembly in the migrating primordium. This could be done by immunohistochemistry experiments using antibody against F-actin.

All the defects characterized in the lateral line system are gross defects in development. However, given the similarity between the filopodia in growth cone of neurons and stereocilia in hair cells of the neuromasts as well as the inner ear, it is plausible that Fmn2b plays a role in the development of the stereocilia too. These minute defects are yet to be studied. FESEM studies of the inner ear and neuromasts will help visualize the hair cells in these organs and study the length and number of stereocilia on them.

As the primary function of the lateral line system is mechanosensation, defects in the development of lateral line will also cause defects in mechanosensation in the Fmn2b knockdown larvae. Hence, a behavioural assay needs to be developed in order to study mechanosensation in these larvae.

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