Evaluation of the role of Formin-2 in the development and function of the posterior lateral line in zebrafish larvae

BSMS thesis submitted towards the partial fulfillment of

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 "**Evaluation of the role of Formin-2 in the development and function of the posterior lateral line in zebrafish larvae**" towards partial fulfilment of BSMS dual degree programme at Indian Institute of Science Education and Research Pune represents the study carried out by Manjari Prakash at IISER Pune under the guidance of Dr. Aurnab Ghose, Associate Professor, Department of Biology, IISER Pune during the academic year of January 2019 - December 2019.

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Declaration

I hereby declare that the matter embodied in this dissertation entitled "**Evaluation of the role of Formin-2 in the development and function of the posterior lateral line in zebrafish larvae**" are the study/work carried out by Manjari Prakash at IISER Pune under the guidance of Dr. Aurnab Ghose, Associate Professor, Department of Biology, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abstract

Interconnected neural circuits give rise to behavior. For the formation of these neural circuits, axonal guidance during development is essential. Axonal guidance requires the remodelling of the actin cytoskeleton that helps the neurons in reaching their respective targets to form neural circuits. Formin-2 (Fmn2) is an actin cytoskeleton remodelling protein. Zebrafish larvae have Fmn2 selectively enriched in the brain, spinal cord, and the retinal ganglionic cells. The knockdown of Fmn2 in zebrafish larvae results in balance defects. The larvae frequently fall on their sides and swim with reduced directionality while receiving tactile stimuli. Zebrafish have different organs for mediating balance and posture that are the lateral line, inner ear, axial muscles, pectoral fins, and the swim bladder. The lateral line helps in the orientation of the larvae towards water current that is essential for keeping balance in the water. The lateral line has a set of mechanosensory organs known as neuromasts that sense mechanical stimuli. The neuromasts are deposited along the trunk and the tail of the larvae by a migrating group of cells known as the primordium. The neuromasts are innervated by sensory neurons. The lateral line system has two branches the anterior lateral line and the posterior lateral line. The goal of the project is to investigate whether any defect in the posterior lateral line contributes to the balance defect observed by Fmn2 knockdown. Our results show that Fmn2 is not involved in mediating the migration of the posterior lateral line primordium and the deposition of the neuromasts. Preliminary data show that the functionality of the neuromasts may get affected by the knockdown of Fmn2. From our study, the overall development and the functionality of the lateral line does not seem to get affected by Fmn2 knockdown. The development and functionality of the lateral line neurons also has to be studied further to examine if the mechanical stimuli is received by the sensory neurons of the lateral line. In order to observe the morphology of the lateral line neurons, we cloned the transgenic constructs HuC:mVenus-CAAX and HuC:mKOFP2-CAAX using gateway cloning. If Fmn2 knockdown does not affect the lateral line balance circuit other balance related organs in zebrafish larvae have to be studied in future.

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Chapter 1: Introduction

1.1 Zebrafish as a model system for studying neurodevelopment

Various psychiatric disorders such as epilepsy, PTSD, anxiety are caused due to genetic or environmental factors or due to defects linked to neuronal connectivity. It is essential to study neuronal connectivity to provide effective treatment for these disorders. Zebrafish (*Danio rerio*) is a useful model organism to study neural development and disorders related to CNS (Kalueff, Stewart, and Gerlai 2014). The transparency seen in earlier stages and the ease in breeding in large numbers make zebrafish a suitable model system for in vivo imaging studies. The posterior lateral line development in zebrafish larvae can provide insights into collective cell movement that can be used for research related to metastatic cancers and molecular mechanisms involved in organogenesis (Olson and Nechiporuk 2018).

1.2 Formin-2

The remodelling actin cytoskeleton is essential for various cell functions including cell motility, division, axon outgrowth, etc. Formins are a family of proteins involved in actin cytoskeleton remodelling (Fig 1.1) (Xu et al., 2004; Lu et al., 2007; Ramabhadran et al., 2012). Formin-2 or Fmn2 is an actin nucleator, F-actin bundler and F-actin elongator (Pfender et al., 2011). Fmn2 is an ortholog of Drosophila formin Cappuccino protein. Fmn2 has three domains for its function, the FH1 domain which binds to profilin bound actin monomers, FH2 domain which helps in actin elongation and the FSI domain which is involved in actin spire interaction (Pfender et al., 2011). The FH2 domains forms a dimer that binds to F-actin and helps in the elongation. Fmn2 is found to be enriched in the brain and the nervous system in chick, mice, and zebrafish (Leader and leder, 2000, unpublished data from lab). Mutations in Fmn2 in humans is known to cause intellectual and cognitive disabilities. It also causes reduced synaptic density in neuronal cells in mice (Law et al, 2014) Recent studies also have shown the role of Fmn2 mutations in causing increased risk of Alzheimer's disease and memory impairment with aging in mice (Agís-Balboa et al. 2017).

The study of Fmn2 knockdown in chick embryos has shown reduced filopodial dynamics in neuronal growth cones. Fmn2 knockdown affects the filopodia numbers, elongation and length. Fmn2 also plays a role in stabilizing focal adhesions in fibroblasts (Sahashrabhudhe et al,2016).

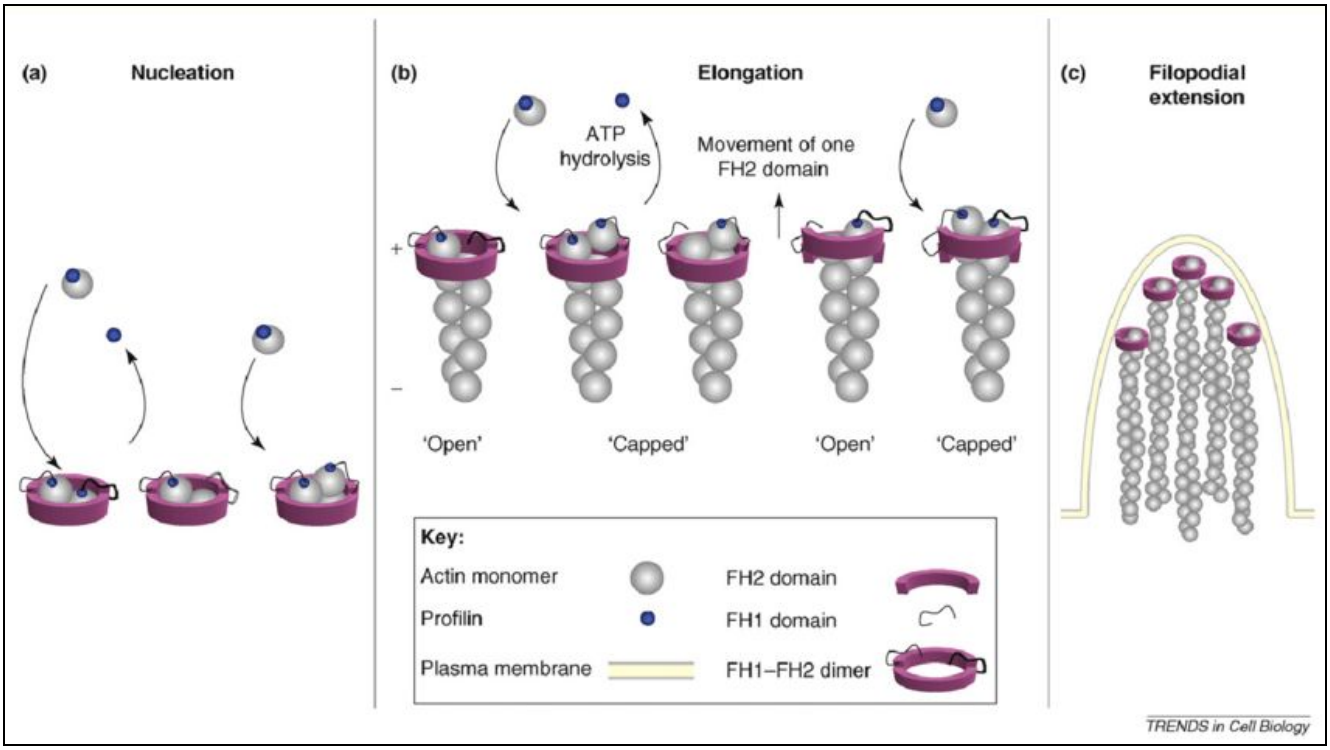


Fig 1.1: Schematic showing Formins function in actin nucleation, elongation and bundling and the elongation F-actin in filopodia (Reproduced from Renault, Bugyi, and Carlier 2008).

1.3 Maintenance of posture in zebrafish

Balance and Posture are maintained by the collective involvement of different organs in zebrafish. Zebrafish is an excellent model organism to study defects in balance. Zebrafish has to maintain its posture against constant nose-down torque and the buoyancy in water(Bagnall and McLean 2014). Sensing the hydrodynamics is essential for orientation of the body to the direction of water flow. Balance is mediated by the lateral line, the inner ear, the swim bladder and also the differential activation of the motor neurons of the axial muscles

coupled with the pectoral fins (Suli et al. 2012; Whitfield et al. 1996; Bagnall and McLean 2014; Ehrlich and Schoppik 2019).

1.4 Fmn2 knockdown leads to balance defects in zebrafish larvae

Studies from the lab by Dhriti Nagar have shown that the knockdown of Fmn2 causes a balance defect in zebrafish larvae. While receiving a tactile stimulus, the Fmn2 morphant larvae would often fall on their sides and take longer time to gain upright position .The larvae end up swimming with reduced directionality compared to the control larvae that would swim away from the stimuli.

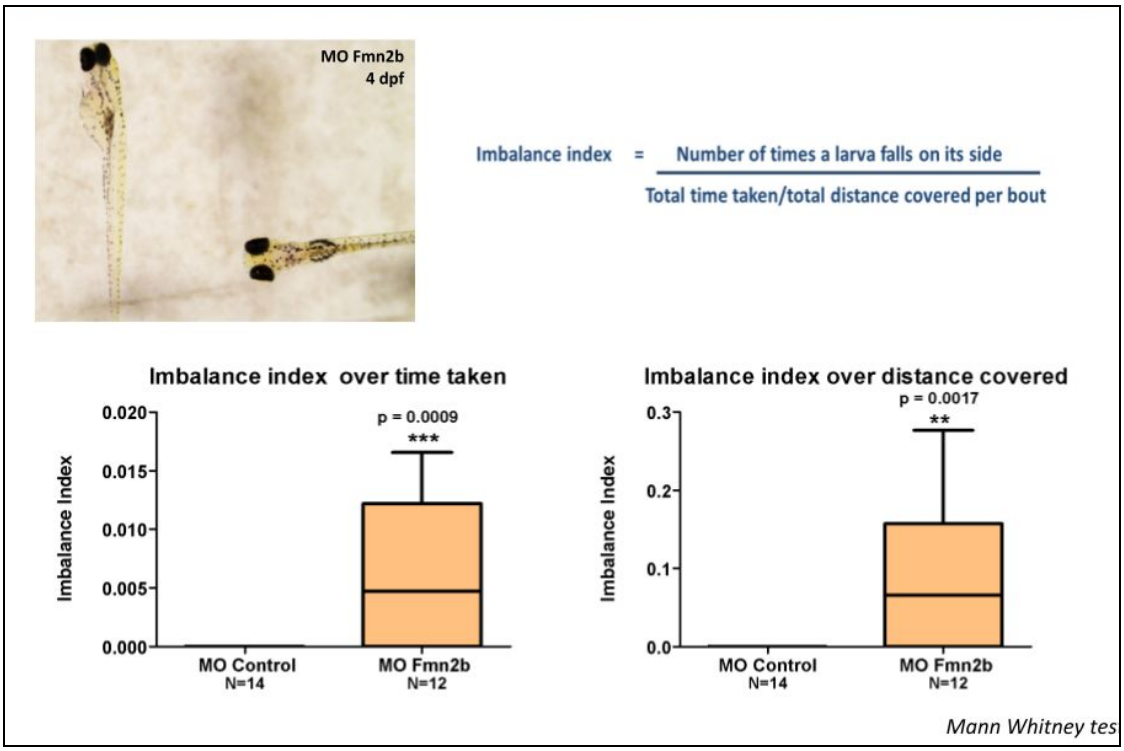


Fig 1.2: Fmn2 morphant larvae show balance defects. Imbalance index is calculated as the number of times the larvae would fall on their sides divided by the time taken. The imbalance index of Fmn2 larvae is significantly higher than that of the control larvae (Credits: Dhriti Nagar).

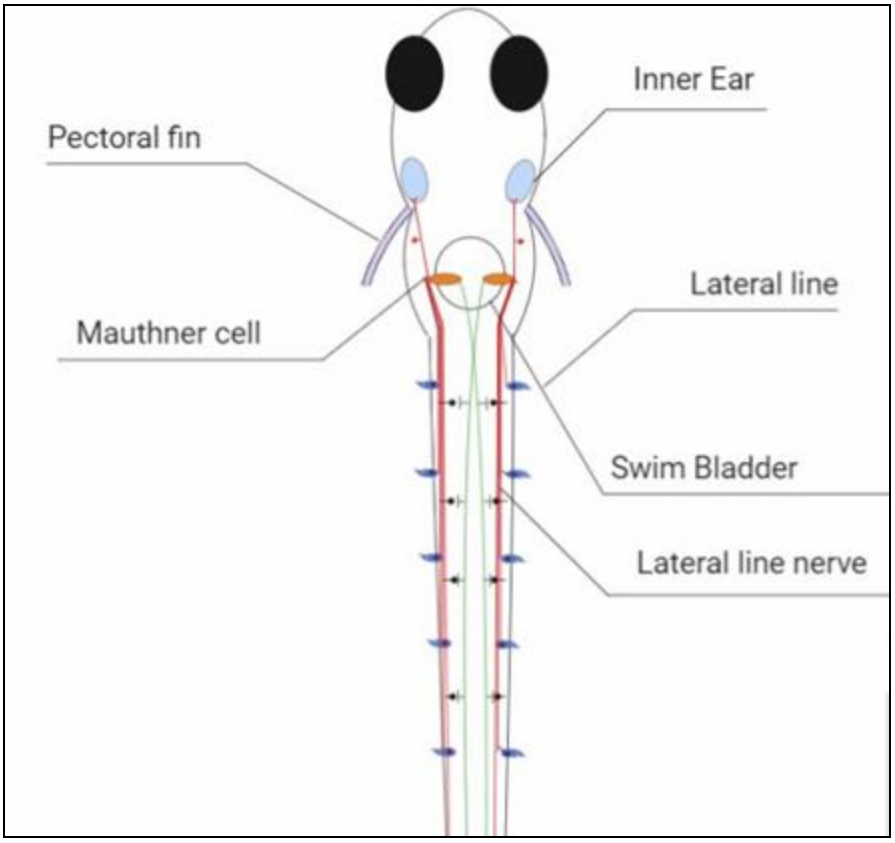


Fig 1.3: Schematic representation of the balance circuit in zebrafish larvae.The hair cells, lateral line afferent neurons, swim bladder, Inner ear and the mauthner cell in the hindbrain are shown.

1.5 Balance related organs in zebrafish

1.5.1 Inner ear

Inner ear is necessary for sensing the acoustic stimuli and for maintaining balance. The inner ear consists of three semicircular canals with patches of hair cells filled with an endolymph to sense rotation in three directions and three maculae with patches of hair cells overlaid by an otolith for sensing linear acceleration in three directions (Kimmel et al., 1995). The hair cells of the inner ear respond to acoustic stimuli. They are innervated by the neurons of the statoacoustic ganglion which sends its central axons to the mauthner cell which is located in

the hindbrain that is involved fast escape responses (Whitfield et al,1996; Whitfield et al, 2002).

1.5.2 Swim bladder

The swim bladder is an air-filled organ that helps in maintaining buoyancy in water. The amount of air in the bladder can be controlled according to need. In larval zebrafish, the swim bladder develops from 1dpf to 3dpf. The inflation and deflation of the swim bladder at appropriate times is necessary for maintaining buoyancy in water (Robertson et al. 2007).

1.5.3 Axial muscles

The dorsal and ventral axial muscles are under the control of parallel microcircuits that produce the necessary torque for self-righting behavior. Larval zebrafish show upright behavior when subjected to nose down torque which is because the center of mass and center of volume are acting at different locations in the body. The differential activation of the motor neurons innervating the dorsal and ventral muscles is essential for producing the torque for self-righting behavior or for the roll to maintain posture. For example for a fish lying by its side, the motoneurons in the ventral side of ear side up and the dorsal side of ear side down was activated that produced the torque for upright behavior (Bagnall and Schoppik, 2018; Bagnall and McLean 2014).

1.5.4 Pectoral fins

Rhythmic swimming by zebrafish larvae and also the turning and feeding requires the pectoral fins that are present on either side of the body. While swimming at a slow speed there is the rhythmic beating of the pectoral fins with up and down movement of the body. While swimming in fast speed pectoral fins remain closer to the body (Green, Ho, and Hale 2011). Pectoral fins helps in maintaining posture during upward climbs during swimming (Ehrlich and Schoppik 2019). Pectoral fins have two main muscles the abductor muscle and the adductor muscle. The abductor muscle is present on one side of the endoskeletal disc that helps expand the fin and the adductor muscle is present on the other side that helps in

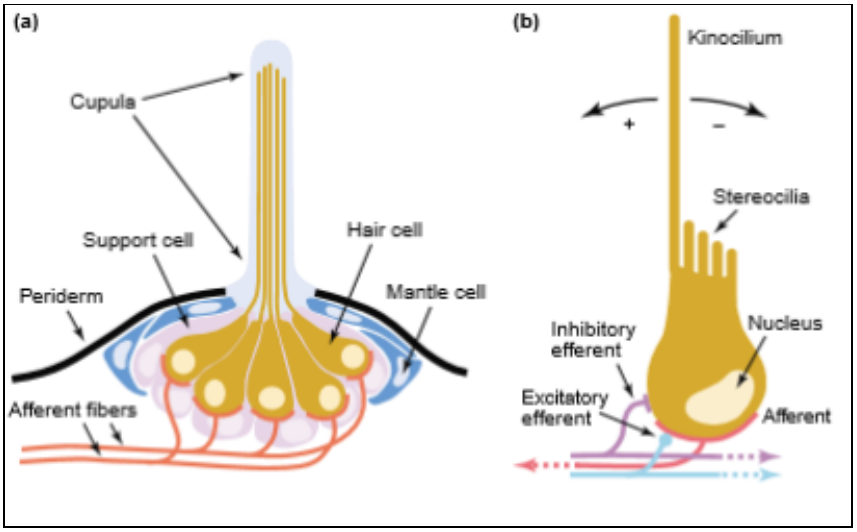
retraction. These muscles are innervated by 4 motor neurons. Pectoral fin muscles and the neurons innervating them develop by 5 dpf. (Thorsen and Hale, 2007).

1.6 Lateral line

The lateral line is a mechanosensory system in zebrafish that is used to sense the mechanical stimuli in water. It helps in maintaining posture, rheotaxis, predator avoidance and schooling (Suli et al. 2012; Pujol-Martí and López-Schier 2013). Larval zebrafish use the lateral line as a gradient sensor by sensing the vorticity of the vector field of water for rheotaxis. This detects the flow dynamics and flow direction of water (Oteiza et al. 2017).

The lateral line consists of mechanosensory organs known as neuromasts that are made up of hair cells surrounded by support cells (Engelmann et al, 2000; Ghysen and Dambly-Chaudière, 2007). Hair cells consists of a long kinocilia made up of microtubules and a set of stereocilia arranged in stepwise fashion made of actin. Kinocilia and stereocilia are connected to each tip links (Kindt, Finch, and Nicolson 2012). The deflection of the stereocilia towards the kinocilia pushes open the mechanosensitive channels which let the potassium ions in which causes depolarisation of the hair cell. The opening of potassium channels, in turn, leads to the opening of the calcium channels and the entry of calcium into the cells. Ca2+ entry causes the release of neurotransmitters to the sensory neurons innervating the neuromasts (Purves et al., 2001). The lateral line consists of 2 branches, the anterior lateral line with neuromasts along with the head and the jaw and the posterior lateral line with neuromasts along the trunk and tail (Gompel et al., 2001; Kimmel et al., 1995).

a)


(a)
Cupula
Support cell
Hair cell
Periderm
Mantle cell
Afferent fibers
(b)
Kinocilium
+
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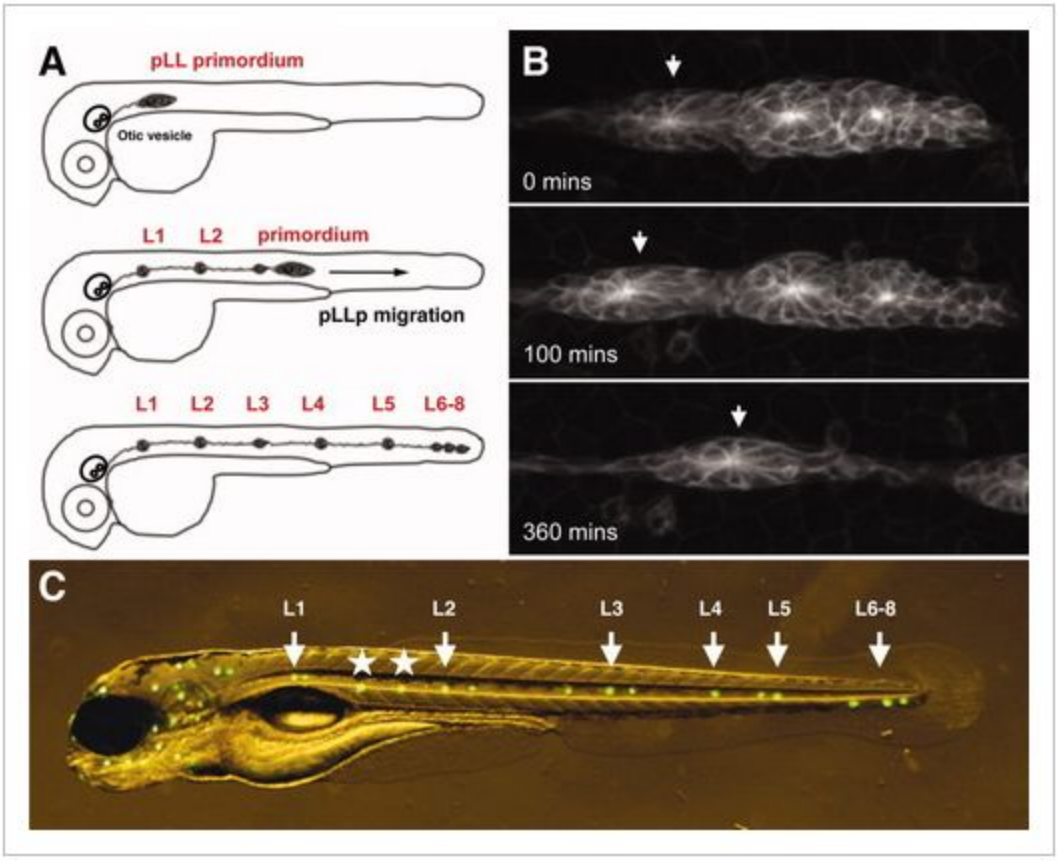
Nucleus
Excitatory efferent
Afferent

b)


A
pLL primordium
Otic vesicle
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pLLp migration
L1 L2 L3 L4 L5 L6-8
B
0 mins
100 mins
360 mins
C
L1
L2
L3
L4
L5
L6-8

Fig 1.4: Neuromasts and posterior lateral line primordium : a) Schematic of a neuromast, the hair cells, support cells, kinocilia, and stereocilia are labelled. b) Schematic to the left shows the deposition of neuromasts by the primordium to the right shows confocal images of the migrating primordium depositing a neuromast labelled by transgenic line Claudin:lyn-GFP (Reproduced from Dambly-Chaudiere et al. 2003, Chitnis et al., 2012)

1.6.1 Molecular signalling involving lateral line primordium migration and deposition of neuromasts

The neuromasts of the posterior lateral line are deposited by a migrating cluster of cells known as the primordium which originates posterior to the otic vesicle and starts migration around 20 hpf (hours post fertilization) (Metcalfe,1985; Kimmel et al, 1995). The primordium periodically deposits a group of eight cellular rosettes which eventually matures into a neuromast. The primordium's leading end (the migratory end) is dominated by Wnt signalling that produces the ligands FGF10 and FGF3 which diffuses to the trailing end activating FGF signalling and the antagonist of FGF signalling Sef prevents FGF signalling in the leading end. Hence the migrating leading end of primordium has dominant Wnt signalling that leads to mesenchymal morphology of cells while the trailing end has FGF signalling that leads to the formation of epithelial rosettes. (Nechiporuk and Raible, 2008; Lecaudey et al. 2008) .

There is a differential expression of cell adhesion molecules N-cadherin and E-cadherin in the leading end and the trailing end (Matsuda and Chitnis 2010). The leading end shows a dominant expression of N-cadherin can help in the migration while the trailing end has dominant E-cadherin that helps in the deposition of the cellular rosettes (Pujol-Martí and López-Schier,2013; Matsuda and Chitnis,2010).

The migration of the primordium is dependent on the expression of CXCL12/SDF (Stromal Derived Factor 1a) in the horizontal myoseptum. The leading end has a dominant expression of CXCR4 receptor for CXCL12/SDFa that helps in the protrusion of the cells. The trailing end shows a dominant expression of CXCR7 receptor that inturn depletes

CXCL12 in the horizontal myoseptum and leads to lesser protrusive activity and deposition of epithelial rosettes (Valentin, Haas, and Gilmour 2007, Haas and Gilmour 2006).

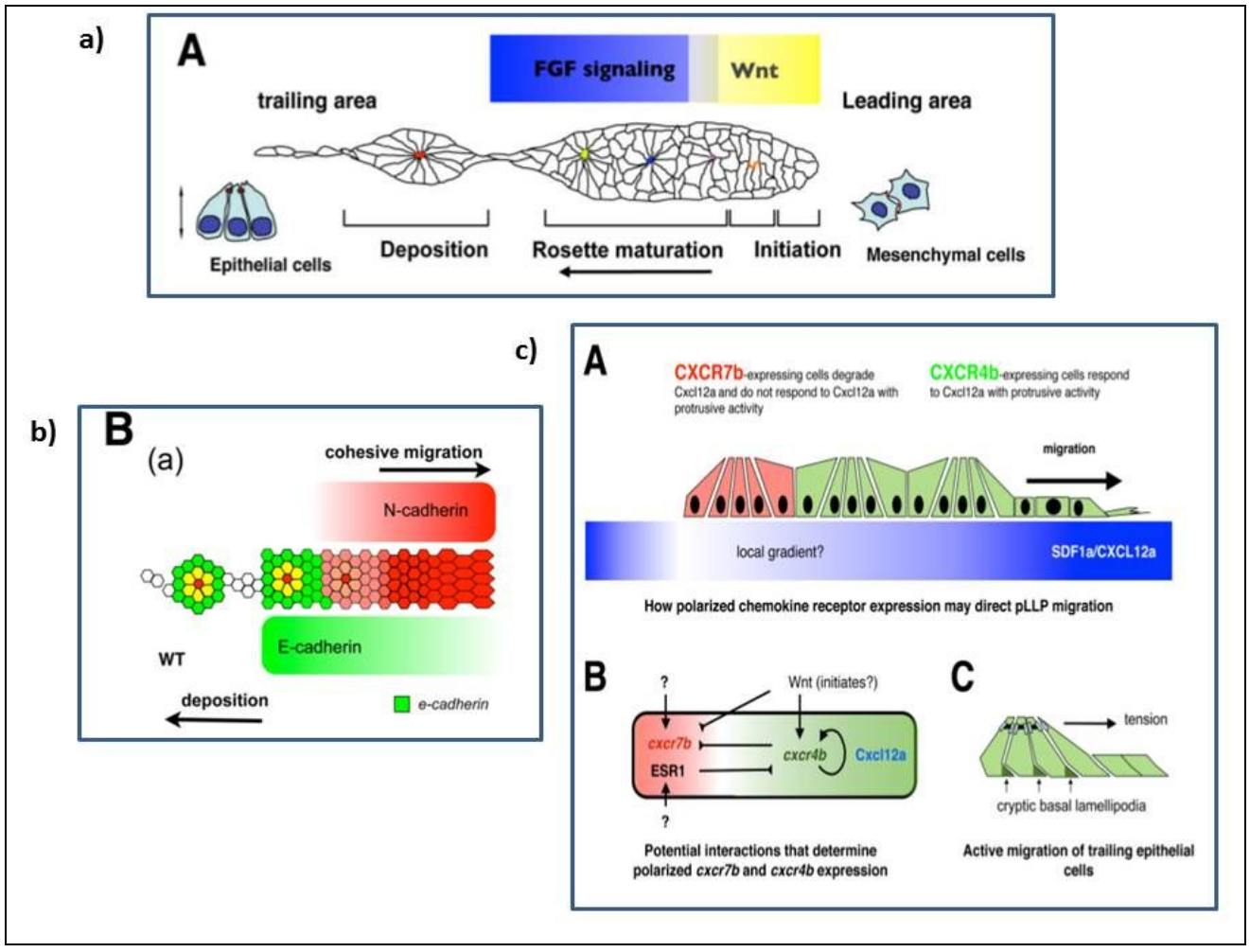

Fig 1.5: Schematic of molecular signalling in the primordium of the posterior lateral line a) Differential Wnt-FGF signalling in the leading and the trailing end of the primordium, leading end has cells with mesenchymal morphology and the trailing end has cells with epithelial morphology; b) Differential expression of cell adhesion molecules N-cadherin and E-cadherin in the primordium that helps in the migration of primordium and the deposition of rosettes; c) Expression of chemokine receptors CXCR4 and CXCR7 in the primordium and the possible downstream signalling pathway (Reproduced from Chitnis et al.,2012)

1.7 Lateral line neurons

The posterior lateral line primordium leaves behind a set of 20 cells which forms the lateral line ganglion. The hair cells of the lateral line are innervated by the neurons of the posterior lateral line ganglion which sends its central axons to the mauthner cells situated in the hindbrain. The mauthner cell helps in mediating fast escape responses (Haehnel, Taguchi, and Liao 2012). The central axons also have a somatotopic representation in the medial octavolateralis nucleus in the hindbrain (Gompel, Dambly-Chaudière, and Ghysen 2001).

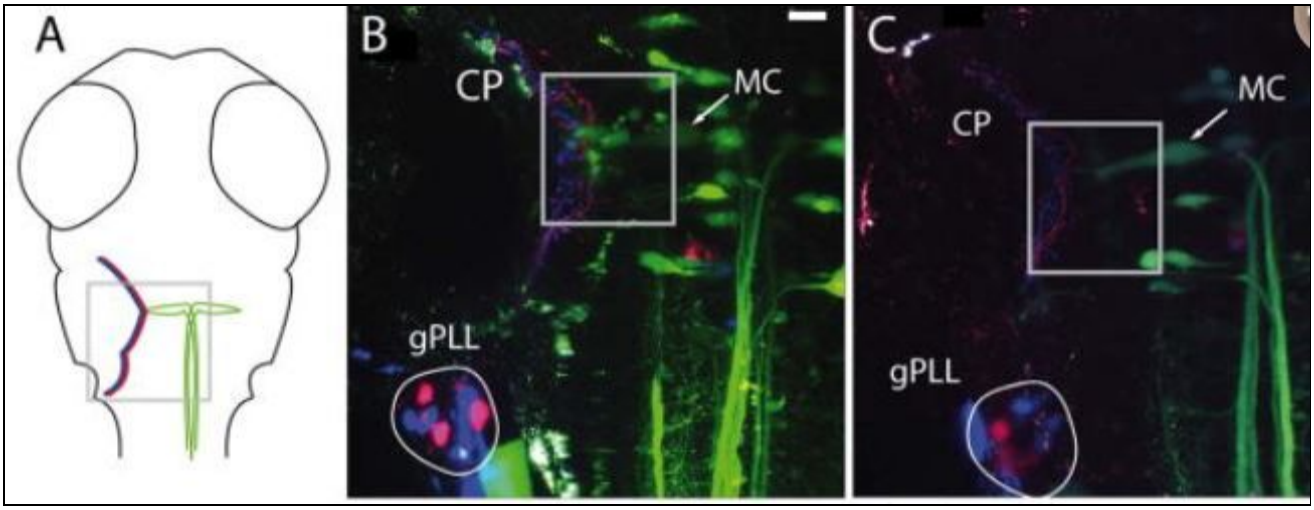

Fig 1.6: Central axons of the posterior lateral line contact the mauthner cell in the hindbrain A) Schematic representation of the lateral line neurons contacting the mauthner.(Mauthner in green, lateral line afferents in blue) B,C) Confocal images of the central projections (CP) of the lateral line afferents contacting the mauthner cell (MC), the posterior lateral line ganglion is also labelled (Reproduced from Haehnel, Taguchi, and Liao 2012).

1.8 Objectives

The objective of this project is to investigate whether the balance defect observed due to Fmn2 knockdown in zebrafish larvae is due to defects in the posterior lateral line balance circuit. For studying the contribution of the lateral line to the balance defect, we are studying

a) The functionality of the lateral line neuromasts

   Rationale: The deflection of stereocilia towards the kinocilia activates the hair cells. The stereocilia and the kinocilia are connected by tip links that push open the mechanosensitive channels due to deflection. The kinocilia are made of microtubules and the stereocilia are made of actin (Kindt, Finch, and Nicolson 2012). Since neuronal growth cones require Fmn2 for actin cytoskeleton remodelling (Sahasrabudhe et al.,2016). Defects in the structure and functioning of the kinocilia or stereocilia due to Fmn2 knockdown can cause the balance defect that is observed.

b) The migration of the lateral line primordium and the deposition of the lateral line neuromasts

   Rationale: The migration and the deposition of the lateral line primordium and the timely deposition of neuromasts depends on collective cell migration and cell-cell adhesion. Fmn2 stabilizes focal adhesions in fibroblasts (Fig 1.2). If Fmn2 knockdown causes a defect in the migration of the primordium or the deposition of the neuromasts, that can lead to either late deposition of neuromasts or fewer neuromasts and failure in the reception of mechanical stimuli which can lead to balance defects.

c) Development of afferent neurons of the posterior lateral line

   Rationale: Fmn2 localizes along actin the filopodia of growth cones and aids in actin polymerisation at the leading end of neuronal growth cones (Sahashrabhudhe et al,2016). The sensory neurons which innervate the hair cells of the neuromasts have their axons migrate following the migrating primordium to reach their respective destination (Metcalfe 1985). Fmn2 depletion is known to causes reduced spine density in neurons and defects in axonal outgrowth (Law et al, 2014; Sahasrabudhe 2016). Fmn2 knockdown can affect the migration of axons to the appropriate hair cells and also their innervation of the hair cells.

Chapter 2: Materials and Methods

2.1 Materials

35 mm coverslip bottom dishes, E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and methylene blue), 1 % low gelling agarose, 1 X tricaine (0.03%) , 1 X PTU (0.003 % phenyl thiourea) , FM4-64 dye (2 µM, (*N*-(3-Triethylammoniumpropyl) -4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide)), Plasmids gateway cloning - (p5E-HuC, pME-mVenus-CAAX, pdestTol2CG) from Addgene, Morpholino constructs - (Control morpholino 5'-CCTCTTACCTCAGTTACAATTTATA -3', Fmn2 Splice Blocking morpholino 5'-ACAGAAGCGGTCATTACTTTTTGGT -3')

2.2 Zebrafish maintenance: Zebrafish transgenic lines TAB5 WT, Claudin-b:lyn-GFP and Brn3c: GAP43-GFP were maintained in standard conditions. Crosses were set overnight and embryos were collected and kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and methylene blue) at 28.5°C on a 14h-10h light-dark cycle. PTU was added at 1 dpf to prevent pigmentation.

2.3 Morpholino injections: Embryos were injected with control morpholino (0.125mM, 2nL) and splice blocking morpholino (0.125mM, 2nL) in one cell stage by Dhriti Nagar. The injected embryos were kept in the E3 medium at 28.5°C on a 14h-10h light-dark cycle. The splice blocking morpholino is a nucleic acid construct that binds between an exon and an intron of Fmn2 mRNA which prevents the normal splicing and the presence of a stop codon in the incorrectly spliced mRNA results in a protein which is truncated without its functional domains that is required actin nucleation and elongation.

2.4 Transgenic lines used

Brn3c:GAP43-GFP : This transgenic line is driven by brn3c promoter has a GAP43 gap junction protein fused to GFP which is membrane-targeted. The hair cells of the neuromasts

and the inner ear are labelled using this transgenic line (gifted by Dr. Shawn Burgess) (Xiao et al., 2005).

Claudin-b:lyn-GFP (created by Darren Gilmour): This transgenic line is driven by claudin B promoter and has the expression of lynEGFP which is membrane-targeted. The epithelial cells get labelled by this line and can be used to visualize the support cells of the neuromasts and the posterior lateral line primordium.

2.5 Methods

2.5.1 Counting neuromasts and imaging: Number of neuromasts were counted at 3 dpf (days post fertilization) using claudin-b:lyn-GFP and at 5 dpf (days post fertilization) using Brn3c: GAP43-GFP. GFP positive larvae were screened from control and Fmn2 morphants and were placed on 2.5% agarose plate for fluorescence imaging in Olympus MVX10 epifluorescence stereoscope. The length of the larvae was measured from head to tail using ImageJ. The number of neuromasts was normalised to the total length.

2.5.2 FM4-64 FX dye staining and imaging: GFP screened larvae of Brn3c:GAP43-GFP at 3dpf and 4dpf were anesthetized using 0.3% tricaine and was immersed in 2 µM of FM4-64 (molecular probes) dye for 30 seconds. The larvae were rinsed twice in E3 medium and mounted using 1% low melting agarose in 35 mm coverslip bottom plates for imaging. Only neuromasts with intact kinocilia and stereocilia were selected for imaging. Imaging was done using confocal mode in Zeiss multiphoton with 25X oil immersion objective. Images were analysed using Imaris. The total intensity sum of the red channel (dye 514 nm) was normalised to the total volume using the surface option in Imaris.

2.5.3 Time-lapse imaging of migrating primordium: Imaging of the migrating primordium was done for 1.5dpf claudin-b:lyn-GFP larvae of control (N=2) and Fmn2 morphants (N=2). The larvae were anaesthetised by using 0.03% tricaine and mounted in 1% low melting agarose on coverslip bottom plates. 40 stacks were taken over the course of 400 minutes

with 10 minutes time interval using confocal mode in Zeiss multiphoton. Images were processed as Maximum intensity projection using Zeiss zen software. The velocity of the primordium was measured by the plugin manual tracking in ImageJ.

2.5.4 Calculation of distance migrated by primordium: Control (N=20) and Fmn2 (N=20) morphant larvae were dechorionated at 1.5dpf. Larvae were mounted in 2 % methylcellulose containing 0.024% tricaine in 35 mm dishes and imaged using 2.5 X objective of Olympus MVX10 epifluorescence microscope. The distance transversed by the primordium was calculated using the ImageJ line tool and was normalised to the total length of the larvae.

2.5.5 Gateway Cloning: Gateway cloning of HuC:mVenus-CAAX was done for mosaic labelling of the afferent nerves of the posterior lateral line. The entry clones p5E-HuC (5'-entry promoter) (HuC cloned to p5E-MCS by Dhriti Nagar) , pME-mVenus-CAAX (Middle entry - reporter protein tagged to membrane-bound protein) , p3E-PolyA (3' - PolyA tail) was cloned to the destination vector pdestTol2CG which expresses cmlc2 GFP in the heart of the larvae. An LR reaction was set overnight at room temperature with the entry clones (75 ng) and the destination vector (75 ng) with LR clonase. The reaction product was transformed into Dh5alpha competent cells. Four positive clones were obtained which was later confirmed with EcoRI digestion which has a cut site in the insert as well as pdestTol2CG. The same procedure was repeated for the cloning of HuC:mKOFP2-CAAX into pdestTol2CG. One positive colony was obtained after EcoRI digestion screening.

2.5.6 Imaging HuC:mVenus-CAAX and HuC:mKOFP2-CAAX

The cloned plasmid of HuC:mVenus-CAAX (40 ng) was injected into TAB5WT in one-cell stage and larvae were screening for mVenus expression at 1.5dpf using Olympus MVX10 epifluorescence microscope. The mVenus positive larvae were selected for confocal imaging was imaged at 488nm using Zeiss multiphoton microscope. The cloned plasmid of HuC:mKOFP2-CAAX (40 ng) was injected into Brn3c:GAP43-GFP in one-cell stage and was screened for mKOFP2 expression using RFP channel in Olympus MVX10 epifluorescence

microscope. The ones showing mKOFP2 expression in the lateral line neurons were selected for confocal imaging at 561 nm using Zeiss multiphoton. For mounting the larvae were kept between two coverslips that were stuck together with vacuum grease (coverslip sandwich) . The coverslip sandwich with the larvae was later stuck to the glass slide using vacuum grease for imaging using 40x objective of Zeiss multiphoton microscope.

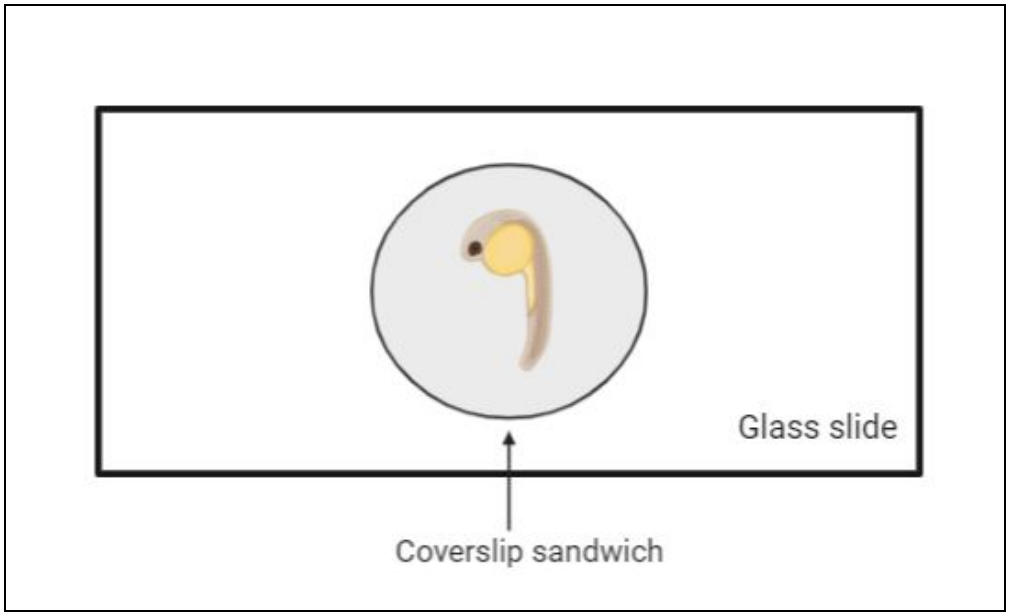

Fig 2.1: Schematic representation of mounting larvae using coverslip sandwich method

Chapter 3: Results

3.1a: Total number of posterior lateral line neuromasts deposited is not affected by Fmn2 knockdown

The posterior lateral line primordium starts migration around 20 hpf depositing about 5-8 neuromasts periodically along the trunk and the tail. The number of posterior lateral line neuromasts were counted using the transgenic line Claudin-b:lyn-GFP (labels support cells of neuromasts) at 3 dpf (days post fertilization (Fig 3.1a), the time point at which the first primordium (primI) has deposited all the neuromasts. At 5 dpf (days post fertilization) , when the primordium II or the prim II has started migration and deposition of neuromasts, the neuromasts were counted using Brn3c:GAP43-GFP (labels hair cells of neuromasts) (Fig 3.2a). (Gompel, Dambly-Chaudière, and Ghysen 2001). The total numbers that were quantified were normalised to the total length of the larvae. There was no significant difference between the number of neuromasts deposited between the control and the Fmn2 morphant larvae at 3 dpf (Fig 3.1b) and at 5 dpf (Fig3.2b). There was a significant difference in the length of the larvae due to Fmn2 knockdown at 3 dpf (Fig 3.1 c) but not at 5 dpf (Fig 3.2 c). The total number of neuromasts normalised to the total length shows no significant difference between the control and the Fmn2 morphants (Fig 3.1 d, Fig 3.2 d). There is a significant difference in the length of the larvae and the number of neuromasts normalised to length between the wild type and the control, this could be due to the smaller sample space of wild type data collected (Fig 3.2 c, Fig 3.2 d).

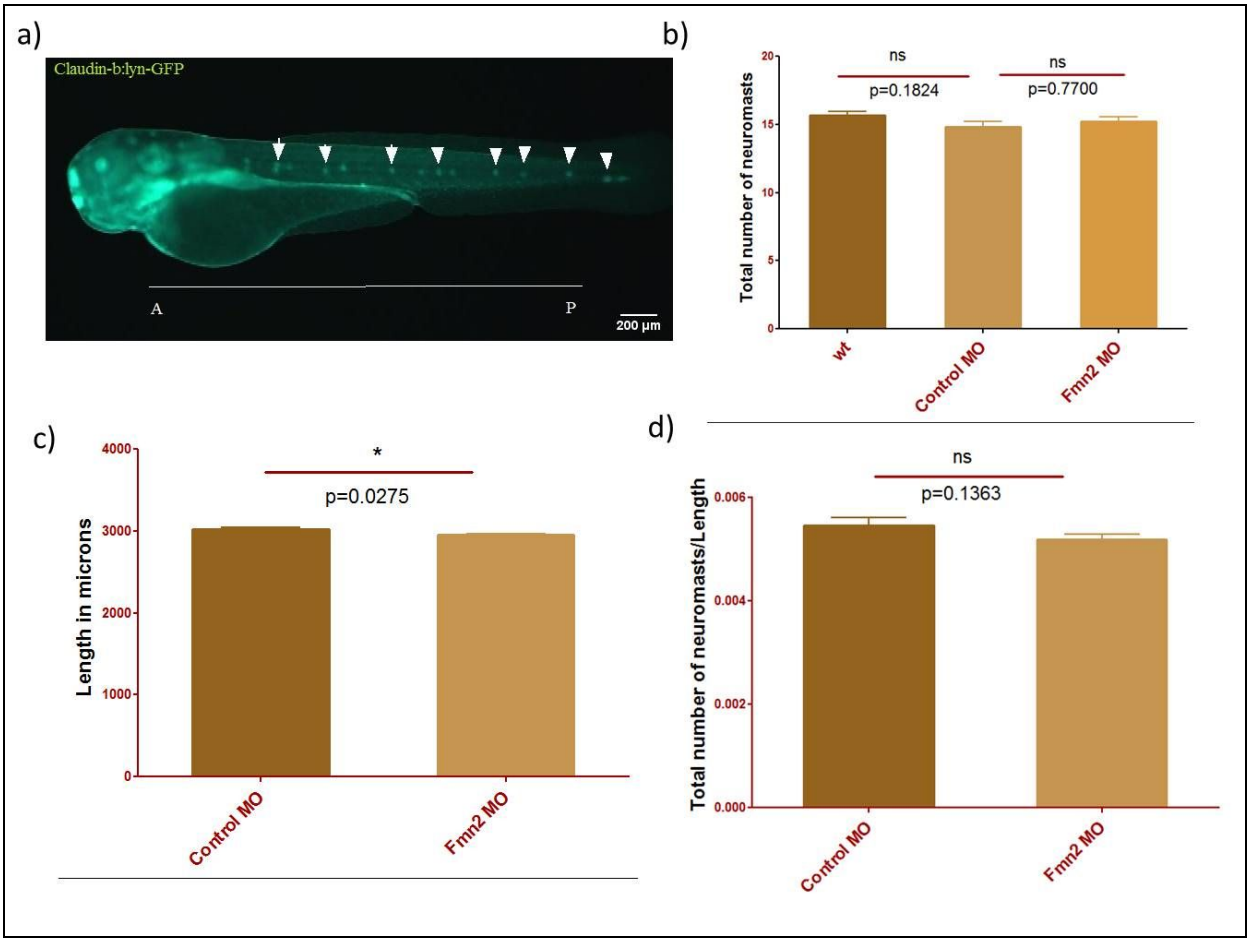

Fig 3.1:Total number of posterior lateral line neuromasts deposited at 3dpf is not affected by Fmn2 knockdown a) Representative image of Claudin-b:lyn-GFP larvae at 3dpf, neuromasts are labelled by arrows, A - Anterior, P - Posterior b) Total number of neuromasts from both sides counted from wild type WT (n=35), control morpholino (MO,n= 48) and Fmn2 splice blocking morpholino injected larvae (Fmn2 MO, n= 23) c) Fmn2 MO (n= 21) larvae has reduced length compared to Control MO (n= 20) d) Total number of neuromasts normalised to the length of larvae for Control MO (n=20) and Fmn2 MO (n=21). Scale = 200 µm.

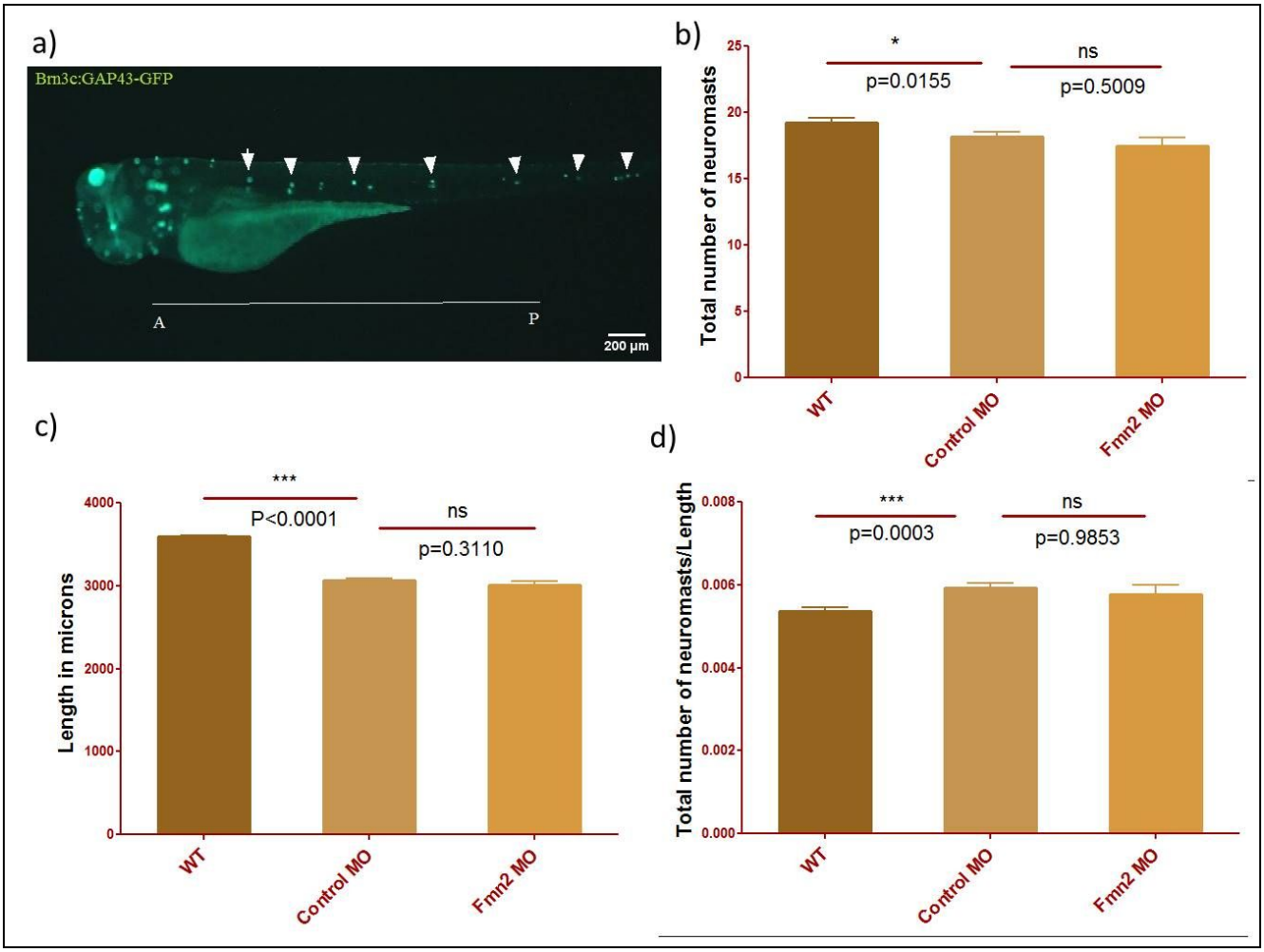

Fig 3.2 :Total number of posterior lateral line neuromasts deposited is not affected by Fmn2 knockdown at 5dpf a) Representative image of Brn3c:GAP43-GFP larvae at 5dpf, neuromasts labelled by arrows; b) Total number of neuromasts from either sides of wild type (WT, n=11), control morpholino injected larvae (Control MO, n=20), Fmn2 splice blocking morpholino (Fmn2 MO, n=13); c) Length of larvae of WT (n=11), Control MO (n=20) and Fmn2 MO (n=13); d) Total number of neuromasts divided by length of larvae of larvae for Control MO (n=20), WT (n=11) and Fmn2 MO(n=13) . Scale = 200 µm.

3.2b The migration of the posterior lateral line neuromasts is unaffected by knockdown of Fmn2 knockdown

The posterior lateral line primordium consists of a group of 100 cells that originates from a placode posterior to the otic vesicle that starts migration around 20 hpf depositing a group of eight cellular rosettes periodically that later matures into a neuromast (Kimmel et al., 1995). The migration of the primordium was visualised using live time lapse imaging in confocal mode using Claudin-b:lyn-GFP (labels support cells of neuromasts) . The posterior lateral line primordium of Fmn2 morphants did not display any gross defect in the migration compared to the control (Fig 3.4, Fig 3.5. The distance migrated by the primordium was also characterised for the control and the Fmn2 morphants at 1.5 dpf using Claudin-b:lyn-GFP . The number for larvae for which the primordium has migrated beyond the yolk sac is not significantly different between control larvae and Fmn2 morphants (Fig 3.3 c). The distance migrated by the primordium divided by the total length of the larvae for the Fmn2 morphants showed no significant difference from the control larvae (Fig 3.3 d).

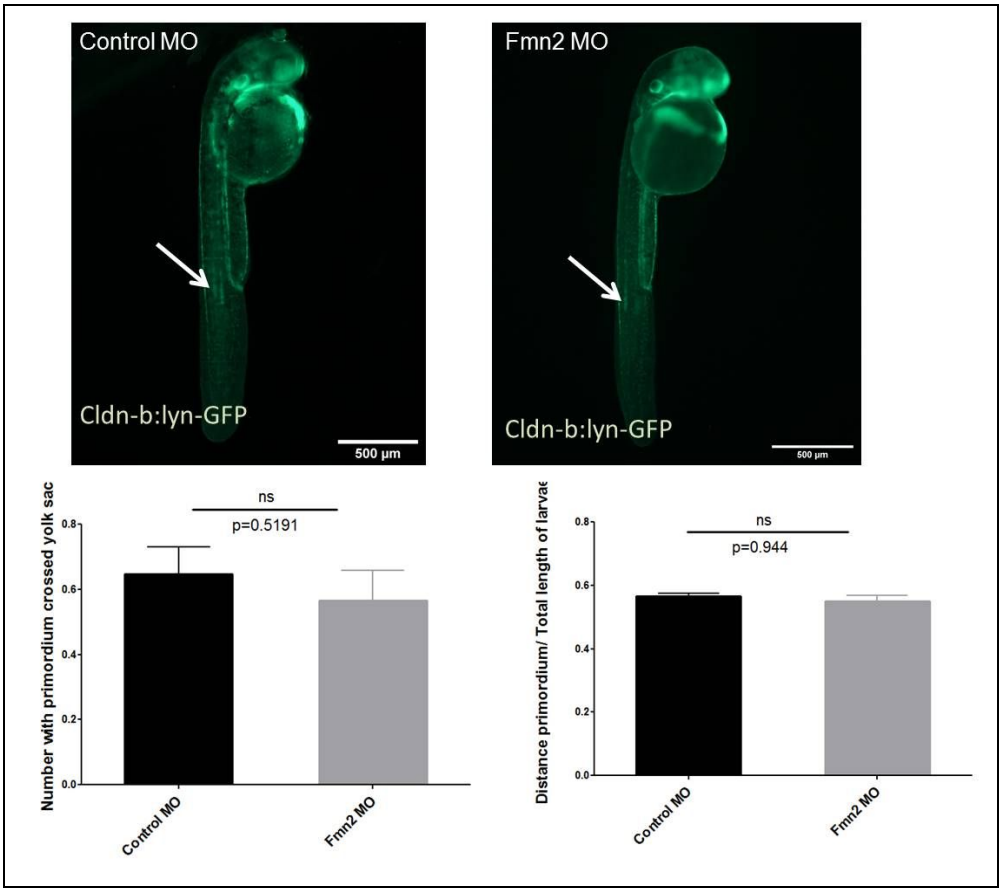

Fig 3.3: The migration of the posterior lateral line neuromasts is unaffected by knockdown of Fmn2, a) Representative image of control morpholino injected claudin-b:lyn-GFP (Control MO) at 1.5dpf, b) Representative image of Fmn2 morphant of claudin-b:lyn-GFP (Fmn2 MO)at 1.5dpf, c) Plot of number of larvae with primordium migrated beyond the yolk sac of control MO (N=30) is not significantly different from Fmn2 MO (N=34), d) Plot of the distance migrated by the primordium of control MO (N=20) is not significantly different from Fmn2 MO (N=20) .Scale = 500 um (White arrow points to the primordium). Scale = 500 μm.

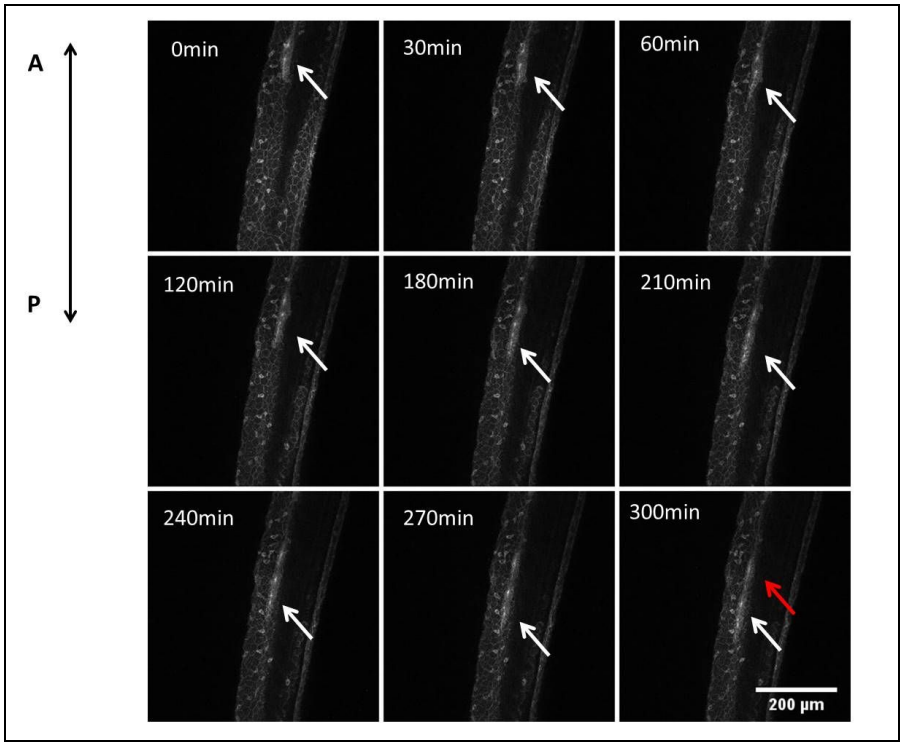

Fig 3.4: Time-lapse Imaging of the migrating primordium at 1.5 dpf control claudin-b: lyn-GFP. The posterior lateral line of 1.5 dpf control claudin-b:lyn-GFP larvae was visualised using confocal imaging over 7 hours in live larvae. The primordium migrates

depositing a set of eight cellular rosettes that later matures into a neuromast. Migration rate - 0.107µm/min . White arrowhead points to the migrating primordium. A separated rosette from the primordium can be seen (bottom right red arrow) (N=2) .Scale = 200 µm.

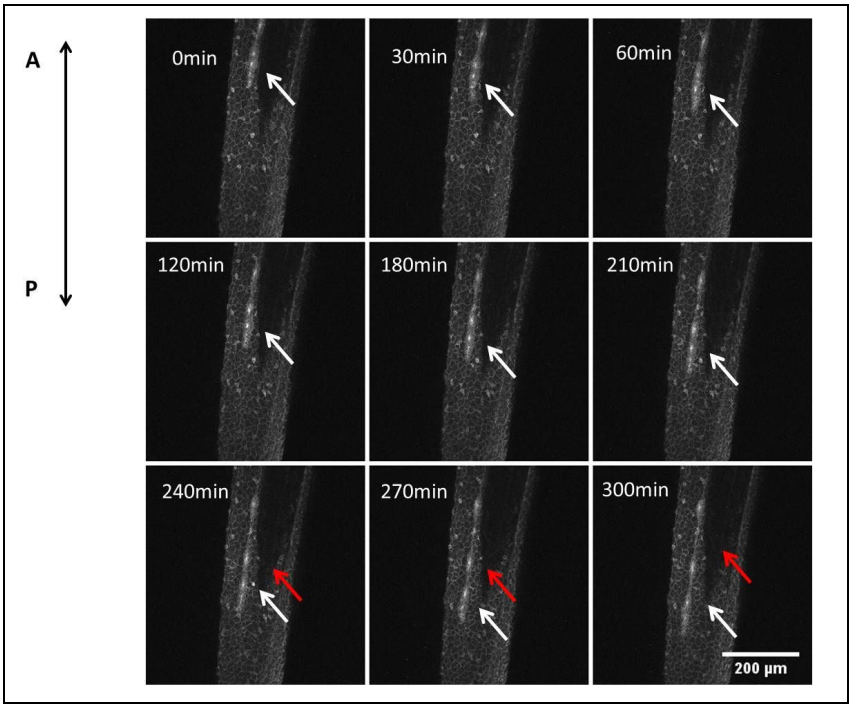

Fig 3.5: Time-lapse Imaging of the migrating primordium in 1.5dpf Fmn2 MO claudin-b: lyn-GFP. The posterior lateral line of 1.5 dpf Fmn2 morphant claudin-b:lyn-GFP larvae was visualised using confocal imaging over 7 hours in live larvae. The primordium migrates depositing a set of eight cellular rosettes that later matures into a neuromast. Migration rate - 0.113 µm/min . White arrowhead points to the migrating primordium. A separated rosette from the primordium can be seen (bottom right red arrow) (N=2) .Scale = 200 µm.

3.3 Fmn2 knockdown may affect the functionality of the posterior lateral line neuromasts

FM4-64 is a styryl dye that can be used to visualize synaptic vesicle recycling. The dye has 3 domains, the lipophilic tail for binding to the membrane, two aromatic rings with a bridge in between for fluorescence and a charged head group that prevents permeability across the membrane. The dye is taken in by the hair cell during endocytosis that happens compensatory to the exocytosis of the cell during stimulation of the hair cell. The dye will exit the hair cell during exocytosis of the vesicles. FM4-64 labelling of hair cells can be used to assess the functionality of the hair cells of the posterior lateral line neuromasts (Iwabuchi et al. 2014). FM4-64 staining was carried out for control and Fmn2 morphant larvae of Brn3c:GAP43-GFP. The dye concentration was standardized at 2 µM after several trials with different concentrations. There was a significant difference (*p=0.02) between the control larvae and the Fmn2 morphant larvae(Fig 3.6 c). This points out that the functioning of the posterior lateral line neuromasts may affected by Fmn2 knockdown.

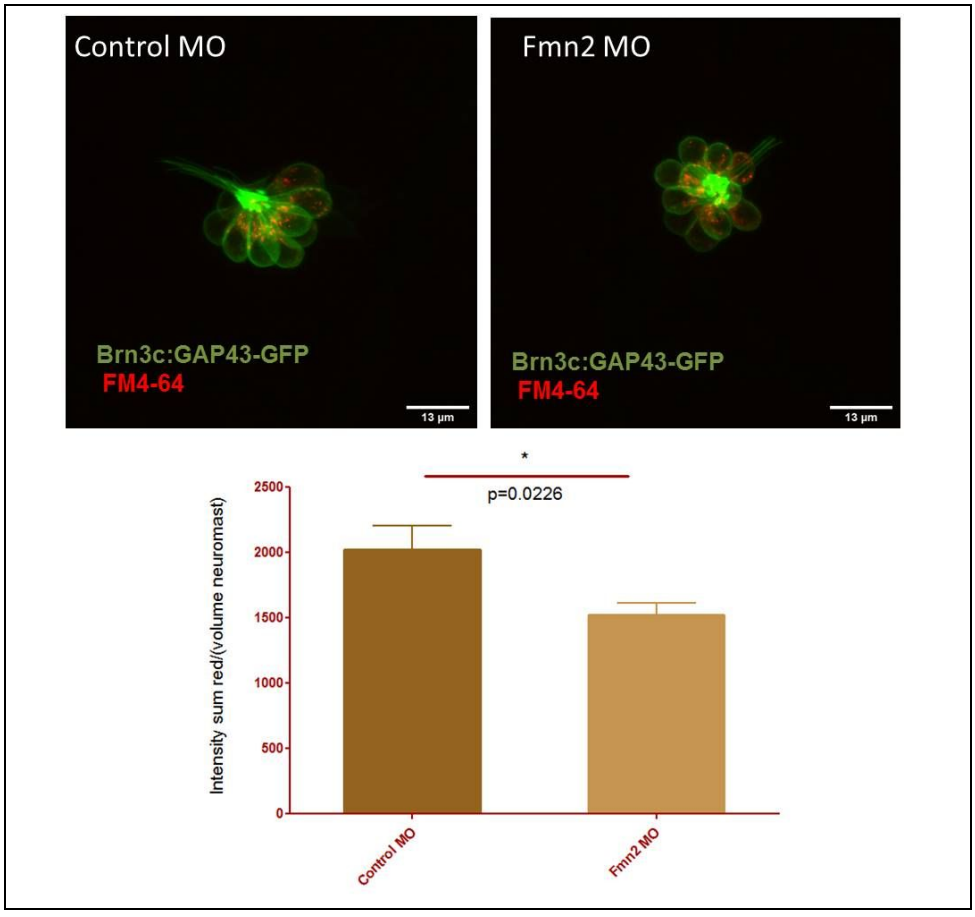

Fig 3.6 : Fmn2 knockdown may affect the functionality of the posterior lateral line neuromasts, a) Representative image of FM4-64 labelled hair cells of 3 dpf control Brn3c:GAP43-GFP (Control MO) b) Representative image of FM4-64 labels hair cells of 3 dpf of Fmn2 morphant Brn3c:GAP43-GFP (Fmn2 MO) , c) Dye uptake quantified using the intensity sum of the red channel (FM4-64) /Total Volume of neuromast (measured using the green channel). Plot of Dye uptake (Intensity sum of FM4-64 uptake/ Volume of the neuromast) of control (Control MO, n=34) and Fmn2 morphants (Fmn2 MO,n=36) show that there is a significant difference in the dye uptake between the Control MO and Fmn2 MO larvae. Quantification was done using Imaris. Scale=12 μm.

3.4 Gateway cloning of HuC:mVenus-CAAX and HuC:mKOFP2-CAAX to visualise the lateral line neurons

HuC is a pan neuronal promoter that expresses in differentiating neurons. A transgenic construct with HuC promoter that drives the expression of a fluorescent protein will label the neurons of the lateral line (Faucherre et al. 2009) . HuC:mVenus-CAAX plasmid can be injected into zebrafish embryos for mosaic labelling of the lateral line neurons. HuC promoter is used to drive the expression of membrane-bound fluorescent proteins mVenus-CAAX and mKOFP2-CAX that labels the lateral line neurons. For the generation of HuC:mVenus-CAAX and HuC:mKOFP2-CAAX gateway cloning was employed. Gateway cloning uses site specific recombination of attL ,attR,attB and attP sites for the generation of clones. The entry clones p5E-HuC (5' promoter with flanking attL4 and attR1 sites) , pME-mVenus-CAAX (Middle entry with flanking attL1 and attR2 sites) , p3E-PolyA (3' entry with flanking attL2 and attR3 sites) was cloned to the destination vector pdestTol2CG with flanking attL4 and attR4 sites that expresses cmlc2 GFP in the heart of the larvae (Fig 3.7 a, c). Similarly for the cloning of HuC:mKOFP2-CAAX the entry clones p5E-HuC, pME-mKOFP2-CAAX, and p3E-PolyA were cloned to the destination vector pdestTol2CG (Fig 3.7d). The cloned

plasmids of HuC:mVenus-CAAX and HuC:mKOFP2-CAAX were injected into TAB5 WT (Wild type) and Brn3c:GAP43-GFP transgenic lines respectively at the one-cell stage. The HuC:mVenus-CAAX injected larvae of TAB5 WT (Wild type line) was imaged at 1 dpf to check for expression. HuC:MKOFP2-CAAX injected into Brn3c:GAP43-GFP was imaged at 2 dpf to check for expression. Plasmid expression of HuC:mKOFP2-CAAX was seen in lateral line neurons (Fig 3.8 c,d,e,f) and that of HuC:mVenus-CAAX was seen in Rohon beard neurons (Fig 3.8 a,b).

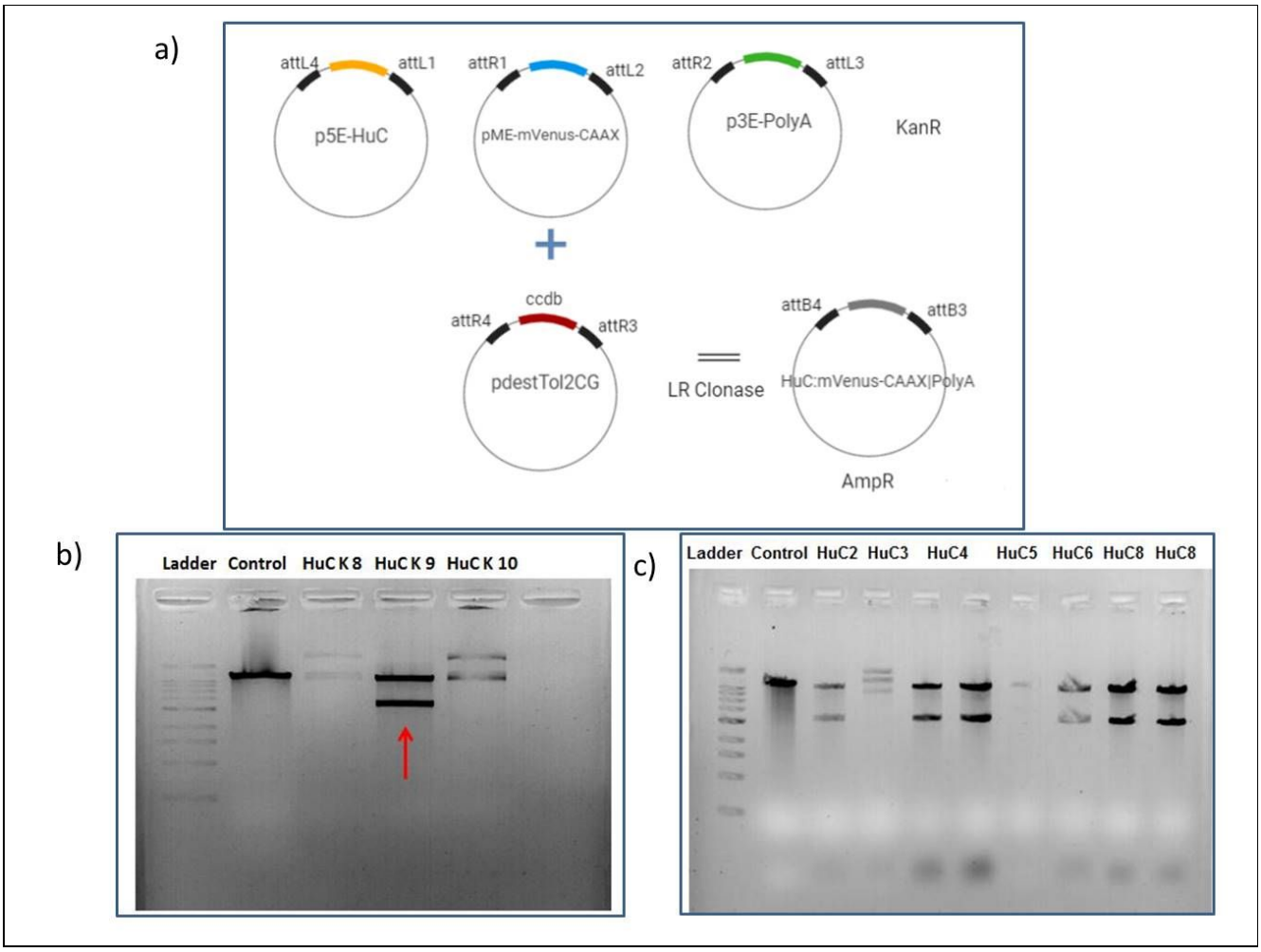

Fig 3.7 : Gateway cloning of HuC:mVenus-CAAX and HuC:mKOFP2-CAAX a) Schematic representation of gateway cloning of HuC:mVenus-CAAX to pdestTol2CG, the entry vectors p5E-HuC, pME-mVenus-CAAX and p3E-PolyA have attL and attR sites that

are recombined with the attL and attR sites in pdestTol2CG destination vector to give attB and attP sites in the cloned plasmid b) Digestion by EcoRI of HuC:mKOFP2-CAAX , positive clones have an additional EcoRI cut site. Hence shows 2 bands compared to control, red arrow shows positive clone c) Digestion of HuC:mVenus-CAAX clones by EcoRI of. Positive clones have an additional EcoRI cut site. HuC 2, HuC 4, HuC 6, HuC 8 are the positive clones.

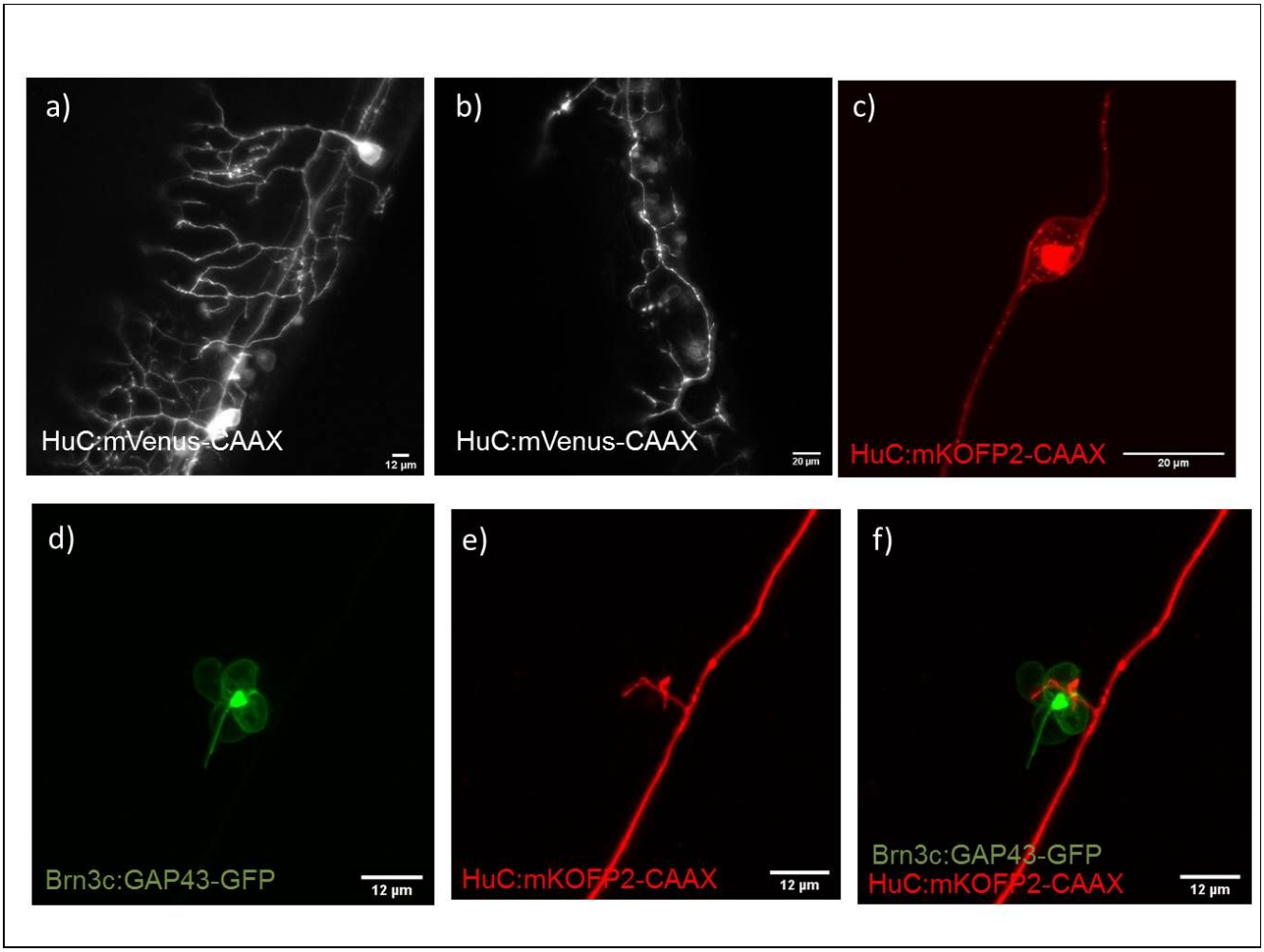

Fig 3.8: Expression of HuC:mVenus-CAAX and HuC:mKOFP2-CAAX, a,b) HuC:mVenus-CAAX injected to TAB5 WT imaged at 1 dpf. HuC:mVenus-CAAX

expression seen in Rohon beard neurons c) HuC:mKOFP2-CAAX expression in Brn3c:GAP43-GFP WT shows labelling of posterior lateral line ganglion; d,e,f) HuC:mKOFP2-CAAX expression in a lateral line neuron innervating the hair cells of the neuromasts, d) Hair cells of a neuromast labelled by Brn3c:GAP43-GFP, e) Lateral line neuron labelled by HuC:mKOFP2-CAAX, f) Merge of both d) and e). Scale=12 um.

Chapter 4 : Discussion and Future Directions

4.1 Conclusion

In this project, we have tried to investigate the role of Fmn2 in the development and the function of posterior lateral line in zebrafish larvae. We arrive at the following conclusions from the results:

a) Fmn2 knockdown does not affect the migration of the primordium
b) Fmn2 is not involved in mediating the deposition of the lateral line neuromasts
c) Fmn2 knockdown may affect the functionality of the lateral line neuromasts

4.2 Fmn2 is not involved in mediating the migration of the posterior lateral line primordium and the deposition of neuromasts

The migration of the lateral line primordium is dependent on the collective migration of cells and also the deposition of rosettes at appropriate time points. Fmn2 knockdown does not affect the number of neuromasts deposited or the migration of the primordium. This could emphasize the presence of actin remodelers such as Arp ⅔ (Actin related protein), other formins and cell adhesion molecules that are involved in mediating this process.

4.3 Fmn2 knockdown may affect the functionality of the lateral line neuromasts

Mechanotransduction by hair cells depends on the deflection of stereocilia towards the kinocilia that results in the stimulation of the hair cells. The functionality that is measured by the dye uptake shows a slight reduction (*p=0.022) between the control and the Fmn2 morphants. The mechanical stimuli is received by the hair cells in spite of Fmn2 knockdown. Fm4-64 labelling can be used to assess the functionality of lateral line neuromasts but is not a substitute for electrophysiology. The caveats in the FM4-64 labelling of neuromasts can be solved by using electrophysical studies that could provide higher resolution about functionality.

4.4 Future Directions

After assessing the development and the functionality of the lateral line neuromasts the next step is to study the lateral line neurons that innervates the neuromasts.Transgenic constructs HuC:mKOFP2-CAAX and HuC:mVenus-CAAX was developed for the mosaic labelling of these neurons.

The future experiments are as follows:

a) to study the innervation of neuromasts by HuC:mKOFP2-CAAX labelled lateral line neurons at 2 dpf.
b) to study the development of the lateral line neurons at 1.5 dpf. The lateral line nerve co-migrates along with the lateral line primordium during development. To study the development of the lateral line neurons, the co-migration of the lateral line nerve with the primordium can be studied using live-time lapse imaging.
c) Assessing the functionality of lateral line neurons using calcium imaging of the transgenic lines NeuroD:Gcamp or NBT:Gcamp5.

The hair cells of the lateral line are also innervated by the efferent neurons from the CNS. Efferent modulation is necessary to not sense the stimulus produced by the motor activity of the larvae. This can help in providing increased sensitivity to stimuli that are not self-generated. The morphology and the activity of the lateral line efferent neurons should also be checked for Fmn2 morphants. (Lunsford, Skandalis, and Liao 2019) .

The lateral line neurons send its axonal projections to the mauthner cell in the hindbrain for fast escape responses. Studies by Dhriti Nagar has shown that the mauthner cell morphology is unaffected by Fmn2 knockdown. The motor neurons stimulated by the mauthner cell also have to be studied in the future. After carrying out the experiments that are listed out, if the lateral line is not contributing to the balance defect observed ,other organs that are involved in mediating balance that are the pectoral fins, axial muscles for self-righting torque, swim bladder and the inner ear has to be studied in detail to provide more insight into the reason behind the balance defect observed by Fmn2 knockdown.

References

1. Almuqbil, M., Hamdan, F. F., Mathonnet, G., Rosenblatt, B. & Srour, M. De novo deletion of FMN2 in a girl with mild non-syndromic intellectual disability. Eur J Med Genet 56, 686–688 (2013)
2. Agís-Balboa, R. C. *et al.* Formin 2 links neuropsychiatric phenotypes at young age to an increased risk for dementia. *EMBO J.* 36, 2815–2828 (2017)
3. Bagnall, M. W. & McLean, D. L. Modular organization of axial microcircuits in zebrafish. *Science* **343**, 197–200 (2014)
4. Bagnall, M. W. & Schoppik, D. Development of vestibular behaviors in zebrafish. *Current Opinion in Neurobiology* **53**, 83–89 (2018)
5. Ehrlich, D. E. & Schoppik, D. A primal role for the vestibular sense in the development of coordinated locomotion. *Elife* **8**, (2019).
6. Faucherre, A., Pujol-Martí, J., Kawakami, K. & López-Schier, H. Afferent neurons of the zebrafish lateral line are strict selectors of hair-cell orientation. *PLoS ONE* **4**, e4477 (2009)
7. Gompel, N. *et al.* Pattern formation in the lateral line of zebrafish. *Mech. Dev.* **105**, 69–77 (2001)
8. Green, M. H., Ho, R. K. & Hale, M. E. Movement and function of the pectoral fins of the larval zebrafish (Danio rerio) during slow swimming. *Journal of Experimental Biology* **214**, 3111–3123 (2011)
9. Haehnel, M., Taguchi, M. & Liao, J. C. Heterogeneity and dynamics of lateral line afferent innervation during development in zebrafish (Danio rerio). *J. Comp. Neurol.* **520**, 1376–1386 (2012)
10. Haas, P. & Gilmour, D. Chemokine signaling mediates self-organizing tissue migration in the zebrafish lateral line. *Dev. Cell* **10**, 673–680 (2006)
11. Iwabuchi, S., Kakazu, Y., Koh, J.-Y., Goodman, K. M. & Harata, N. C. Examination of synaptic vesicle recycling using FM dyes during evoked, spontaneous, and miniature synaptic activities

12. Kalueff, A. V., Stewart, A. M. & Gerlai, R. Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol. Sci.* **35**, 63–75 (2014)
13. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995)
14. Kindt, K. S., Finch, G. & Nicolson, T. Kinocilia mediate mechanosensitivity in developing zebrafish hair cells. *Dev. Cell* **23**, 329–341 (2012)
15. Law R, Dixon-Salazar T, Jerber J, et al. Biallelic truncating mutations in FMN2, encoding the actin-regulatory protein Formin 2, cause nonsyndromic autosomal-recessive intellectual disability. Am J Hum Genet. 2014;95(6):721-8.
16. Leader, B., and Leder, P. (2000). Formin-2, a novel formin homology protein of the cappuccino subfamily, is highly expressed in the developing and adult central nervous system. Mech. Dev. 93, 221–231
17. Lecaudey, V., Cakan-Akdogan, G., Norton, W. H. J. & Gilmour, D. Dynamic Fgf signaling couples morphogenesis and migration in the zebrafish lateral line primordium. *Development* **135**, 2695–2705 (2008)
18. Lunsford, E. T., Skandalis, D. A. & Liao, J. C. Efferent Modulation of Spontaneous Lateral Line Activity During and afterLunsford, E. T., Skandalis, D. A. & Liao, J. C. Efferent Modulation of Spontaneous Lateral Line Activity During and after Zebrafish Motor Commands. http://biorxiv.org/lookup/doi/10.1101/769547 (2019)(2019)
19. Matsuda, M. & Chitnis, A. B. Atoh1a expression must be restricted by Notch signaling for effective morphogenesis of the posterior lateral line primordium in zebrafish. *Development* **137**, 3477–3487 (2010)
20. McClenahan P, Troup M, Scott EK (2012) Fin-Tail Coordination during Escape and Predatory Behavior in Larval Zebrafish. PLoS ONE 7(2): e32295. doi:10.1371/journal.pone.0032295
21. Metcalfe, W.K., Kimmel, C.B., and Schabtach, E. 1985. Anatomy of the posterior lateral line system in young larvae of the zebrafish. J. Comp. Neurol. 233: 377–389
22. Metcalfe, W. K. Sensory neuron growth cones comigrate with posterior lateral line primordial cells in zebrafish. *J. Comp. Neurol.* **238**, 218–224 (1985)

23. Nechiporuk, A. & Raible, D. W. FGF-dependent mechanosensory organ patterning in zebrafish. *Science* **320**, 1774–1777 (2008)
24. Olson, H. M. & Nechiporuk, A. V. Using Zebrafish to Study Collective Cell Migration in Development and Disease. *Front Cell Dev Biol* **6**, 83 (2018)
25. Oteiza, P., Odstrcil, I., Lauder, G., Portugues, R. & Engert, F. A novel mechanism for mechanosensory-based rheotaxis in larval zebrafish. *Nature* **547**, 445–448 (2017)
26. Pujol-Martí, J. & López-Schier, H. Developmental and architectural principles of the lateral-line neural map. *Front Neural Circuits* **7**, 47 (2013)
27. Purves, D. *et al.* Hair Cells and the Mechanoelectrical Transduction of Sound Waves. *Neuroscience. 2nd edition* (2001)
28. Pfender, S., Kuznetsov, V., Pleiser, S., Kerkhoff, E. & Schuh, M. Spire-type actin nucleators cooperate with Formin-2 to drive asymmetric oocyte division. *Curr. Biol.* **21**, 955–960 (2011)
29. Renault, L., Bugyi, B. & Carlier, M.-F. Spire and Cordon-bleu: multifunctional regulators of actin dynamics. *Trends Cell Biol.* **18**, 494–504 (2008)
30. Robertson, G. N., McGee, C. a. S., Dumbarton, T. C., Croll, R. P. & Smith, F. M. Development of the swimbladder and its innervation in the zebrafish, Danio rerio. *Journal of Morphology* **268**, 967–985 (2007)
31. Sahasrabudhe, A., Ghate, K., Mutalik, S., Jacob, A. & Ghose, A. Formin 2 regulates the stabilization of filopodial tip adhesions in growth cones and affects neuronal outgrowth and pathfinding in vivo. *Development* **143**, 449–460 (2016)
32. Suli, A., Watson, G. M., Rubel, E. W. & Raible, D. W. Rheotaxis in Larval Zebrafish Is Mediated by Lateral Line Mechanosensory Hair Cells. *PLOS ONE* **7**, e29727 (2012)
33. Thorsen, D. H. & Hale, M. E. Neural development of the zebrafish (Danio rerio) pectoral fin. *J. Comp. Neurol.* **504**, 168–184 (2007)
34. Valentin, G., Haas, P. & Gilmour, D. The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Curr. Biol.* **17**, 1026–1031 (2007)

35. Whitfield, T. T., Riley, B. B., Chiang, M.-Y. & Phillips, B. Development of the zebrafish inner ear. *Developmental Dynamics* **223**, 427–458 (2002)
36. Whitfield, T. T. *et al.* Mutations affecting development of the zebrafish inner ear and lateral line. *Development* **123**, 241–254 (1996).
37. Xiao, T., Roeser, T., Staub, W. & Baier, H. A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development* **132**, 2955–2967 (2005)